

Impact of tumor-associated myeloid cell depletion on T-cell leukemia development

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ABSTRACT

T-lymphoblastic leukemia/lymphoma (T-ALL/LBL) is an aggressive malignancy originating from immature T-cell precursors, which depends not only on cell-intrinsic genetic alterations, but also extrinsic stromal cell signals. Our group has previously shown that, compared to healthy thymi, thymic lymphomas from ETV6-JAK2 fusion transgenic (also called TEL-JAK2; TJ2-Tg) mice exhibit an increased content in dendritic cells (DCs), alongside with macrophages presence. Although recent studies revealed that both DCs and macrophages promote proliferation or survival of leukemic T cells *in vitro*, whether myeloid cells promote leukemogenesis *in vivo* remains to be determined.

Hence, we hypothesized that tumor-associated myeloid cells play an important role in TJ2-induced leukemogenesis. Here, we aimed to determine the myeloid cell composition in diseased TJ2-Tg mice and to deplete myeloid lineage cells *in vivo* before T-ALL/T-LBL development and study its impact on disease development.

First, we performed a characterization of myeloid populations in the TJ2-induced leukemia/lymphoma mice, by flow cytometry and immunofluorescence analysis. We found that both thymic and splenic tumor microenvironments in TJ2-Tg mice exhibited structural alterations, accompanied by increased myeloid components, and different myeloid subsets, namely conventional dendritic cells (cDCs) were increased.

Next, in order to deplete myeloid populations *in vivo*, we generated LysM-Cre/R26iDTR mice, by cross-breeding Lysozyme M (LysM)-Cre transgenic and ROSA26-loxP-STOP-loxP-DTR (R26iDTR) strains. We confirmed that the compound mice expressed the simian Diphtheria Toxin (DT) Receptor (DTR) in LysM-positive myeloid cells, due to loss of the STOP sequence mediated by Cre recombinase. To confirm that DTR-expressing cells could be eliminated, LysM-Cre/R26iDTR mice were injected with variable doses of DT, following different administration regimens, and myeloid cell depletion/recovery was assessed by flow cytometry. DT-mediated myeloid cell ablation was efficient when frequent and high doses of DT were administrated, followed by lower maintenance doses. In TJ2-Tg/LysM-Cre[+]/R26iDTR mice with leukemia, long-term DT treatment resulted in efficient depletion of thymic CD8⁺ cDCs, which was associated with significant lower thymic tumor burden and higher dissemination of leukemic cells to other organs. However, survival analysis showed no differences in leukemia onset between TJ2-Tg/LysM-Cre[+]/R26iDTR and TJ2-Tg/LysM-Cre[-]/R26iDTR treated mice. On the other hand, DT-mediated depletion of cDCs and macrophages did not impair TJ2⁺ cells colonization in splenic and lymph node microenvironments.

Collectively, our results suggest that CD8⁺ cDCs play a central role in the thymic lymphoma microenvironment of TJ2-Tg mice, concomitantly with leukemia dissemination and accumulation in the secondary lymphoid organs.

RESUMO

A leucemia linfoblástica aguda de linfócitos T (LLA-T) é uma doença agressiva originada a partir de precursores imaturos. Para além de várias alterações genéticas intrínsecas, sinais extrínsecos produzidos por células estromais também promovem o desenvolvimento da LLA-T. O nosso grupo verificou anteriormente que células dendríticas (DCs) estão expandidas no microambiente de linfomas tímicos de murganhos transgénicos ETV6-JAK2 (ou TEL-JAK2; TJ2-Tg), juntamente com a presença de macrófagos. Outros estudos revelaram que células dendríticas e macrófagos promovem a proliferação e/ou a sobrevivência da leucemia T *in vitro/ex vivo*, ficando por averiguar a função daquelas células *in vivo*.

Com este projeto pretendemos determinar a composição em células mieloides e avaliar o papel destas no processo de desenvolvimento de leucemia e linfoma induzido por TEL-JAK2 *in vivo*. Para tal, pretendemos deletar as células em questão, previamente ao desenvolvimento da doença.

Primeiramente, realizamos uma caracterização das populações mieloides em linfomas/leucemias TEL-JAK2 por análise de citometria, e observamos que diferentes células mieloides, nomeadamente, células dendríticas convencionais (cDCs), estavam aumentadas em órgãos linfoides de murganhos leucémicos TEL-JAK2. Além disso, análises por imunofluorescência revelaram que os microambientes tímico e esplénico de murganhos transgénicos TEL-JAK2 mostraram alterações estruturais acompanhadas de um incremento de suporte mioide estromal.

De seguida, para deletar células mieloides gerámos murganhos LysM-Cre/R26iDTR, cruzando a estirpe transgénica Lysozyme M (LysM)-Cre com a estirpe ROSA26-loxP-STOP-loxP-DTR (R26iDTR). Estes animais expressam o recetor para a toxina diftérica (DTR) em células mieloides (LysM positivas) após a remoção da sequência STOP, mediada pela recombinase Cre. Desta forma, células que expressam o DTR podem ser eliminadas com a administração da toxina diftérica. Os murganhos LysM-Cre/R26iDTR foram então injetados com doses variáveis de DT, segundo diferentes regimes de administração, e a monitorização da deleção/recuperação de células mieloides foi feita por análise de citometria. Os resultados revelaram que células mieloides eram mais eficientemente deletadas quando doses mais elevadas e frequentes de DT eram injetadas numa 1ª fase, seguidas de doses mais baixas para manutenção. Em contexto de leucemia, o tratamento prolongado com DT em murganhos TJ2-Tg/LysM-Cre[+]/R26iDTR promoveu a deleção eficiente de cDCs CD8⁺ no timo destes animais, a qual parece estar associada com uma diminuição significativa da carga tumoral tímica e com uma maior disseminação

de células leucémicas. No entanto, não foram observadas diferenças relativas à sobrevivência entre murganhos TJ2-Tg/LysM-Cre⁺/R26iDTR e TJ2-Tg/LysM-Cre⁻/R26iDTR. Por outro lado, a ablação de cDCs e macrófagos em órgãos linfoides secundários (baço e gânglios linfáticos) não impediu a invasão e acumulação de células TJ2⁺ nestes microambientes tumorais.

Em conclusão, estes resultados sugerem que as cDCs CD8⁺ parecem desempenhar um papel fundamental no microambiente tímico de murganhos transgênicos TEL-JAK2 e, concomitantemente, na disseminação de células leucémicas e invasão de órgãos linfoides secundários.

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LIST OF ABBREVIATIONS

Aire - Autoimmune Regulator

ALL - Acute Lymphoblastic Leukemia

alloHSCT - allogenic Hematopoietic Stem Cell Transplant

AML - Acute Myeloid Leukemia

APCs - Antigen Presenting Cells

Arg1 - Arginase 1

B-ALL - B-cell Acute Lymphoblastic Leukemia

BCL-2 - B-cell Lymphoma 2

bHLH - Basic Helix-Loop-Helix

BM - Bone Marrow

BMP4 - Bone Morphogenetic Protein 4

BSA - Bovine Serum Albumin

CCL2 - C-C Motif Chemokine Ligand 2

CCL19 - C-C Motif Chemokine Ligand 19

CCL21 - C-C Motif Chemokine Ligand 21

CCR7 - C-C Motif Chemokine Receptor 7

CCR9 - C-C Motif Chemokine Receptor 9

CD40L - CD40 ligand

cDCs - Conventional/classical DCs

CDKN2A - Cyclin-Dependent Kinase Inhibitor 2A

CDPs - Common DC Progenitors

CLPs - Common Lymphoid Progenitors

cMOPs - Common Monocyte Progenitors

CMPs - Common Myeloid Progenitors

CNAs - Copy Number Abnormalities

CNS - Central Nervous System

CR - Complete Remission

CSF-1 - Colony-Stimulating Factor 1

cTECs - cortical Thymic Epithelial Cells

CXCL5 - C-X-C Motif Chemokine Ligand 5

CXCL12 - C-X-C Motif Chemokine Ligand 12
CXCR4 - C-X-C Motif Chemokine Receptor 4
DAMPs - Damage-Associated Molecular Patterns
DCs - Dendritic Cells
DGAV - Direção Geral de Alimentação e Veterinária
DLL4 - Delta-like Ligand 4
DN1 - Double-Negative 1
DN2 - Double-Negative 2
DN3 - Double-Negative 3
DN4 - Double-Negative 4
DP - Double-Positive
DT - Diphtheria Toxin
DTR - Diphtheria Toxin Receptor
EF-2 - Elongation Factor 2
EGIL - European Group for the Immunologic Classification of Leukemia
Eos - Eosinophils
ETPs - Early Thymic Progenitors
ETP-ALL - Early T-cell Precursor Acute Lymphoblastic Leukemia
EYFP - Enhanced Yellow Fluorescent Protein
GM-CSF - Granulocyte–Macrophage Colony-Stimulating Factor
GMPs - Granulocyte-Monocyte Progenitors
HB-EGF - Heparin-Binding EGF-like Growth Factor
HD - Heterodimerization Domain
HIF1 α - Hypoxia Inducible Factor 1 Alpha
HSCs - Hematopoietic Stem Cells
ICAM-1 - Intercellular Adhesion Molecule-1
ICN1 - Notch1 Intracellular Domain
ICOSL - Inducible T-cell Co-Stimulator Ligand
IFN $\alpha\beta$ - Interferon Alpha/Beta
IFN γ - Interferon Gamma
IGF1R - Insulin-like Growth Factor 1 Receptor

IL-7 - Interleukin-7
IL-4R α - Interleukin-4 Receptor Alpha
IL-7R - Interleukin-7 Receptor
IP - Intraperitoneally
ISP - Immature Single-Positive
JAK - Janus Kinases
Krt⁻ - Keratin-negative
Krt5 - Keratin 5
Krt8 - Keratin 8
LAMs - Leukemia-Associated Macrophages
LFA-1 - Lymphocyte Function-associated Antigen-1
LIC/LSC - Leukemia-initiating Cell/Leukemia Stem Cell
LIF - Leukemia Inhibitory Factors
LMO - LIM-domain-only
LMPPs - Lymphoid-primed Multipotent Progenitors
LNs - Lymph Nodes
LP - Lamina Propria
LPMs - Large Peritoneal Macrophages
LT $\alpha\beta$ - Lymphotoxin $\alpha\beta$
LysM - Lysozyme M
M ϕ - Macrophages
mDCs - myeloid DCs
MDPs - Monocyte/macrophage and Dendritic Cell Progenitors
MDS - Myelodysplastic Syndromes
MDSCs - Myeloid-Derived Suppressor Cells
MEPs - Megakaryocyte-Erythrocyte Progenitors
MHC - Major Histocompatibility Complex
M-MDSCs - Monocytic Myeloid-Derived Suppressor Cells
MoDCs - Monocyte-derived DCs
MOMF - Monocyte-derived Macrophages
Mono Gr-1⁺ - Monocytes Gr-1-positive

Mono Gr-1⁻ - Monocytes Gr-1-negative

MPN - Myeloproliferative Neoplasms

MPPs - Multipotent Progenitors

MRD - Minimal Residual Disease

MSC - Mesenchymal Stromal Cell

mTECs - medullary Thymic Epithelial Cells

mTOR - mammalian Target of Rapamycin

MZ - Marginal Zone

NF1 - Neurofibromin 1

NF-κB - Nuclear Factor-κB

NK - Natural Killer Cells

NO - Nitric Oxide

ORR - Overall Response Rate

OS - Overall Survival

PAMPs - Pathogen-Associated Molecular Patterns

PB - Peripheral Blood

pDCs - Plasmacytoid DCs

PK1 - Phosphoinositide-Dependent Kinase 1

PD-L1 - Programmed Death-Ligand 1

PGE2 - Prostaglandin E2

PI3Ks - Phosphoinositide 3 Kinases

PIP2 - Phosphatidyl-P2

PIP3 - Phosphatidyl-inositol P3

PMNs - Polymorphonuclear Cells

PMN-MDSCs - Polymorphonuclear Myeloid-Derived Suppressor Cells

PRC2 - Polycomb Repressive Complex 2

PRR - Pattern Recognition Receptor

PSGL-1 - P-selectin Glycoprotein Ligand-1

PTEN - Phosphatase and Tensin Homolog

RANKL - Receptor Activator of Nuclear Factor-kappa Beta Ligand

RAS-MAPK - RAS/Mitogen-activated Protein Kinase

ROS - Reactive Oxygen Species

RP - Red Pulp

RT - Room Temperature

S1P - Sphingosine-1-Phosphate

S1P1 - Sphingosine-1-Phosphate Receptor

SCF - Stem Cell Factor

SOX13 - SRY-box 13

SP - Single Positive

SPMs - Small Peritoneal Macrophages

sUPIDs - segmental Uniparental Isodisomies

TADCs - Tumor-Associated DCs

T-ALL/T-LBL - T-cell Acute Lymphoblastic leukemia/T-cell Lymphoblastic Lymphoma

TAMs - Tumor-Associated Macrophages

TCR - T-cell Receptor

TdT - Terminal deoxynucleotidyl Transferase

TGF- β - Transforming Growth Factor Beta

Th - T helper

TipDCs - Inducible Nitric Oxide Synthase (iNOS)-producing DCs

TME - Tumor Microenvironment

TNF - Tumor Necrosis Factor

TNFSF4 - TNF Ligand Superfamily Member 4

ToIDCs - Tolerogenic DCs

Tregs - Regulatory T cells

TSAs - Tissue-associated Antigens

TSPs - Thymic Seeding Progenitors

VEGF - Vascular Endothelial Growth Factor

WBC - White Blood Cells

WHO - World Health Organization

WP - White Pulp

WT - Wild-Type

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INTRODUCTION

1. T-cell acute lymphoblastic leukemia/T-cell lymphoblastic lymphoma

T-cell acute lymphoblastic leukemia/T-cell lymphoblastic lymphoma (T-ALL/T-LBL) is an aggressive malignancy originating from immature T-cell precursors (1). To understand how T-ALL/T-LBL arises and what are the mechanisms of disease, it is important to understand normal T-cell development. Therefore, I will briefly allude to some key aspects of T-cell development before I delve into the intricacies of T-ALL/T-LBL.

1.1. Normal T-cell development

1.1.1 Lymphopoiesis in mice

Hematopoietic stem cells (HSCs) residing in the bone marrow (BM) are responsible for the generation of all blood cells (erythrocytes, myeloid cells, megakaryocytes, and lymphocytes) due to their self-renewal and differentiation capacity into multiple lineages (2, 3). This process is regulated by cell-to-cell interactions, besides growth factors that induce expression of transcription factors responsible for lineage commitment (4). Regarding lymphoid lineage, several stages of differentiation convert HSCs into multipotent progenitors (MPPs) that gradually lose their self-renewal capacity, thus turning first into oligopotent precursors and then into lineage-committed precursors (5).

In mice, the lymphoid development pathway (lymphopoiesis) is initiated on lymphoid-primed multipotent progenitors (LMPPs) harboring full lymphoid but only limited myeloid potential. These differentiate into common lymphoid progenitors (CLPs), that further generate T, B, natural killer (NK), and dendritic cells (DCs) (4, 6) (Figure 1). Within LMPPs and CLPs, only a specific subset of cells is able to home and settle in the thymus to differentiate into T cells (7). Indeed, thymic seeding progenitors (TSPs) present upregulation of P-selectin glycoprotein ligand-1 (PSGL-1) expression together with chemokine (C-C motif) receptors 7 (CCR7) and 9 (CCR9) (7, 8). TSPs then give rise to early thymic progenitors (ETPs) that initiate the T-cell development pathway (7, 9) (Figure 2). Animal experiments have been highlighting the bipotency of ETPs to induce T- or myeloid cell development (10, 11). Nevertheless, a major commitment towards T-cell lineage occurs in the thymus, being ETPs responsible for only a small fraction of thymic non-T cells (11). The mechanisms behind ETP cell fate decision remain to be fully elucidated but the intra-thymic microenvironment is thought to play an important role.

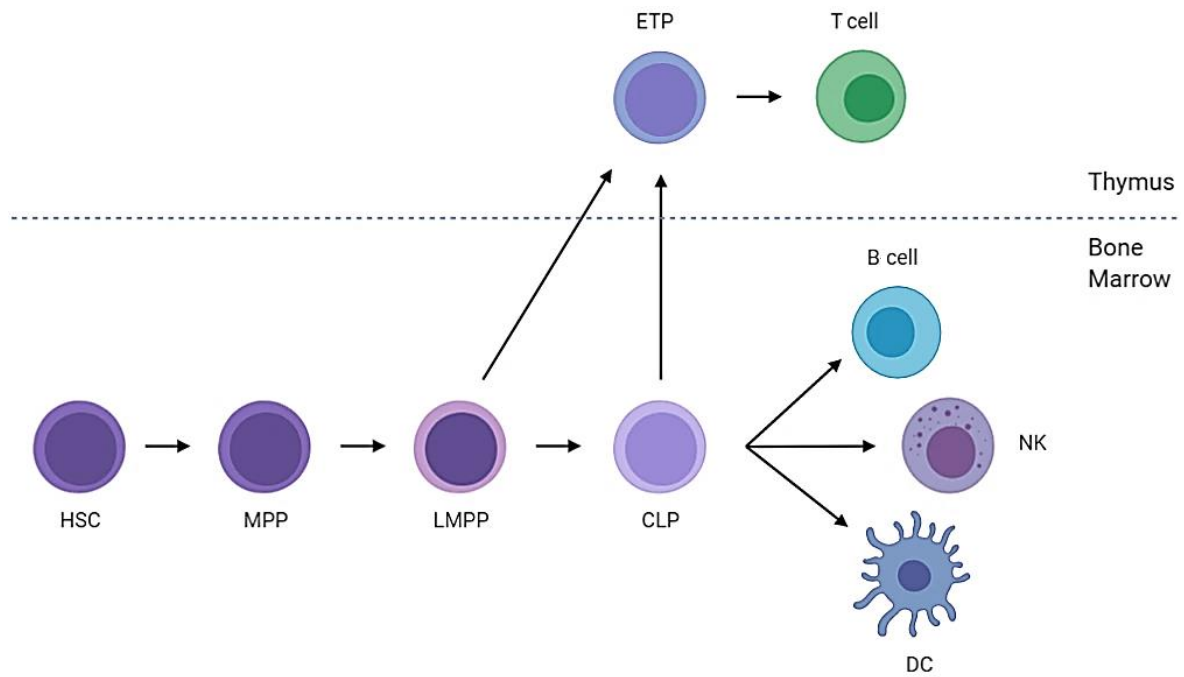


Figure 1. Lymphopoiesis in mice. BM-residing HSC differentiate into MPP, which in turn give rise to LMPP harboring both lymphoid and myeloid potential. The latter further divides into lymphoid committed progenitors (CLP) and generates T, B, NK and dendritic cells. Multipotent progenitors egress the BM and home to the thymus where they differentiate into early thymic progenitors (ETP), initiating T-cell production. Figure created with BioRender.com.

1.1.2 T-cell development in the thymus

The thymus is a mediastinal organ, localized behind the sternum, responsible for T-cell development from incoming early progenitors (3, 12). Histologically, the thymus is divided into a central medulla, a peripheral cortex, and a cortico-medullary junction, where uncommitted lymphoid progenitor cells enter through blood vessels to initiate T-cell differentiation (Figure 2) (3). The latter is characterized by different stages with different profiles of CD25, CD44, CD4, and CD8 expression (13). The ETP uncommitted progenitors differentiate first into the double-negative 1 (DN1) stage ($CD44^+$, $CD25^-$, $CD4^-$, $CD8^-$) and then into DN2 cells in the cortex ($CD44^+$, $CD25^+$, $CD4^-$, $CD8^-$), which suffer a proliferative clonal expansion followed by loss of B- and NK cell potential (14, 15). When cells proceed to DN3 stage ($CD44^-$, $CD25^+$, $CD4^-$, $CD8^-$) they commit definitively to the T-cell lineage and initiate RAG-mediated T-cell receptor (TCR) loci rearrangements (14, 15). NOTCH ligands together with cytokine interleukin-7 (IL-7) yielded by cortical thymic epithelial cells (cTECs) induce T-cell development till DN3 stage. Simultaneously, these immature thymocytes promote differentiation of stromal cells in the thymus and contribute for the cortical-epithelial microenvironment formation (13).

When DN3 thymocytes migrate through the cortex towards the subcapsular region, they begin to rearrange TCR loci as mentioned before. Only those that successfully rearrange TCR- β chains are able to proceed with the differentiation process, by assembling with the invariant pre-TCR α protein to form the pre-TCR signaling complex (13). In addition to TCR β chain rearrangements, γ and σ chain rearrangements can occur. The functional expression of $\gamma\sigma$ TCR complex at the surface together with SRY-box 13 (SOX13) drives cells into the $\gamma\sigma$ cell lineage (16), generating a distinct T-cell subtype involved in innate-like immune responses. Chemokine receptors such as CXCR4, CCR9 and CCR7 are required for translocation of DN thymocytes to the subcapsule and therefore for early T-cell development (13). Pre-TCR, CXCL12/CXCR4 axis, and Notch signals (namely Delta-like ligand 4 (DLL4)) induce proliferation and further differentiation of DN3 cells into DN4 (CD44⁻, CD25⁻, CD4⁻, CD8⁻) which undergo a transitional immature CD8 single-positive (ISP) stage before turning to CD4⁺, CD8⁺ double-positive (DP) thymocytes (3, 13, 15).

The newly generated DP thymocytes express TCR $\alpha\beta$ complex at their surface, resulting from RAG-mediated rearrangements of the TCR α locus (3). These DP thymocytes are highly motile through the cortex, where they engage in TCR-mediated interactions with major histocompatibility complex (MHC) molecules bound to self-peptides expressed by stromal cells (cTECs and DCs). These thymocytes undergo a selection process based on TCR signaling strength (13). In particular, cTECs and DCs act as antigen presenting cells (APCs), which take up and digest proteolytically antigens (proteins), transforming them into smaller fragments (peptides). The latter are presented at the APC surface, associated with MHC molecules, MHC class I or MHC class II, to interact with TCR associated with CD8 or CD4 co-receptors, respectively (17). Thus, when TCRs recognize MHC coupled with low affinity self-peptides, low input signaling is triggered and DP cells survive and mature into CD4 or CD8 single-positive (SP) thymocytes, in a so-called positive selection process, generating functionally competent T cells. On the other hand, overtly auto-reactive T-cells (i.e., interacting with high affinity self-peptides bound to MHC) will be eliminated by apoptosis through a negative selection process to avoid autoimmunity. Similarly, the non-self-reactive T cells (non-selection) because they fail to express a functional TCR or the TCR does not interact with any self-peptide bound to MHC, will not trigger any signaling and die, so-called “death by neglect” (13, 15). After the selection process at the cortex (mainly positive selection and non-selection), positively selected DP thymocytes migrate by chemotaxis towards the medulla for further maturation. This chemotaxis is dependent on CCR7 expression by DP thymocytes, which are attracted to the medulla where CCR7 ligands, chemokine (C-C motif) ligands 19 (CCL19) and 21 (CCL21), are produced by medullary thymic epithelial cells (mTECs) (13, 18). Simultaneously, medulla formation

occurs, triggered by positively selected thymocytes, which express tumor necrosis factor (TNF) family ligands (receptor activator of nuclear factor-kappa beta ligand (RANKL), CD40 ligand (CD40L) and lymphotoxin $\alpha\beta$ (LT $\alpha\beta$)) (19), and stimulate TNF family receptors and nuclear factor- κ B (NF- κ B) -mediated signaling in mTECs (20). Once DP thymocytes differentiate into CD4 or CD8 SP thymocytes in the medulla, they undergo a maturation process to become functional, characterized by CD62L and CD69 expression profiles. At this stage, T cells experience a second wave of selection (negative selection) in the medulla, allowing for self-reactive thymocytes to be depleted, which promotes central tolerance to tissue-associated antigens (TSAs), namely because the latter are promiscuously expressed by mTECs (expression partially mediated by transcription factor autoimmune regulator (Aire) (21, 22)) (13). Also reinforcing the self-tolerance, is the production of regulatory T cells (Tregs) from medullary thymocytes recognizing self-antigens presented by mTECs, despite the conflicting data about a possible role of the cortex in Treg development (23).

Exit of mature SP thymocytes from the thymus is dependent upon G-protein-coupled receptors, namely sphingosine-1-phosphate (S1P) receptor (S1P1) whose expression is acquired during maturation (24, 25). Besides S1P-mediated emigration, it was reported that interleukin-4 receptor alpha (IL-4R α) expression by the thymic stroma also regulates thymocytes egress by a distinct mechanism (26). In newborn mice, CCR7 signals appear to display an important role in T-cell emigration (27). These mature thymocytes then enter into circulation through perivascular spaces, towards lymphoid and non-lymphoid organs (13).

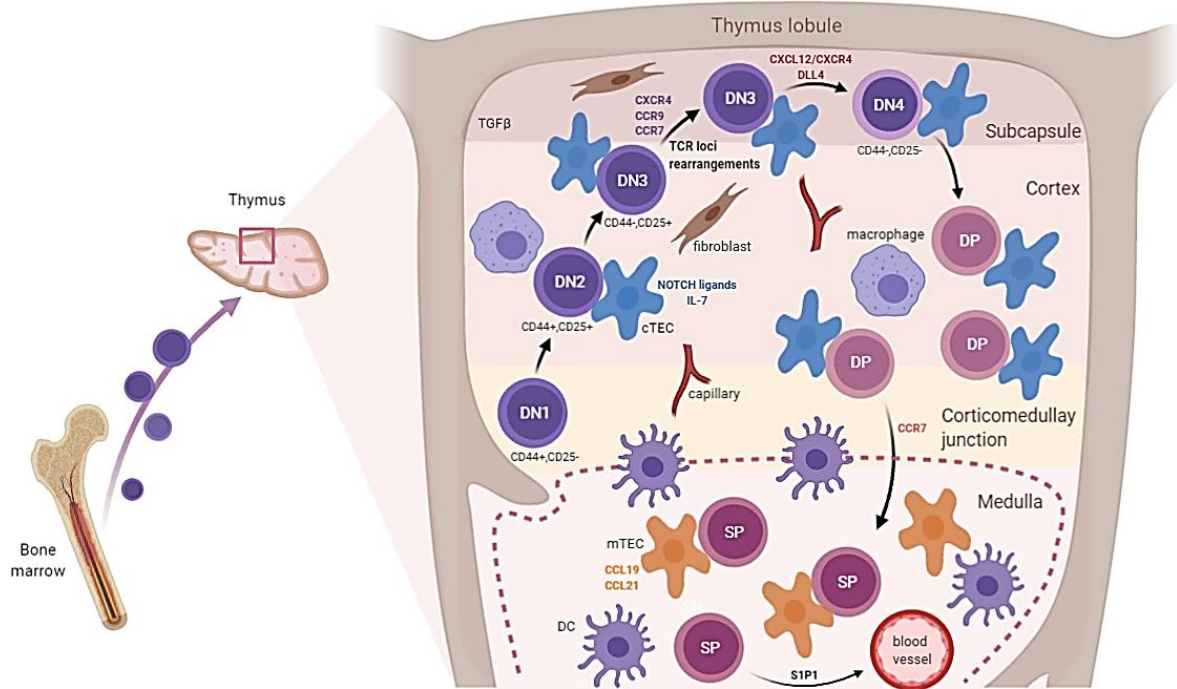


Figure 2. T-cell development pathway: interactions with stromal compartments. Circulating T-cell precursors enter the thymus to initiate a highly intricate process that culminates with T-cell production. Uncommitted DN1 progenitors differentiate into DN2 thymocytes that after clonal expansion commit to T-cell lineage and initiate TCR loci rearrangements (DN3). Thymocytes that successfully rearrange TCR β chain proceed the differentiation process assembling with TCR α chain and further differentiating into DN4 stage. DN4 thymocytes undergo a transitional immature CD8 SP stage before becoming DP thymocytes. Newly generated TCR $\alpha\beta$ -expressing DP thymocytes experience a selection process that promotes maturation into SP thymocytes in the medulla. Functional T cells egress the thymus through bloodstream towards lymphoid and non-lymphoid organs. T-cell stage progression is regulated by interactions between thymocytes and stromal cells as represented schematically. Figure created with BioRender.com.

1.2. Hematological malignancies: focusing on Acute Lymphoblastic Leukemia

Hematological malignancies are a group of neoplasms originated in hematopoietic and lymphoid tissues whose clinical manifestation occurs in the form of leukemia, lymphoma, or myeloma (28). According to the World Health Organization (WHO) classification, these malignancies are classified depending on the immunophenotype, morphology, genetic and molecular signatures, and clinical features. Basically, they are categorized according to cell lineage (myeloid or lymphoid), maturational/differentiation profile (immature or mature phenotype), disease behavior (acute or chronic) and localization (leukemia or lymphoma) (29).

Myeloid malignancies include Myeloproliferative Neoplasms (MPN), Myelodysplastic Syndromes (MDS), Myelodysplastic/Myeloproliferative Neoplasms, when there is an overlap of morphological, genetic, and clinical features of both, eosinophilia-associated Myeloid/Lymphoid neoplasms, and Acute Myeloid Leukemias (AML) (30).

Lymphoid malignancies are divided into two principal groups: neoplasms derived from precursors of B or T cells and neoplasms originated from mature B, T or NK cells. In particular, Acute Lymphoblastic Leukemia (ALL) is included in the group of neoplasms arising from lymphoid progenitors, being divided into B- and T-lymphoblastic leukemia/lymphoma (30-32).

ALL is thought to result from the malignant transformation of immature lymphoid cells and is characterized by the uncontrolled proliferation of these cells in the bone marrow, peripheral blood (PB) and extramedullary sites (33). The vast majority of ALL cases occurs in children (80%), representing the most common pediatric malignancy (12, 33). In 2017, approximately 64 200 new cases were reported worldwide (34).

1.2.1 T-ALL/T-LBL epidemiology and risk factors

In particular, T-cell acute lymphoblastic leukemia (T-ALL) is less frequent than B-cell acute lymphoblastic leukemia (B-ALL), accounting for about 10-15% of the total cases of ALL (1, 3). T-ALL is namely diagnosed in children and adolescents, arising also in aged adults (25% of cases) (3). On the contrary, the big majority of lymphoblastic lymphomas (LBL) are from T-cell lineage, accounting T-LBL for 85-90% of all LBL cases (1). In particular, T-LBL represents 20% of pediatric and 2% of adult non-Hodgkin lymphomas (35).

Regarding risk factors, males display a threefold increased risk of developing T-ALL comparing to females (36). Such difference may be due to deletions or inactivating mutations of tumor suppressor genes localized on chromosome X (12). Moreover, within environmental factors, only ionizing radiation has been proven to cause ALL (37). There are other exogenous factors such as parental smoking, household chemicals, among others, but none has provided convincing data proving its contribution in childhood ALL incidence (38).

1.3. T-ALL/T-LBL clinical features and classification

T-ALL/T-LBL is characterized by the non-controlled production of malignant T-cell precursors both in the BM and/or thymus (2). Clinically, patients may present a mediastinal mass emerging from the thymus (60% of cases) together with extra-medullary infiltration of other lymphoid and non-lymphoid organs (Figure 3) (2, 12). This proliferation is associated with hyperleukocytosis (50% of cases), BM function suppression, respiratory problems, and neurological disorders. In particular, spread of immature T cells in the BM impairs hematopoiesis which is associated with clinical manifestations such as pyrexia, lethargy, recurrent infections, skin and mucosa hemorrhages as well as bone pain (12). Patients may also present central nervous system (CNS) infiltration at the diagnosis (3-9%) experiencing

headache, seizures, nausea and mental disturbances (39). As mentioned before, half of the patients exhibit hyperleukocytosis (above $100 \times 10^9/L$) which is associated with several complications including tumor lysis syndrome (12).

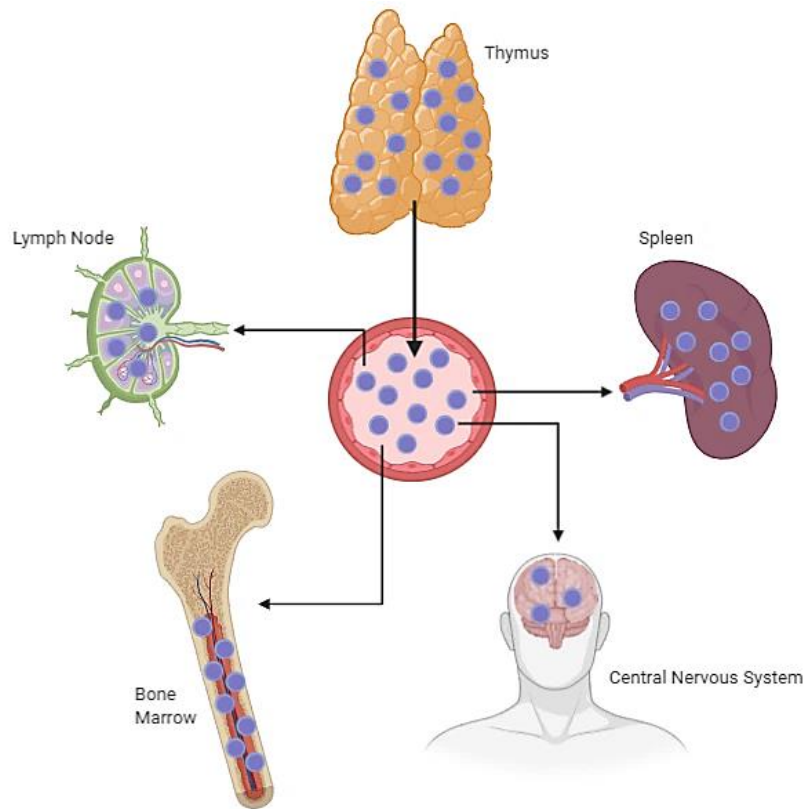


Figure 3. Egress of T-ALL blasts from thymus to lymphoid and non-lymphoid organs. Malignant immature T-cells exit the thymus through the bloodstream and infiltrate secondary lymphoid (spleen, lymph nodes, bone marrow) and non-lymphoid organs (e.g., central nervous system). Figure created with BioRender.com.

1.3.1 Immunophenotype of T-ALL/T-LBL

T-ALL/T-LBL diagnosis and further discrimination from other lymphoblastic malignancies implies an immunophenotypic analysis that consists in the identification of intracellular or cell surface antigens (12). In particular, T cells are characterized by the expression of CD1a, CD2, CD3 (both at surface and intracellular), CD4, CD5, CD7 and CD8 (31). Even though CD2, CD5 and CD7 mark for immature T cells, they are not lineage-specific, therefore T-ALL/T-LBL diagnosis should be based on CD3 expression (12, 31). CD3 is important since its association with TCR results in complexes that are required for T-cell survival in the thymus. CD4 and CD8 co-receptor proteins assist the TCR receptor upon interaction with MHC. CD1 is involved in antigen presentation, CD5 in immune response modulation and CD2 and CD7 participate in cell-to-cell interactions and adhesion (12). The terminal deoxynucleotidyl transferase (TdT) is expressed in 90-95% of lymphoblastic lymphoma/leukemia and CD10 in around 25% of cases (31, 40). Moreover, CD34 can also

be expressed in T-ALL as well as myeloid antigens, CD13 and CD33, in approximately 20-30% of patients (12, 31). The expression of these differentiation markers allows discrimination of four T-ALL subtypes. The European Group for the Immunologic Classification of Leukemia (EGIL) classifies T-ALL into pro-T-ALL, pre-T-ALL, cortical/thymic T-ALL, and mature T-ALL (1, 41). This classification is also applicable to T-LBL, although the frequency of each subtype is distinct (see below). The subtypes and its respective key markers are listed in Table 1.

Recently, a new infiltrative subtype of T-ALL mimicking early T-cell progenitor phenotype, the ETP-ALL, has been identified as an aggressive subtype with very poor prognosis (2). Indeed, the 10-year overall survival (OS) in patients with ETP-ALL is around 19% while in patients without ETP-ALL is 84% (42). Within T-ALL, 5% to 15% of the cases are ETP-ALL (12). Immunophenotypically, this subtype is part of the pro-T-ALL subtype and characterized by the expression of cCD3, CD7, and CD5 (below 75%) and absence of CD1a and CD8 expression. Over 25% of ETP-ALL blasts also express at least one stem or myeloid cell markers such as CD33, CD13, CD34, CD11b, CD65, CD117 and HLA-DR (1).

Table 1. Major immunophenotypes in T-ALL.

Adapted from (1, 31, 41, 43)

T-ALL Subtype	Key markers
Pro-T-ALL (T-I)	cCD3+, CD7+, CD1a-, CD34±, CD28-, CD4-, CD8-
Pre-T-ALL (T-II)	cCD3+, CD7+, CD5/CD2+, CD1a-, CD34±, CD28+, CD4-, CD8-
Cortical/Thymic T-ALL (T-III)	cCD3+, sCD3±, CD1a+, CD7+, CD34-, CD28+, CD4+, CD8+
Mature T-ALL (T-IV)	cCD3+, sCD3+, CD1a-, CD7+, CD34-, CD28+, CD4+ or CD8+

1.3.2. Differences between T-ALL and T-LBL

As mentioned before, T-ALL and T-LBL are immature T-cell malignancies, often considered as different manifestations of the same disease, mainly distinguished by the level of BM involvement: T-ALL implies presence of over 25% of BM blasts, which means that BM infiltration under 25% is considered T-LBL (12, 41). Microarrays transcriptomic analysis of pediatric T-ALL/T-LBL samples allowed the separation of these malignancies due to different gene expression profiles, with T-ALL overexpressing CD47 and T-LBL overexpressing MML1 (41, 44). Regarding T-LBL, the thymic subtype is the most common in children (≈70%), however, there is a big slice (40-50%) that cannot be phenotyped.

Similarly, in adults, no immunophenotypes were distinguished. On the other hand, in adult T-ALL, pro-T/pre-T and mature subtype proportions are more prevalent in comparison to childhood T-ALL, being associated with worse prognosis (41). The thymic subtype, in turn, exhibits a better outcome, being the most predominant in pediatric T-ALL patients (41).

Nowadays, it is assumed that T-ALL arises from more immature stages, involving BM or PB, while T-LBL arises from more mature thymocytes exhibiting a mediastinal mass in most cases (90%). In T-ALL, the percentage of mediastinal tumors depends on the immunologic phenotype: in pro-T/pre-T T-ALL, 44% of patients present a mediastinal mass while in thymic or mature T-ALL the percentage increases to 79% (41).

Concluding, T-ALL and T-LBL are mainly distinguished by BM involvement and more recently by differences in genetic profile. Nevertheless, both malignancies reveal similar immunophenotype, clinical features and response to therapy, therefore they are not seen by the WHO as different entities but more as localized versus systemic disease.

1.4. Genetic alterations and signaling pathways involved in T-ALL leukemogenesis

Over the last years, several genetic and epigenetic alterations were identified within the T-ALL leukemogenic process. Approximately, 55% to 75% of T-ALL cases reveal karyotype abnormalities being the majority of them structural, often solitary, chromosome alterations (reviewed in (12)). According to Mitelman database, comprising all cytogenetic abnormalities found in T-ALL pediatric cases, the most common alterations are TCR loci translocations or inversions ($\approx 30\%$), followed by deletions on the long arm of chromosome 6 ($\approx 20\%$), unbalanced translocations or deletions in 9p ($\approx 15\%$), 11q deletions, trisomy of chromosome 8, and 12p loss (5-10%) (45). In particular, deletions in 9p are functionally important since they promote the loss of Cyclin-dependent kinase inhibitor 2A (*CDKN2A*), a tumor suppressor gene that regulates cell cycle (46, 47). In ETP-ALL, the deletion of *CDKN2A* is much less common, whilst chromosomal deletions including 5q, 11q and 13q are frequent (1).

Regarding TCR abnormalities in T-ALL, it is known that many of the genes recombining illegitimately with TCR loci are homeobox, LIM-domain-only (LMO) and class II basic helix-loop-helix (bHLH) transcription factor genes. These transcription factors regulate transcription activity, which means that TCR translocations in T-ALL result mainly in aberrant gene expression (12). Indeed, T-ALL can be divided in subgroups that exhibit specific gene expression patterns depending on the TCR translocation partner gene (*LMO1/2*, *LYL1*, *HOXA*, *TLX1/3* or *TAL1*) (48, 49).

Cytogenetic and genomic analyses revealed the occurrence of at least 75 fusion genes in T-ALL. Half of them encode transcription factors while a smaller part (18%) encodes for tyrosine kinases (reviewed in (12)). The latter are particularly interesting when it comes to targeted therapy, because of their potential “drugability” (50-52). In particular, *ETV6*, *KMT2A* (both transcriptional regulators) and *ABL1* (a tyrosine kinase) are some of the most frequent genes involved in T-ALL gene fusions (reviewed in (12)). Within translocations involving *ETV6*, we note the fusion gene *ETV6-JAK2*, which is found in T cell childhood acute lymphoblastic leukemia with low frequency (< 1%) (53), because we used an *ETV6-JAK2* transgenic mouse model for our work.

Analyses of pediatric T-ALL samples by large-scale sequencing and SNP arrays revealed that the big majority ($\approx 92\%$) of them carried copy number abnormalities (CNAs), and that almost half of the cohort ($\approx 42\%$) presented segmental uniparental isodisomies (sUPIDs) (46). The most common CNAs in T-ALL are deletions ($\approx 78\%$) (46), which usually target tumor suppressor genes, such as *CDKN2A* deletions (over 70%), often but not always in concomitance with *CDKN2B* deletion (46, 54). Besides *CDKN2A/B*, deletions involving *PTEN* (55) and *WT1* (56) (tumor suppressors), *LEF1* (57) (transcription factor), *PTPN2* (58) (tyrosine phosphatase), *BCL11B* (59) (transcriptional repressor) and genes related to epigenetic regulation such as *EZH2* (60) and *PHF6* (61), are also common. To note that, excluding *CDKN2A/B*, many of these genes are also frequently inactivated in T-ALL by mutations, regardless of the other allele bearing the deletion or not (62). Functionally, deletions not always end up in loss of gene function, they can also result in promoter swapping (e.g., *TAL1*) or removal of negative regulatory elements (e.g., *LMO2*), leading to overexpression of the target genes. On the other hand, *MYB* gain is identified in 10% of T-ALL patients as a result of tandem duplication within the *MYB* locus (63).

Regarding gene mutations, over 50% of T-ALL cases exhibit activating *NOTCH1* mutations (12, 64, 65). These are also present in ETP-ALL, albeit much less frequently (1). *NOTCH1* is a transmembrane heterodimeric receptor important for T-cell outcome and differentiation. Under physiological conditions, it interacts with ligands such as Delta-like 1/3/4 or Jagged 1/2 via the extracellular domain, which leads to *NOTCH1* cleavage, followed by translocation of the intracellular subunit (ICN1) to the nucleus where it acts as a transcriptional regulator (Figure 4) (12). *NOTCH1* is involved in the regulation of genes participating in cell growth, metabolism and cell cycling (65). In particular, *NOTCH1* promotes the activation of PI3K-AKT-mTOR pathway in T-ALL via modulation of *MYC*, *HES1* and *IL7R α* expression (64, 65). Besides, it also regulates cell cycle progression and survival of T-ALL blasts by upregulating *CCND3* and NF- κ B related genes, respectively (65-67). The signaling pathway is terminated upon phosphorylation of the ICN1 PEST domain

and recruitment of the FBXW7 ubiquitin protein ligase, which promotes proteasomal degradation of activated NOTCH1 (12). In T-ALL, most NOTCH1 mutations reside in the heterodimerization domain (HD) ($\approx 40\%$) connecting the extra- and intracellular units, or in the PEST domain ($\approx 15\%$), which lead to ligand-independent activation or impaired NOTCH1 degradation, respectively. *FBXW7* mutations have the same effect as PEST mutations and are present in 15% of T-ALL cases. There are also a few cases (20%) harboring dual mutations that involve HD and PEST, or *FBXW7*, exhibiting overexpression of NOTCH1 signaling (12, 65).

Furthermore, 20% to 30% of T-ALL patients present activating mutations in Interleukin-7 receptor (IL-7R), Janus kinases 1 and 3 (JAK1/3) and/or STAT5 (64). IL-7/IL-7R signaling is fundamental for normal lymphoid development. The binding of IL-7 to its receptor interacting with JAK1 and JAK3, induces conformational changes that drive kinases transphosphorylation, creating docking sites for signaling molecules such as STAT5 or phosphoinositide 3 kinases (PI3Ks) to bind and become activated, modulating transcription activity (68). In particular, STAT5 regulates the transcription of B-cell lymphoma 2 (BCL-2) protein family members, promoting a survival advantage in developing T cells (69). Nevertheless, in T-ALL blasts there is an alternative IL-7-mediated survival mechanism in which STAT5 upregulates cell cycle regulator *PIM1* (70). Besides IL-7R/JAK/STAT cascade, PI3K-AKT, RAS/mitogen-activated protein kinase (RAS-MAPK) and NF- κ B pathways were also found to be constitutively activated in T-ALL (66, 71). The abnormal activation of the PI3K pathway in T-ALL is mostly driven by deletions/inactivating mutations of its negative regulator, phosphatase and tensin homolog (PTEN) (64). On the other hand, RAS/MAPK hyperactivation in T-ALL patients results from mutations in *RAS* oncogene and/or neurofibromin 1 (*NF1*) tumor suppressor, regulating the RAS cascade (71). Regarding NF- κ B signaling, even though very few mutations were reported in T-ALL, both NF- κ B canonical and noncanonical pathways were shown to be activated in human T-ALL samples and several T-ALL mouse models (72-76). The major activator of NF- κ B signaling in T-cell acute leukemia is Notch1 and the crosstalk between these two pathways is fundamental for Notch1-induced transformation and leukemia maintenance (72, 77).

In particular and similarly to T-ALL, ETP-ALL usually exhibits activating mutations in genes involved in cytokine receptor (JAK1/3, IL7R) and RAS pathways (KRAS, NRAS, BRAF), besides inactivating mutations that impair hematopoiesis (RUNX1, GATA3, ETV6, IKZF1) (1).

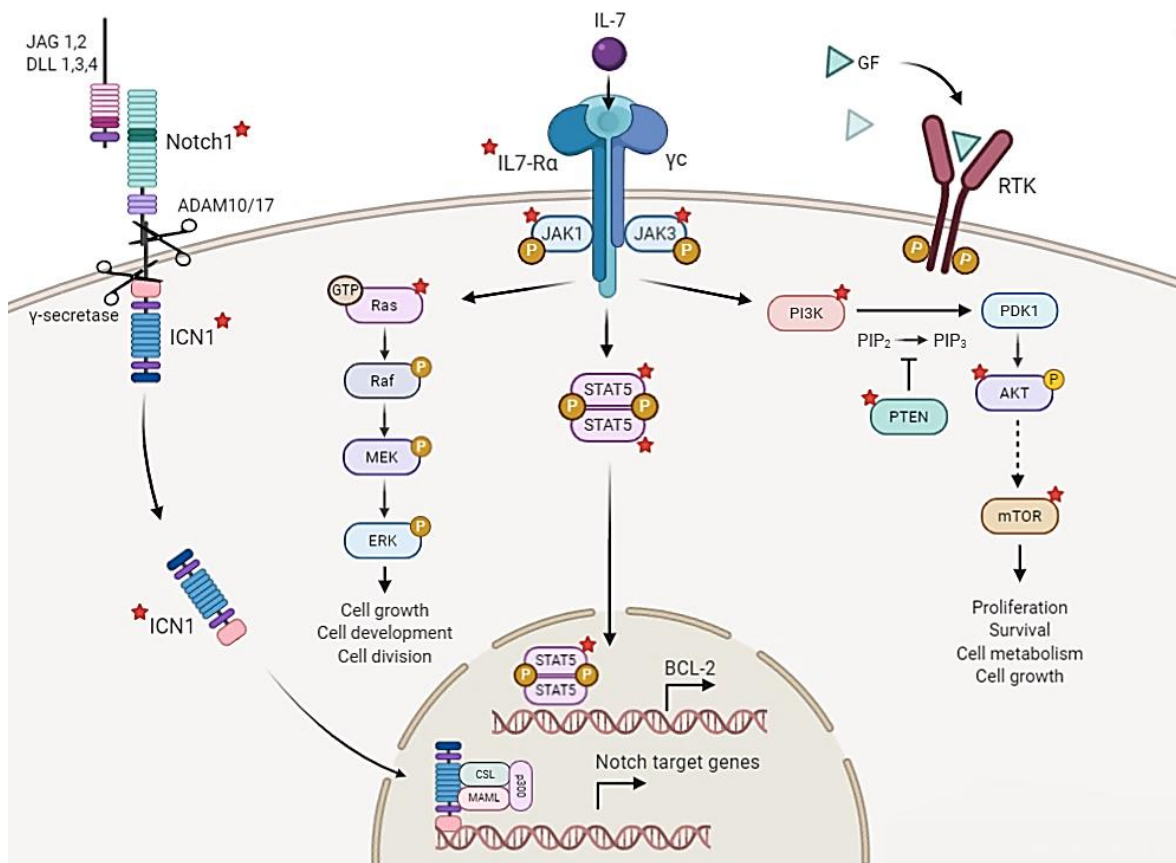


Figure 4. Major signaling pathways altered in T-ALL. Representation of different oncogenic mechanisms involved in T-ALL leukemogenesis. The binding of IL-7 to IL-7R promotes JAK1-JAK3 reciprocal phosphorylation, allowing for STAT5 recruitment, which dimerizes and translocates to the nucleus, where it modulates BCL-2 transcription. IL-7 can also activate PI3K-AKT and RAS/MAPK cascades (64). Activated PI3Ks convert phosphatidyl-P2 (PIP2) into phosphatidyl-inositol P3 (PIP3), enabling the engagement of effector molecules such as phosphoinositide-dependent kinase 1 (PDK1) and AKT. The latter will secondarily activate the mammalian target of rapamycin (mTOR), resulting in a pro-survival and proliferative signal (71). Regarding RAS/MAPK pathway, and upon GTP-mediated activation of small G-protein RAS, it recruits/activates the serine/threonine protein kinase, RAF (MAP3K), which in turn phosphorylates the MEK (MAPK2K) and consequently the ERK1/2 (MAPK). Then, ERK can translocate to the nucleus and regulate cell growth, division and differentiation (78, 79). Constitutive activation of Notch1 signaling results from binding of Notch ligands (Delta/Jagged) to the extracellular domain of NOTCH1, which induces endomembrane cleavage of the receptor, first by an ADAM metalloproteinase followed by the γ-secretase, releasing the intracellular domain (ICN1) to the cytosol. ICN1 then translocates to the nucleus, where it associates with CSL-DNA binding protein and transcriptional co-activators, MAML and p300m, inducing gene expression (80, 81). Figure created with BioRender.com.

★ Proteins that are mutated in T-ALL

Not only genetic alterations contribute for leukemogenesis, as T-ALL display specific epigenetic modifications (82). Furthermore, genetic alterations in epigenetic regulators have been identified in more than half of pediatric T-ALL cases (83). In particular, the polycomb repressive complex 2 (PRC2) - *EZH2*, *SUZ12*, *EED*, and *RBBP4* – harboring histone

methyltransferase activity, is affected by loss-of-function mutations or deletions namely in ETP-ALL subtype (84). In addition, mutations or deletions in the chromatin regulator, *PHF6*, are also exhibited in 38% and 16% of adult and pediatric T-ALL cases, respectively (85). On the other hand, mutations in histone acetyltransferases genes such as *EP300* and *CREBBP* are also reported in T-ALL (12, 62). Similarly, a subset of microRNAs targeting tumor suppressor genes (*PTEN*, *BIM*, etc.) was found to be highly expressed in T-ALL, in which the miR223 stands out for its differential upregulation. The later also controls FBXW7 ligase, promoting *NOTCH1*-driven T-ALL (86).

1.5. T-ALL/T-LBL treatment

1.5.1 Standard therapies

In the clinical practice, ALL patients are treated with a combination of chemotherapy regimens administered in several phases including: induction, consolidation, delayed intensification, and maintenance with CNS prophylaxis (2, 87, 88). Indeed, standard systemic chemotherapy does not penetrate in CNS and testes, so these are privileged sites for ALL cells to reside. Therefore, most patients perform a lumbar puncture followed by intrathecal methotrexate and/or cytarabine administration to support induction and consolidation treatments (87-89). Low-dose CNS-directed radiation therapy as prophylaxis is only considered in high-risk ALL cases (89, 90). Children achieve a complete remission (CR) rate of 97-99% while adults present a lower CR rate between 75% and 90%. Once normal hematopoiesis is restored, patients proceed to consolidation and maintenance treatments to eliminate disease. Nevertheless, ALL patients usually relapse within several months to years after achieving remission for the first time. Reports have shown that when maintenance regimens are prolonged for 2 or 3 years, ALL relapse is less frequent (91, 92). Besides, extended treatment also increases the possibility of eliminating slow-growing leukemic clones or eventually kill occult disease, namely in the CNS (87).

Nevertheless, while ALL pediatric treatment has achieved a cure rate of about 90%, in adults the cure rate varies between 20% and 40% (87). Several studies have shown that young adults actually respond better to pediatric-intensive chemotherapy treatments than adult-intensive chemotherapy regimens (43). In addition, the biological mechanisms behind ALL progression are different depending on age group, and older people, for instance, cannot tolerate aggressive chemotherapy treatments or transplantation procedures as younger patients do (43, 87). All together contribute for the poor outcome observed in older patients.

In ALL, allogeneic hematopoietic stem cell transplant (alloHSCT) is recommended in first remission for high-risk patients (93, 94). Moreover, minimal residual disease (MRD)

response has been influencing alloH SCT recommendation (88, 93). There are several studies showing the beneficial role of SCT namely from an HLA-matched sibling in high-risk ALL patients during remission (95-98). However, if no HLA-matched donor is available, ALL patients can undergo an allogeneic SCT from an HLA-matched unrelated donor or participate in clinical trials (87, 99, 100). Regarding autologous HCT, there are not sufficient evidence proving its advantage over chemotherapy treatments (43, 87). In T-LBL, both allogeneic and autologous SCT are not usually required given its better prognosis but can be suggested in a high-risk condition (101).

The majority of ALL patients (80%) relapse within 2 years after being diagnosed, with leukemic cells reappearing in the CNS, BM, or testes (87, 88). For relapsed or refractory disease, ALL patients are suggested to undergo allogeneic SCT or novel therapies (87). Nelarabine, a chemotherapeutic agent, is also used for both T-ALL and T-LBL relapsed/refractory disease inducing an overall response rate (ORR) around 46% in adults, with a 3-year OS of 36% when conjugated with SCT (43, 88). The identification of specific genetic alterations in the majority of T-ALL patients, drew attention for the possibility of targeted therapy.

1.5.2 Novel therapeutic agents based on genetic alterations

The standard treatment combining chemotherapy and CNS prophylaxis has significantly improved T-ALL/T-LBL survival rates in pediatric patients. Nevertheless, these procedures are associated with severe toxicity and the possibility of secondary malignancies. Besides, around half of the adult patients still suffer from relapsed or refractory disease, underlining the need for new therapies (3). Since the majority of T-ALL patients frequently harbor specific genetic aberrations, such as *NOTCH1/FBXW7* mutations, the targeted therapy has arisen as a promising therapeutic option. Several reports, including clinical trials, using γ -secretase inhibitors (102-105), which prevent Notch cleavage, as well as DLL4-neutralizing antibodies, have shown promising therapeutic effects, namely in relapsed disease (106, 107). Similarly, tyrosine kinase inhibitors, such as tofacitinib, have been suggested as an alternative treatment for T-ALL patients bearing activating *JAK3* mutations (108). Moreover, the blockade of PI3K-AKT pathway, using small molecules against PI3K (109), AKT (110), mTOR (111) or dual PI3K-mTOR antagonists (112), has revealed increased apoptotic activity and survival benefit in T-ALL cases exhibiting PTEN deletion/suppression.

1.6. Microenvironmental cues modulating T-ALL development

Besides intrinsic genetic and epigenetic alterations, the input from hematopoietic niches also seems to be critical for both leukemia initiation and progression. Indeed, there are two non-mutually exclusive hypotheses regarding the role of stroma in leukemogenesis: niche

alterations may have an oncogenic potential and lead to malignancy development, and malignant cells can remodel the surrounding niche in its favor, promoting disease progression (113). For instance, myeloid malignancies have been more associated with the niche-predisposing factor theory. Several genetic abnormalities in niche compartments led to pre-leukemic conditions in mice, such as myeloproliferative or myelodysplastic disorders (114-116). On the other hand, in lymphoid malignancies, niche remodeling seems to play the biggest role. In here, I present three well studied tissue microenvironments and their contribution to T-ALL.

1.6.1. Bone marrow niche contribution towards T-ALL development

Like healthy HSC, leukemia-initiating cell/leukemia stem cell (LIC/LSC) fate and activity also depend on signals yielded by BM stromal cells regulating hematopoiesis. Indeed, HSCs and LICs share several biologic features with the exception that LICs activate important signaling pathways in an abnormal, unregulated manner. Therefore, the latter can outcompete HSCs and hijack BM niches, creating a “leukemic” niche that supports disease maintenance (117, 118). This corroborates the hypothesis that malignant cells have the ability to remodel the surrounding niche in its favor, promoting leukemogenesis (113).

Experiments using both a Notch-driven T-ALL mouse model and xenografts of human T-ALL revealed that leukemic cells impair normal hematopoiesis through suppression of osteoblast populations (119, 120). Other study demonstrated that BM stromal cells in the B-cell lineage ALL context induces an increase in reactive oxygen species (ROS) levels, which are downregulated by leukemic cells, as an adaptative mechanism, thus favoring chemoresistance and disease recurrence (121). In particular, mesenchymal stromal cell (MSC)-derived adipocytes support ALL BM engraftment through increased production of leptin and chemokine (C-X-C motif) ligand 12 (CXCL12) (122). Similarly, adipocyte-enriched BM decreases T-ALL chemosensitivity by promoting leukemia cells quiescence (123). In addition, IL-18-producing BM stromal cells support T-ALL progression via activation of NF- κ B pathway (124). This protecting role of BM MSCs in ALL thus promotes resistance to drugs and improve malignant cell survival (123-126). Moreover, the adhesion of T-ALL cells to BM MSCs through lymphocyte function-associated antigen-1 (LFA-1)/intercellular adhesion molecule-1 (ICAM-1) binding was shown to be critical for T-ALL survival *in vitro* (127).

The hypoxic microenvironment in the BM plays an important role in HSC quiescence, but it might also support LICs. Indeed, the expansion of hypoxic areas in the BM has been associated with leukemia progression, besides contributing for ALL chemoresistance, as demonstrated *in vitro* (128). Likewise, recent evidence revealed that the hypoxia inducible

factor 1 alpha (HIF1 α), a transcriptional factor induced by hypoxia or PI3K/MAPK survival pathways, is required for T-ALL LIC activity, together with WNT/ β -catenin signaling (129, 130).

Besides WNT/ β -catenin pathway, CXCL12/CXCR4 and Notch signaling are also critical for ALL maintenance. In particular, CXCR4-expressing T-ALL cells require direct interaction with CXCL12-producing BM vascular endothelial cells in order to survive and progress. Deletion of *CXCR4* or *CXCL12* in ALL or vascular endothelial cells, respectively, suppressed disease in mouse models (131, 132). CXCR7, the other receptor for CXCL12, was also found to be more expressed in BM leukemic cells of T-ALL patients, and it appears to potentiate leukemia migration driven by CXCL12/CXCR4 signaling (133). On the other hand, Notch-ligand, DLL4 was found to be expressed in lymphoid organs (BM and spleen) of T-ALL xenografted mice as well as in the BM of T-ALL patients, and its blockade impaired T-ALL growth and survival (107).

1.6.2. Splenic microenvironment role in T-ALL

Besides the BM, the spleen is a major site for extramedullary hematopoiesis, being also involved in leukemia development (4, 134). Indeed, several T-ALL patients present splenomegaly, which was previously associated with poor prognosis (135). Nevertheless, how spleen microenvironment modulates T-ALL remains to be elucidated. By using a Notch1-induced T-ALL mouse model it was revealed that CCR7-expressing T-ALL cells are mainly recruited to the spleen due to increased levels of CCL19. Besides, splenic microenvironmental cells potentiated T-ALL cells proliferation and migration *in vitro*, while *in vivo* the splenectomy of T-ALL-bearing mice prolonged their survival (136). Moreover, the spleen was recently identified as a relapse site for BCL-2-dependent T-ALL after treatment with ABT-199, a BCL-2 inhibitor, due to increased expression of IL-6, which altered BCL-2 dependence in the spleen microenvironment (137). More recently, the same team has additionally verified by RNA-seq that splenic T-ALL cells presented a more differentiated phenotype after ABT-199 treatment, which may additionally contribute for the decreased sensitivity of leukemic cells towards the targeted drug (138).

1.6.3 Thymic stroma supporting T-ALL

The role of the thymic microenvironment in T-ALL development has been suggested since the very first thymectomy experiments in T-ALL mouse models (139). In addition, previous reports have shown that mouse leukemic T-cell survival was improved when cocultured with thymic stromal cells (140). This revealed that signals from the thymus microenvironment are fundamental for leukemia maintenance. Besides, several patients with T-ALL/T-LBL

present an enlargement of the thymus at diagnosis, supporting the role of this microenvironment also in human T-cell leukemogenesis (12, 35, 141).

Within the stromal signals, it is known that IL-7, expressed in both thymic and bone marrow stroma, is important not only under physiologic conditions for normal T-cell development, but also in the malignant context (142-144). Indeed, it was shown that survival and proliferation of primary T-ALL cells was promoted by co-culture with TECs or BM-MSCs in an IL-7/IL-7R-dependent manner (145, 146). In particular, IL-7-mediated proliferation is associated with the upregulation of Bcl-2 expression and simultaneous downregulation of p27^{kip1} cdk inhibitor (147, 148). Moreover, AKR/J mice, characterized by spontaneous development of thymic lymphomas, present overexpression of IL-7R in thymocytes (149).

It was previously demonstrated that *Foxn1* haploinsufficiency delayed T-ALL/T-LBL development (150). Besides, *Foxn1*-deficient mice were shown to lack thymic expression of Notch ligand DLL4, chemokines CXCL12 and CCL25, and cytokine stem cell factor (SCF) (151). The FoxN1 transcription factor is an important regulator of TEC differentiation, whose downregulation is associated with thymic degeneration allied to loss of specific TEC subsets and decreased T-cell production (152). Therefore, it appears that TECs are responsible for the production of several stimulatory signals already identified in leukemogenesis (SCF), growth/survival (DLL4) and trafficking/proliferation (CXCL12, CCL25) of T-ALL cells (106, 107, 153-155). Nevertheless, reports from our group and other authors demonstrated that T-ALL tumorigenesis induces major alterations in the thymic epithelium and other stroma compartments. Indeed, thymic lymphomas from ETV6-JAK2 fusion transgenic (also known as TEL-JAK2; TJ2-Tg) and LN3 transgenic mice, exhibit an expansion of mTECs in detriment of cTECs, besides epithelial-free regions associated with leukemic cell expansion (150, 156). In particular, these proliferative sites were enriched in DCs, namely the Sirpα⁺ subset, fibroblasts and blood vessels (150, 156). Moreover, RelB-deficient mice, characterized by defects in thymic architecture, namely mTEC absence, experience a delayed TEL-JAK2-driven leukemia onset (74). The fact that RelB is expressed in several cell types found increased in thymic lymphomas suggests that besides TECs, other stromal cells within thymic tumor microenvironment (TME), may support leukemia survival/progression.

In summary, we can conclude that the crosstalk between leukemic cells and different microenvironments is fundamental for disease establishment and progression. Further investigation regarding communication between T-ALL cells and stroma may allow the discovery of novel molecular mechanisms and targets to fight leukemia.

2. Myeloid Cell Development and Their Involvement in Cancer

2.1. Myeloid cell ontogeny

Abundant evidence supports the critical role of stromal cells in T-ALL leukemogenesis and progression. Within the microenvironmental cells (epithelial, mesenchymal, endothelial, immune cells), there is one type in particular, the myeloid cells, whose relevance in both solid and hematological malignancies has been uncovered over the last years. Indeed, several studies demonstrated how these innate immune cells modulate the whole tumorigenic process, from tumor initiation to metastasis formation, as well as treatment responses, appearing as attractive therapeutic targets (157-165).

As mentioned before, BM-residing hematopoietic stem cells can proliferate and differentiate into common myeloid (CMPs) or lymphoid progenitors, under the influence of secreted regulatory cytokines and cellular interactions (87, 166, 167). In particular, CMPs originate megakaryocyte-erythrocyte progenitors (MEPs) and granulocyte-monocyte progenitors (GMPs) (Figure 5) (168). The latter give rise to granulocytes, including neutrophils, eosinophils, and basophils, and further differentiate into monocyte/macrophage and dendritic cell progenitors (MDPs), responsible for the generation of several monocyte/macrophage and DC subsets (166, 168). Nevertheless, recent evidence showed that mouse MDPs can be directly originated from CMPs and that both MDPs and GMPs produce functionally distinct monocytes (169). In the bone marrow, MDPs further differentiate into common monocyte progenitors (cMOPs) or common DC progenitors (CDPs) (166, 170). To note that besides the BM, monopoiesis can also occur in the spleen of adult mice under an inflammatory context, namely in cancer (171-173). Indeed, the adult spleen presents a pool of monocytes that is recruited to sites of infection to help resolve the inflammatory process (174).

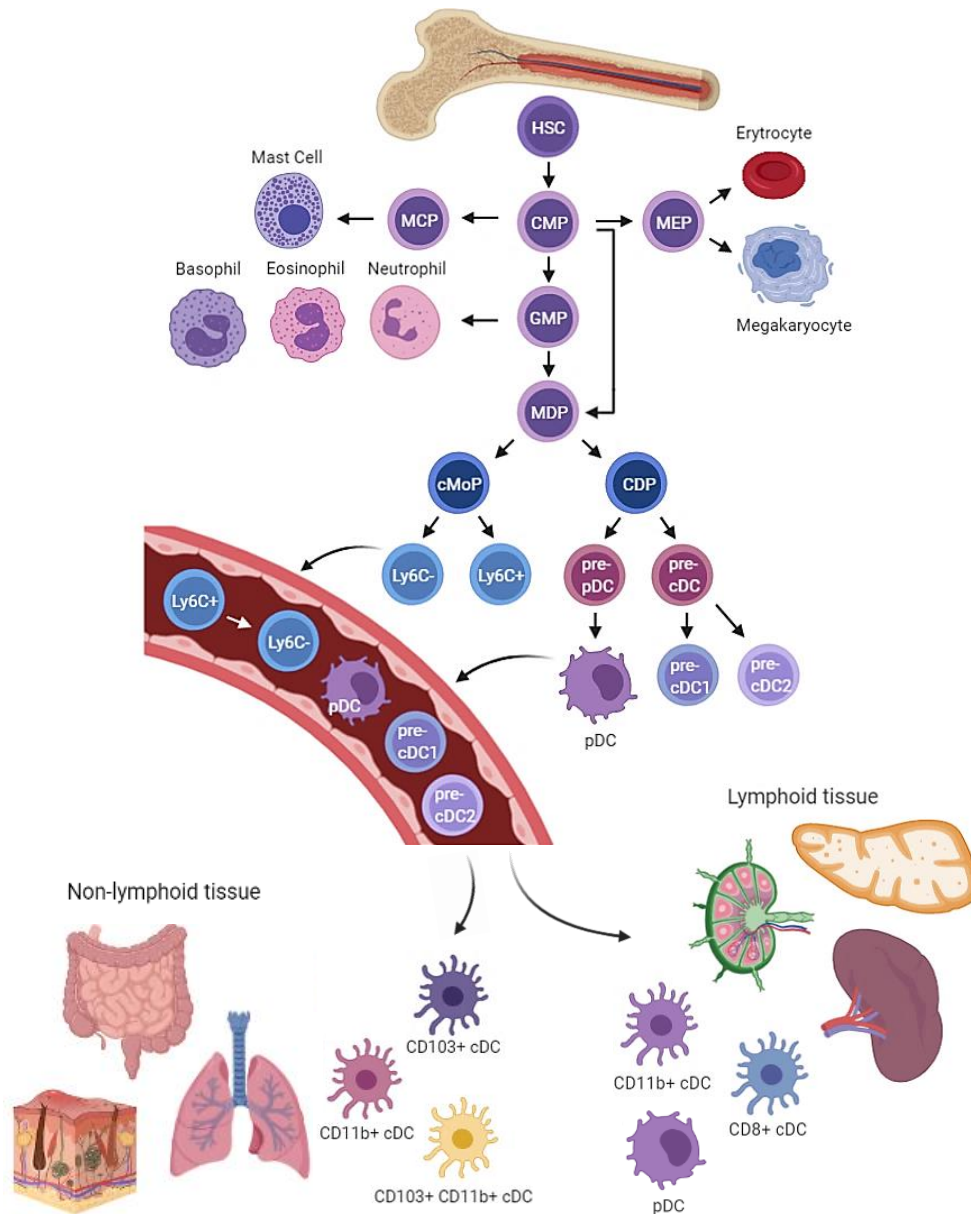


Figure 5. Myeloid cell differentiation in mice under steady-state conditions. The figure illustrates how HSC commit to the myeloid lineage, differentiating first into CMP and further giving rise to a network of myeloid progenitors responsible for generating erythrocytes, megakaryocytes (platelets), granulocytes and several monocyte/macrophage and dendritic cells subsets. Figure created with BioRender.com.

2.1.1 Monocyte differentiation and function

Newly generated monocytes exit the bone marrow and enter the circulation (166). They are divided into two major groups, both in humans and mice, with distinct functions: the CD14⁺ monocytes in humans, which resemble the Ly6C⁺ subset in mice (classical monocytes), and the CD16⁺ monocytes in humans, which are most similar to Ly6C⁻ monocytes in mice (non-classical monocytes) (167, 175-177). In response to inflammatory stimuli, Ly6C⁺ monocytes migrate from BM and spleen reservoirs to sites of infection, where they control microbial activity and posterior tissue repair, by generating monocyte-derived inflammatory

macrophages and DCs (e.g., TNF/ inducible nitric oxide synthase (iNOS)-producing DCs - TipDCs) (166, 167, 170, 176, 177). The Ly6C⁻ monocytes, also known as patrolling or resident monocytes, act as “intravascular housekeepers” due to continuous crawling along the resting endothelium. As such, they are responsible for endothelium repair during homeostasis through recruitment of neutrophils, and have previously been associated with early immune responses (170, 175-178). Recent evidence showed that under steady-state conditions, circulating Ly6C⁺ blood monocytes spontaneously give rise to Ly6C⁻ blood monocytes, acting the former as the major modulator of Ly6C⁻ subset lifespan in the bloodstream (Figure 5) (168, 170, 179). In addition, Ly6C⁺ monocytes also manage the renewal of tissue-resident macrophages in the intestine (180), pancreas (181), dermis (182) and heart (183).

2.1.2. Dendritic cell differentiation and function

Regarding the DC development pathway, CDPs originate both plasmacytoid (pDC) and conventional/classical (cDC) dendritic cell-restricted progenitors in the bone marrow (pre-pDCs and pre-cDCs, respectively) (Figure 5) (184). While pre-pDCs produce pDCs, pre-cDCs commit to CD8 α ⁺ (CD103⁺) cDC1 (pre-CD8⁺ DC) or CD11b⁺ cDC2 (pre-CD11b⁺ DC) lineage in the marrow before differentiating into mature CD8 α ⁺ and CD11b⁺ cDCs, respectively, in lymphoid organs (thymus, spleen, and lymph nodes (LNs)) (184, 185). In particular, LNs also contain tissue migratory cDCs, which are DCs that have migrated from non-lymphoid tissue towards peripheral LNs, through the lymphatic system (186). Pre-cDCs can also migrate to non-lymphoid tissues (e.g., liver, lungs, skin, kidneys, intestine) to generate CD103⁺CD11b⁻ (resembling the CD8⁺ cDCs present in the lymphoid organs) and CD11b⁺ cDCs (186-188). Moreover, both pre-cDCs and CDPs were shown to generate intestinal CD103⁺CD11b⁺ lamina propria (LP) DCs while Ly6C⁺ monocytes can give rise to CD103⁻CD11b⁺ LP DCs (187, 189). Even though it is widely accepted that DCs derive from CMPs, there are reports showing that in both human and murine hematopoiesis, CLPs can also originate DC subsets (190-192).

Concerning DCs function, while cDC1s are excellent antigen-presenting cells that efficiently induce CD8⁺ cytotoxic T-cell responses, cDC2s are specialized in polarizing CD4⁺ T cells towards T helper 1 (Th1), 2 (Th2) and 17 (Th17) effector cells, promoting both humoral (antibody production via B-cell activation) and cell-mediated immune responses (193-197). Immature cDCs can also migrate from peripheral tissues to lymphoid organs during homeostasis to induce immune tolerance by cross-presenting self-antigens to T cells (194).

pDCs in turn, produce large amounts of type I interferon (IFN) (IFN- α/β) in response to viral infections, which stimulates both innate and adaptive immunity (through secretion of

inflammatory cytokines, enhanced antigen cross-presentation and activation of macrophage phago-cytotoxic activity) (193, 198).

2.2 The fundamental role of the myeloid compartment in cancer

Cancer is seen as a combination of several components, including neoplastic, stromal and immune cells that dynamically communicate with each other, determining disease progression and response to therapy. Myeloid cells in particular, play a major role in the neoplastic niche. They comprise several cells from the innate immune system, including granulocytes, dendritic cells, monocytes/macrophages, and myeloid-derived suppressor cells (MDSCs) (158-162, 199, 200). The latter constitute a very heterogeneous population of cells whose numbers are increased under pathological conditions, such as chronic inflammation, cancer, trauma and autoimmune disorders. In particular, MDSCs act as key players of immune suppression in cancer, besides contributing to tumor angiogenesis, metastasis, and drug resistance (159, 199).

Over the last 20 years, several studies have revealed how the myeloid compartment in cancer is critical for both tumor development and progression (158, 160-162, 200). Indeed, tumor and stromal cells secrete soluble factors that drive in parallel tissue-accumulation of myeloid cells and abnormal myelopoiesis (e.g., CCL2, CCL12, CXCL5, granulocyte–macrophage colony-stimulating factor (GM-CSF), SCF, IL-3, vascular endothelial growth factor (VEGF)). The recruited immature cells undergo an aberrant differentiation process that leads to the production of MDSCs, tumor-associated macrophages (TAMs) and immunosuppressive DCs (158).

2.2.1. Myeloid-derived suppressor cells in cancer

MDSCs, as mentioned before, do not constitute a well-defined subset of myeloid cells, and include both myeloid precursors and immature cells (201). These cells are produced in the BM, from CMPs, and are divided into two major groups, depending on phenotypic and morphological similarities with neutrophils or monocytes: polymorphonuclear MDSCs (PMN-MDSCs) and monocytic MDSCs (M-MDSCs), respectively (159). In tumor-bearing hosts, both MDSC subsets migrate to peripheral organs and tumor tissues, where they initiate different immunosuppressive mechanisms (Figure 6). For most solid tumors, peripheral organs are populated with increased PMN-MDSCs, which induce antigen-specific, contact-dependent, T-cell tolerance mainly through production of ROS and nitrogen species such as nitric oxide (NO) (which inhibit TCR-mediated T-cell activation (202, 203)), depletion of major T-cell nutrition factors (L-arginine (204), L-cysteine (205), L-tryptophan (206)) and blocking of T-cell homing. Also, PMN-MDSCs induce Tregs differentiation. Within the tumor hypoxic niche, M-MDSCs are more predominant than PMN-

MDSCs, and mediated by HIF-1 α these cells induce non-specific T-cell tolerance through production of high amounts of NO and arginase 1 (Arg1) (promotes L-arginine hydrolysis, impairing T-cell survival (207, 208)), increased programmed death-ligand 1 (PD-L1) surface expression, and recruitment of Tregs via release of chemo-attractant chemokines (CCL4 and CCL5). Moreover, hypoxia in the tumor site promotes downregulation of STAT3 activity, which causes differentiation of M-MDSCs to TAMs (209). Other soluble factors, such as IL-6 and leukemia inhibitory factor (LIF) also promoted TAM differentiation (158, 159, 199, 201). Regarding hematological malignancies, MDSCs are also associated with similar immunosuppressive and immune evasion mechanisms (210).

2.2.2. Macrophages

Macrophages, as mentioned before, can be originated from circulating monocytes. Nevertheless, part of the adult tissue-resident macrophages originates from embryonic progenitors (yolk-sac and fetal liver-derived macrophages) capable of sustaining a macrophage pool in the liver (Kupffer cells), lungs (alveolar macrophages), skin (Langerhans cells) and other organs (heart, pancreas, gut, etc.), during adulthood. The microglia (brain macrophages) are the exception since they are thought to be originated predominantly from yolk-sac macrophages (170, 211, 212). Classically, macrophages are cells specialized in the recognition, phagocytosis and elimination of pathogens. They modulate inflammation through secretion of pro-inflammatory cytokines and by acting as APC, promoting antigen-specific (adaptive) immune responses. Moreover, macrophages contribute for homeostasis maintenance through removal of cellular debris and tissue repair (213).

2.2.2.1. Macrophages and cancer

In cancer, macrophages that infiltrate tumor tissues are denominated TAMs (214). These macrophages can display basically two major phenotypes, determined by stimuli from the environment: M1 or “classically activated” macrophages with anti-tumorigenic properties, and M2 or “alternatively activated” macrophages with pro-tumorigenic behavior. M1 macrophages arise from the interaction with microbial/viral molecules (TLR ligands) and IFN γ , whereas M2 macrophages are activated by interleukins (IL-4, IL-10, IL-13), glucocorticoids, and transforming growth factor beta (TGF- β) (158, 211). Anti-tumorigenic TAMs secrete IFN γ , express high levels of MHC class II and costimulatory proteins, and are great activators of Th1 cells, which in turn produce IFN γ and IL-12, creating a positive feedback loop. Further, they enhance NK activity through production of IL-18 and IL-22 (215) (158, 211). On the other hand, TAMs with pro-tumorigenic potential highly express mannose (CD206) and hemoglobin scavenger (CD163) receptors, secrete Arg1 and

produce a subset of chemokines (CCL17, CCL22, CCL24) that attract Tregs, Th2 cells, and granulocytes, favoring an immunosuppressive environment (Figure 6) (158, 211).

Several reports revealed that macrophages, in both human and mice, are modulated under malignant transformation to enable tumor survival and progression (216-219). Moreover, a high number of TAMs, was significantly associated with poor prognosis in patients with solid (lung, breast, oral, head and neck, thyroid, kidney, liver, bladder, pancreas, ovaries) or liquid tumors (Hodgkin lymphoma) (220-223). On the contrary, in gastric and colorectal cancer, an increased content of TAMs was associated with improved survival (163, 220, 223). Part of this discrepancy may be justified by the fact that these studies are based on CD68 expression, a fairly specific marker for murine macrophages, but in humans it may also identify granulocytes, fibroblasts and DCs (211). Indeed, an expression profiling study revealed that TAMs with a specific gene signature are related to primary treatment failure in Hodgkin lymphoma patients (222). Similarly, in follicular lymphoma, a transcriptomic analysis identified a relevant gene signature predictor of inferior outcome in these patients, that comprised genes expressed by macrophages and/or dendritic cells (224). Therefore, TAM activation profile seems to be a more reliable prognostic factor than their density (211).

In most tumors the majority of TAMs are M2-like and besides contributing for a more immunosuppressive environment that favors tumor growth, they also promote malignant cell egress from the primary site by increasing angiogenesis (225, 226), remodeling the extracellular matrix (227, 228) and improving tumor cell mobility/invasion capacity (229) (158, 162, 230). Moreover, TAMs were also associated with tumor resistance to several standard treatments, including chemotherapy, radiotherapy and angiogenic antagonists (165, 231, 232). Thus, several therapeutic approaches targeting important TAM modulators, namely colony-stimulating factor 1 (CSF-1 or M-CSF), which is fundamental for TAM recruitment and polarization towards a pro-tumorigenic phenotype, have been tested and shown improved survival as monotherapy or synergistically with other modalities, in both humans and mice (220, 233-240).

2.2.3. Dendritic cells

Dendritic cells, as previously mentioned, are myeloid cells specialized in the processing and presentation of antigens, modulating immune responses (158, 211). The big majority of DCs arises from BM progenitors (Figure 5) and migrate to peripheral tissues, where they encounter antigens. Without previous stimulation, DCs cannot function as APCs (158, 211), so they undergo a maturation process upon detection of specific stimuli, via pattern recognition receptor (PRR) sensing of pathogen-associated (PAMPs) or damage-associated molecular patterns (DAMPs). This results in the upregulation of MHC and co-

stimulatory molecules (CD80, CD83, and CD86) at DC surface, as well as enhanced migratory and secretory capacity, which allow DCs to become strong activators of T cells and other immune cells (241). Nevertheless, the immature DCs that are not stimulated can contribute for immune tolerance, inducing Treg differentiation (200, 241). Therefore, DCs are important modulators in balancing tolerance and immunity.

2.2.3.1 Dendritic cell role in cancer immunity

In cancer immunity, evidence showed different roles for each DC subtype. It is well-established that cDC1s have anti-tumor behavior (242, 243). Indeed, several studies have shown a correlation between higher expression of cDC1-associated transcripts and overall survival in breast cancer, lung adenocarcinoma, melanoma, and head and neck squamous cell carcinoma (200). They comprise both CD8 α ⁺ lymphoid resident and CD103⁺ migratory DCs. Earlier reports have shown that in the absence of Baft3 transcription factor, mice lack both CD8 α ⁺ and CD103⁺ cDCs, which results in defective priming of CD8⁺ T cells (244, 245) and impaired tumor rejection (245). In particular, CD103⁺ cDCs play a critical role in anti-melanoma immunity, both in human and mice. These cells are able to actively migrate and carry tumor-derived antigens from peripheral tissues towards draining LNs, in a CCR7-dependent manner (246). When in the LNs they induce direct CD8⁺ T-cell stimulation and promote antigen handover to resident myeloid cells. Moreover, CCR7 levels in human melanoma correlate positively with intra-tumoral T-cell infiltration and patient survival (246). Moreover, it was recently shown how CD103⁺ cDCs can affect the efficiency of different cancer therapies. Spranger *et al.* demonstrated that intra-tumoral CD103⁺ cDCs are major producers of CXCL9 and CXCL10, which are required for effector T-cell migration towards the tumor and adoptive T-cell therapy (247). Likewise, Chow *et al.* revealed that CXCL9 production by tumor-residing CD103⁺ cDCs improved anti-PD-1 treatment responses (248).

Regarding cDC2s, there is scarce information about their role in cancer immunity. They have low migratory capacity and are poor stimulators of CD8⁺ T cells. Nevertheless, intra-tumoral cDC2s appear to be specialized in modulating CD4⁺ T-cell-mediated immune responses via MHC class II antigen presentation. Binnewies *et al.* (249) have shown that cDC2s promote Th1 CD4⁺ T-cell priming, and consequently tumor rejection in mice, when Tregs were depleted. Likewise, in melanoma patients, a lower density of Tregs was associated with higher infiltration of CD4⁺ T cells and with anti-PD-1 responsiveness (249). In addition, Laoui *et al.* demonstrated that within tumor-associated DCs (TADCs), cDC2s were able to promote Th17 differentiation (250). Although Th17 role in cancer immunity remains controversial, recent work suggests that its pro- or anti-tumor activity depends on several factors, including cancer type, inflammatory stimuli within the tumor

microenvironment, interaction with other immune cells (e.g., Tregs and CD8⁺ T cells), and therapeutic approaches (251). Nevertheless, an experiment in which mice were vaccinated with tumor-derived cDC2s (without previous stimulation *ex vivo* or loaded with antigens) demonstrated that these cells were able to impair tumor growth, modify TAMs phenotype into M1 type and decrease MDSC levels (250). Overall, it appears that cDC2s can also play an anti-tumorigenic role in cancer.

Despite being highly efficient in inducing pro-inflammatory responses (252), pDCs have been associated with poor prognosis in several cancers, including breast (253, 254), ovarian (255), melanoma (256) and head and neck tumors (257). Indeed, pDCs harbor an antigen processing and presentation machinery (expression of MHC class II, costimulatory molecules and TLR receptors) and are major producers of type I IFN (252). Nevertheless, within the TME, pDCs appear to be poorly activated and mainly instructed to produce TNF ligand superfamily member 4 (TNFSF4) and inducible T-cell co-stimulator ligand (ICOSL), which results in CD4⁺ T-cell differentiation into Th2 and Tregs, respectively. Thus, this phenotype contributes for tumor escape from immunosurveillance and is associated with early relapse in melanoma patients (258).

Finally, monocyte-derived DCs (moDCs), also known as inflammatory DCs, originated in the context of inflammation or cancer, have the capacity to efficiently activate both CD4⁺ and CD8⁺ T cells, being further associated with treatment success in several malignancies (259-262). Indeed, there are many undergoing clinical trials assessing the clinical benefit of using moDC-based immunotherapy as monotherapy or in combination with other cancer therapies (263). On the other hand, intra-tumoral moDCs can also behave as immunosuppressive DCs that highly express TNF- α , iNOS, and IL-10, and impair T-cell proliferation *ex vivo* (250). Therefore, similar to pDCs, moDCs can also display antagonist roles in cancer immunity. Having that in mind, further investigation is required to better understand the influence of TME in skewing DC phenotype.

2.2.3.2. The tumor microenvironment modulates DC functional fate and antitumor activity

It is well known that the TME suppresses DC activation and/or maturation, as well as modulates DC differentiation (Figure 6). This can lead to three possible scenarios: an increased number of intra-tumoral immature DCs; a decline of fully competent mature DCs at the tumor site; and predominance of an immunosuppressive phenotype in tumor-associated DCs. There are several soluble factors secreted within the TME that affect DC differentiation. For instance, VEGF produced by tumor cells impairs DC maturation via blockade of NF- κ B activation in hematopoietic progenitor cells (264). Similarly, tumor-released CSF-1 and IL-6 inhibit DC differentiation by triggering CD34⁺ progenitors to

differentiate instead into immunosuppressive monocytes (265). Moreover, tumor-secreting agents, such as TGF- β , can also directly suppress DC anti-tumor activity, by hindering DC antigen uptake and stimulation of tumor-sensitized T cells *in vitro* (266). In particular, inhibition of TGF- β signaling in tumor-bearing mice has shown enhanced results in immunotherapy treatments (266-268).

On the other hand, the TME metabolism also contributes to DC dysfunction in cancer (259, 269). Growing tumors are characterized by low levels of oxygen, increased concentration of lactate/lactic acid, low pH and accumulation of adenosine, that impair DC functionality (158, 259, 269). In particular, tumor-derived lactic acid was shown to hamper DC differentiation from monocytes in a 3D-tumor model. Gottfried *et al.* demonstrated that when monocytes were cocultured with multicellular tumor spheroids in the presence of GM-CSF and IL-4, they gave rise to a specific type of TADCs, expressing low levels of CD1a and secreting low amounts of IL-12 (270). When lactic acid production was inhibited, the immunosuppressive phenotype induced by TME was reverted to normal. Likewise, in the absence of lactic acid, DCs induced more strongly CD8⁺ T-cell proliferation (270).

Moreover, lipid accumulation in DCs constitutes one of the major mechanisms for DC dysfunction in cancer. Indeed, DCs isolated from tumor-bearing mice and cancer patients revealed high levels of lipids, which was associated with cross-presentation impairment (271-273). In particular, the treatment of mice with radiation-induced thymic lymphomas using an inhibitor of acetyl-CoA carboxylase promoted the normalization of lipid levels and restored DC normal function (273).

More recently, the role of oncogenic pathways in TME modulation was also described, affecting mainly tumor-infiltrating immune cells (274). For instance, the activation of WNT- β -catenin signaling in a melanoma mouse model was associated with decreased production of intra-tumoral CCL4, which resulted in significant decrease of cDC1 recruitment to the tumor site, and consequently defective priming of antigen-specific T cells (275). Likewise, in tumors with elevated COX activity, there is increasing production of prostaglandin E2 (PGE2), which is associated with lower NK cell and cDC1 accumulation at the tumor site, contributing for tumor immune evasion (276).

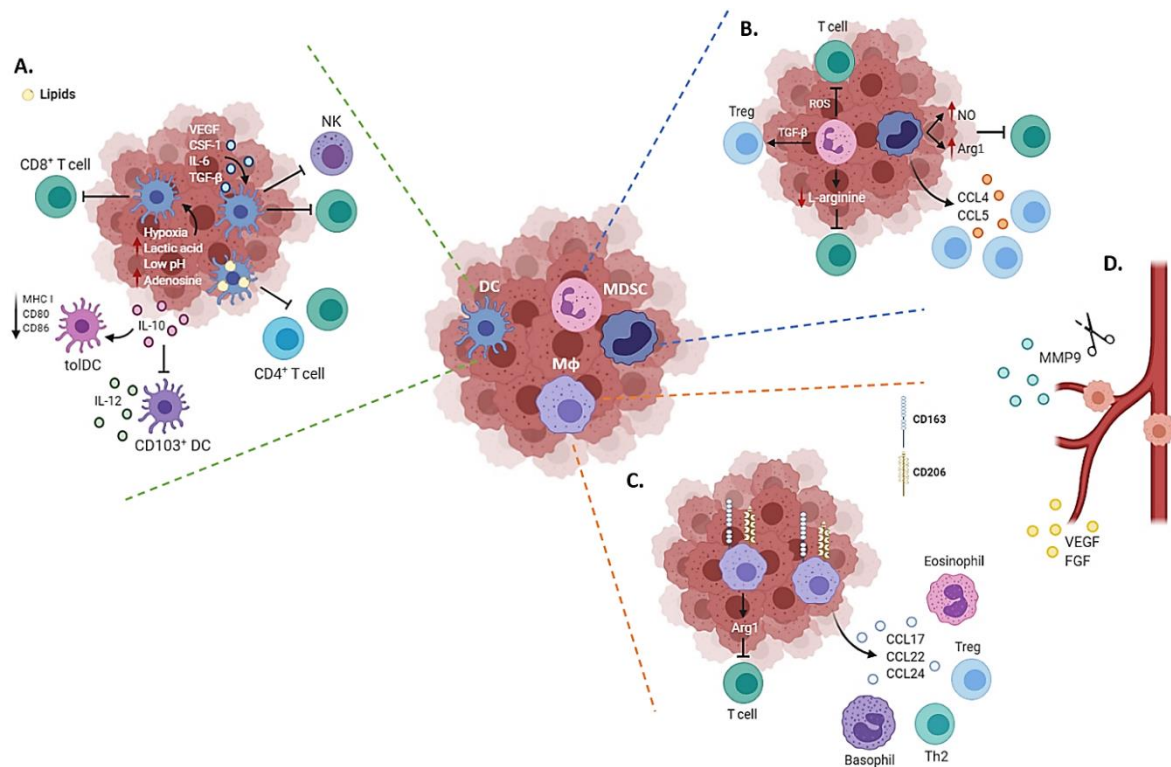


Figure 6. Schematic representation addressing myeloid subsets pro-tumoral activity. This figure summarizes some of the mechanisms described so far concerning the bidirectional crosstalk between cancer cells and intra-tumoral myeloid cells (MDSCs, M2-like macrophages (Mφ; TAMs), tumor-associated dendritic cells), that strongly contributes for tumor survival and progression. **(A)** TME metabolic changes (including lipid accumulation) impair DC functionality. Besides, several tumor-secreting factors, such as VEGF, TGF-β, IL-6, and CSF-1 impair DC differentiation or directly suppress DC activity, compromising both innate and adaptive immunity. Further, TME-derived IL-10 induces differentiation of tolerogenic DCs (tolDCs) (decreased MHC-I, CD80 and CD86 expression and poor stimulatory activity) (277) and inhibits IL-12 secretion by CD103⁺ DCs, required for stronger cytotoxic effect (238). **(B)** MDSCs promote tumor progression through antigen-specific or non-specific suppression. They produce increased levels of ROS, Arginase 1 and NO that inhibit effector T-cell immune responses. Further, MDSCs induce Treg differentiation via TGF-β or even recruit Tregs to tumor site. **(C)** TAMs produce Arginase 1, which inhibits T-cell proliferation, and recruit Th2, Tregs and granulocytes via chemokine secretion (CCL17, CCL22, and CCL24), reinforcing the pro-tumorigenic environment. **(D)** Both TAMs and MDSCs are associated with tumor angiogenesis and metastasis induction via VEGF, FGF and MMP9 expression. Figure created with BioRender.com.

3. Myeloid cell involvement in T-ALL

3.1 Myeloid-derived suppressor cells in ALL

Studies addressing MDSC role in leukemia have been relatively limited comparing to other hematological malignancies, such as lymphoma or multiple myeloma (278). Nevertheless, and regarding ALL, reports have shown an increase of PMN-MDSCs in the PB and BM of pediatric B-ALL patients, whose frequency was correlated with both prognosis and therapeutic response (279, 280). Moreover, patients in remission presented a decrease in

PMN-MDSC numbers accompanied by loss of their immunosuppressive phenotype, which validates the capacity of PMN-MDSCs to modulate immune surveillance in B-ALL (280). Regarding T-ALL, no significant data was found, which means further studies are required to determine the potential MDSC impact on T-cell leukemia.

3.2. Macrophage role in T-ALL survival and progression

Similar to what happens in solid tumors, there is increasing evidence underlining the relevance of TAMs in hematological malignancies (281, 282). These are denominated leukemia-associated macrophages (LAMs), and appear to behave as M2-like macrophages, since the depletion of resident monocytes and macrophages, or the repolarization of LAMs with M1 features delay leukemia progression (283, 284). Nevertheless, depending on the organ and even within the organ, LAMs can present different phenotypic and functional characteristics (284-287). Indeed, in a Notch1-induced T-ALL mouse model, BM and splenic LAMs presented different gene expression profiles, but both induced T-ALL proliferation *in vitro*, although not to the same extent (285). Splenic LAMs were able to stimulate more strongly leukemic cells migration, which underlies the relevance of the organ-specific microenvironment on LAMs (285). More recently, the same authors revealed that Notch1-driven T-ALL splenic LAMs have more M2 characteristics while BM LAMs have more M1 features, which may explain the difference found in LAMs capacity to induce leukemic cell proliferation *in vitro* (284).

Using the same model, Yang *et al.*, have shown gradual hepatomegaly during T-ALL progression in mice (286). Indeed, hepatosplenomegaly is frequently found in T-ALL patients, being associated with poor prognosis (288, 289). This condition results from inflammatory cell infiltration in the liver, during leukemia development, driven in part by increased levels of hepatic LAMs bearing an M1 pro-inflammatory phenotype (286). Peritoneal dissemination of cancer cells was previously described and its effect on tumor progression appears to be modulated by peritoneal macrophages (290-292). Chen *et al.* demonstrated how leukemic cells accumulated in the peritoneal cavity of Notch1-induced T-ALL mice as leukemia evolved (287). Moreover, peritoneal LAMs were divided in two subsets, CD206⁻ and CD206⁺, both with M1 and M2 features. Upon T-ALL cells invasion in the peritoneal cavity it was observed a decrease of CD206⁻ LAMs and an increase of CD206⁺ subset, simultaneously (287). This may suggest that leukemia microenvironment modulates macrophage subsets proportion to favor disease progression. These studies highlight the phenotypic and functional diversity of LAMs and its contribution for tumor progression.

Regarding monocyte/macrophage impact on T-ALL survival, Triplett *et al.* have shown that when cocultured with TME stroma lacking myeloid cells (CD11b⁺CD11c⁻), LN3 mouse T-ALL cells suffered a striking decreased in cell viability (156). Moreover, sorted tumor-infiltrating CD11b⁺ myeloid cells were sufficient by themselves to ensure T-ALL survival *in vitro* (156). Conversely, Yang *et al.* have recently demonstrated that Notch1-driven T-ALL mice exhibit a significant increase of monocyte-derived Ly6C⁺ LAMs in extramedullary organs, which contributed for tumor-associated sterile inflammation (293). In turn, mice deficient for Ly6C⁺ LAMs exhibited an alleviation of sterile inflammation and reduced numbers of T-ALL cells in extramedullary tissues (293). Moreover, the authors revealed that monocyte-derived Ly6C⁺ LAMs express high levels of CCL8 and CCL9/10, and upon the blockade of its receptors, CCR1 and CCR2, hepatosplenomegaly was relieved, and T-ALL extramedullary distribution was inhibited (293). These data accentuate the critical role of myeloid compartment in supporting T-ALL survival and dissemination.

The knowledge regarding LAMs role in T-ALL development and progression remains deficient, especially their role *in vivo*. Therefore, it becomes necessary to determine the function of LAMs *in vivo* and, more importantly, to discover new biological mechanisms and possible therapeutic targets of clinical value. Lyu *et al.* have recently shown that the depletion of myeloid cells *in vivo*, using two different T-ALL mouse models, led to a significant lower leukemia burden in several organs and increased mouse survival (294). Further, this survival effect was dependent on signals provided by myeloid cells, namely macrophages, directly to T-ALL cells. Moreover, transcriptomic and functional analyses revealed that myeloid cell promotion of T-ALL survival was, in part, mediated by activation of insulin-like growth factor 1 receptor (IGF1R) signaling (294). Likewise, PB monocytes or monocyte-derived macrophages supported primary patient T-ALL cells survival *in vitro*, but only macrophages promoted T-ALL survival through IGF1R activation (294). This suggests the existence of additional survival signals/mechanisms provided by myeloid cells to support T-ALL. Moreover, the analysis of transcriptional datasets from pediatric T-ALL patients samples revealed a correlation between enriched macrophage gene signatures and lower event-free survival (294). A similar pattern was observed for monocyte gene signatures.

Our group has previously demonstrated the presence of macrophages in the thymic lymphoma microenvironment of TEL-JAK2 mice (150). Given all the evidence underlining the fundamental role of LAMs in leukemia progression, it would be interesting to uncover the role of LAMs in TEL-JAK2 leukemogenesis *in vivo*.

3.3. Dendritic cell impact on T-ALL development

In most hematological malignancies DCs appear to be decreased or dysfunctional (295). In ALL, in particular a significant decrease in circulating PB myeloid DCs (mDCs) (analogous to cDCs in mice) and pDCs was reported in both pediatric and adult B-ALL patients, while in T-ALL patients, mDC levels were increased in comparison with healthy donors (296, 297). Further, in B-ALL, DC differentiation appears to be impaired, which may contribute to poor anti-tumor immune responses, whereas in T-ALL, DCs exhibit a functional phenotype, which suggests that the T-ALL TME may be modulating DCs in its favor (296, 297). More recently, Valencia *et al.* have confirmed those findings for B-cell leukemia by demonstrating that conditioned medium from B-ALL cells induced the differentiation of human PB monocytes into immunosuppressive moDCs (298). They further revealed that B-ALL cells overexpress bone morphogenetic protein 4 (BMP4), which improved their capacity to generate DCs with immunosuppressive phenotype and M2-like macrophages. Thus, BMP4 might be in part responsible for modulating DC and macrophage differentiation in ALL (298).

Regarding T-ALL, and in accordance with findings from Maecker *et al.* (297), a recent study from Triplett *et al.* has shown that LN3 thymic lymphomas exhibit an increase in both number and percentage of DCs, namely Sirp α^+ DCs (CD11b $^+$ cDCs) (156). In addition, co-cultures of primary T-ALL cells with different tumor-derived stromal cells revealed that DCs were required and sufficient to support T-ALL survival and growth, at both primary and secondary tumor sites. The authors further revealed that DCs support T-ALL survival *ex vivo* via activation of IGF1R signaling (156). Similarly, our group has shown that TEL-JAK2 transgenic mice display an enrichment of DCs within the thymic TME, which is supported by an increased transcriptional activity of DC-related genes, namely concerning the Sirp α^+ subtype, underlining the need to further explore the role of lymphoma-associated DCs in TJ2-Tg leukemogenesis (150).

In line with this, recently, Lyu *et al.* investigated if myeloid cell depletion *in vivo* would affect T-ALL progression as suggested by *in vitro* experiments (294). For that, the authors transplanted mouse T-ALL cells into secondary hosts and performed systemic myeloid cell depletion using two different models (clodronate liposomes or diphtheria toxin-based system – CD11b-DTR transgenic mice). Surprisingly, DC deficiency alone did not delay T-ALL onset. Instead, the depletion of multiple myeloid subsets *in vivo* impaired T-ALL engraftment and prolonged mouse survival (294). This suggests that other leukemia-associated myeloid cells, such as monocytes and macrophages, strongly contribute for T-ALL survival. Still, cDC2s from T-ALL mice and PB monocyte-derived DCs from T-ALL patients were able to support T-ALL *in vitro* (294). This indicates that even though DCs are

not the major player supporting mouse T-ALL *in vivo*, they are part of the main players contributing for T-ALL progression.

Given all the information collected so far, it would be interesting to investigate the role of myeloid cells in different T-ALL mouse models to confirm if myeloid cell-mediated support indeed is fundamental for T-ALL survival/progression *in vivo*. More specifically, it would be interesting to investigate which myeloid cell subsets play the most T-ALL supportive role and through which molecular signals/signaling pathways.

4. Aims

As previously mentioned, the leukemia microenvironment seems to be critical for the malignant transformation of thymocytes and T-ALL/T-LBL progression. Therefore, it is essential to identify key molecular signals yielded by stromal cells, not only to further understand the mechanisms behind T-cell leukemia/lymphoma development, but also for the possibility of using them as therapeutic targets.

In particular, *in vitro* studies strongly suggest that myeloid cells, namely tumor-associated macrophages and DCs, have a fundamental role in T-ALL survival and progression. Our group has already revealed by immunofluorescence and flow cytometry analyses that TJ2-Tg mice exhibit an increased DC content in the thymic lymphoma microenvironment, alongside with macrophages presence (150). Nevertheless, the function of these cells *in vivo* remains to be elucidated.

In line with this, we hypothesized that the myeloid cells within the tumor microenvironment might play an important role in TJ2-Tg leukemogenesis, *in vivo*. Therefore, we aimed to deplete myeloid cells in the TEL-JAK2 transgenic mouse model using a diphtheria toxin-based system (LysM-DTR mice) and evaluate how myeloid deficiency affects leukemia time onset, disease severity and mouse survival. In addition, we aimed to extract RNA from tumors for future analysis of gene expression and identification of myeloid-expressed genes associated with leukemia.

MATERIALS AND METHODS

1. Mouse models, housing and monitoring conditions

Mice were bred and kept at i3S Animal Facility in a specific pathogen-free environment, under a 12 h light/dark cycle with water and food *ad libitum*. A maximum of 5 or 12 animals of the same sex were housed together in conventional type II or type III cages, respectively. All the experiments were performed on C57BL/6 background. The LysM-Cre (B6.129P2-Lyz2tm1(cre)lfo/J) strain was provided by Dra. Salomé Gomes (i3S) while the ROSA26-loxP-STOP-loxP-DTR (R26iDTR; C57BL/6-Gt(ROSA)26Sortm1(HBEGF)Awai/J) strain was acquired from The Jackson Laboratory. LysM-Cre/R26iDTR mice were generated by crossing LysM-Cre transgenic strain with R26iDTR mice. LysM-Cre mice were also crossed with ROSA26-loxP-STOP-loxP-eYFP (R26EYFP) reporter strain.

The EμSRα-TEL-JAK2 (Tg(Emu-ETV6/JAK2)#Ghy; TJ2-Tg) transgenic mice were already described (299), and were always generated with a single copy of the transgene. Upon crossing of TJ2-Tg mice with LysM-Cre/R26iDTR strain, we obtained TJ2-Tg/LysM-Cre[+]R26^{+iDTR} and TJ2-Tg/LysM-Cre[-]R26^{+iDTR}, as control.

Mice were frequently monitored for signs of disease such as lack of mobility, weakness and loss of weight and tail tonus. Pathological manifestations of leukemia/lymphoma development include enlarged and palpable lymph nodes, abdomen swelling, and dyspnea. Humane endpoints were defined to minimize distress and ensure animal welfare. When several of these manifestations were observed, the animals were euthanized by CO₂ inhalation. Blood and organs were collected for further analysis. All the procedures were approved by *Direcção Geral de Alimentação e Veterinária (DGAV)* and performed in accordance with Portuguese (Decreto-lei nº 113/2013) and European legislation (Directive 2010/63/UE).

2. Mouse genotyping

DNA was extracted from ear (before weaning) or heart (after euthanasia) using proteinase K (20 mg/mL) together with lysis buffer (200 mM NaCl, 100mM Tris HCl, 5 mM EDTA, 1% Triton X-100) and incubated overnight, at 55°C. The next day, proteinase K activity was inactivated by 90°C incubation during 10 min. The samples were centrifuged (VWR Micro Star 17R) at 17 000 g, for 15 min, at room temperature (RT), being the supernatant then mixed with 500 µl of isopropanol in a new eppendorf. The samples were centrifuged at 17 000 g for 10 min, the supernatant discarded by inversion and 500 µl of ethanol 70% was added to the tubes. After mixing well, the samples were centrifuged for 3 min at 17 000 g, the supernatant excluded by inversion and the DNA was left to dry on air for 20 min. Finally,

DNA was dissolved in 200 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1mM EDTA) and stored at -20°C.

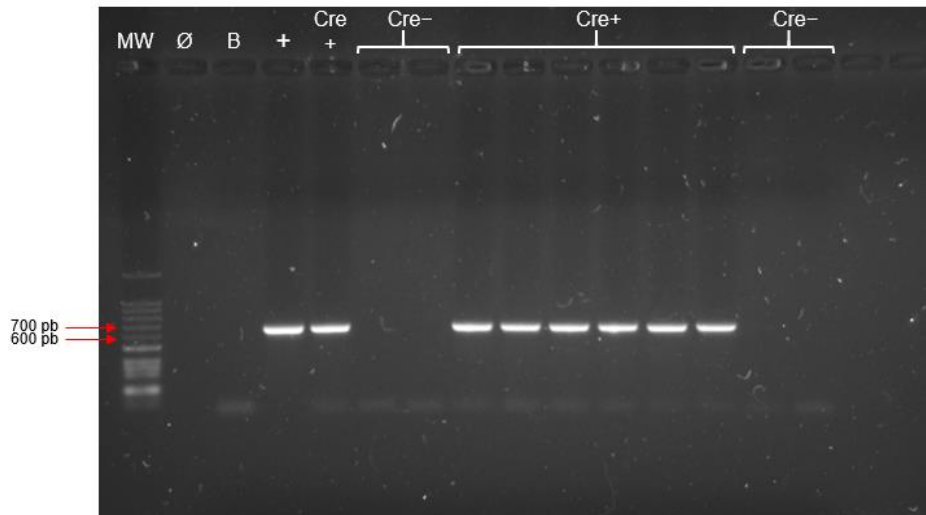
For amplification of both Cre recombinase and TEL-JAK2 transgenes, a total volume of 25 µl was used in PCR reaction containing nuclease-free water, Dream Taq Buffer (Thermo Fisher), 1.5 mM of MgCl₂, 0.2 mM of dNTP, 0.5 mM of forward and reverse primers (Integrated DNA Technologies), 2 U of Dream Taq DNA polymerase (Thermo Fisher) and DNA sample. PCR reactions were performed in a BioRad T100 Thermal Cycler. Samples were run in a 1% Agarose gel at 140V for 30 min. The primer sequences and size of the expected PCR products are indicated in Table 2 while PCR programs used for each protein are described in Table 3.

Table 2. Primers sequences and expected PCR product for Cre Recombinase and TEL-JAK2 proteins.

Primer name	Primer sequence	Expected PCR product
Cre-3F	5' CGG TCG ATG CAA CGA GTG ATG AGG 3'	642 bp
Cre-3R	5' CCA GAG ACG GAA ATC CAT CGC TCG 3'	
TEL-JAK2 +	5' GGG AAG GGA AGC CCA TCA ACC 3'	441 bp
TEL-JAK2 —	5' CCG CAC TGT AGC ACA CTC CC 3'	

Table 3. PCR cycling conditions for Cre Recombinase and TEL-JAK2.

		Cre Recombinase		TEL-JAK2	
		Time	Temperature	Time	Temperature
Steps	Initial Denaturation	8'	95°C	5'	94°C
	Denaturation	30"	94°C	45"	94°C
	Annealing	45"	60°C	45"	63°C
	Extension	1'	72°C	45"	72°C
	Final Extension	10'	72°C	10'	72°C
Number of Cycles		30		33	



Agarose gel electrophoresis of the PCR products for Cre Recombinase protein. MW - molecular weight; Ø – empty well; B – blank; + - positive control

3. Diphtheria toxin *in vivo* treatment

LysM-Cre[+]R26^{+iDTR} and LysM-Cre[-]R26^{+iDTR} (control) mice were treated intraperitoneally (IP) with variable test doses of Diphtheria Toxin (Sigma-Aldrich) per gram of body weight (50 ng/g, 80 ng/g, 100 ng/g, and 200 ng/g) at different time-points to optimize the DT-driven cell ablation protocol. To analyze myeloid cells depletion, mice had their blood collected or were euthanized 1, 2, 4 or 6 days after the last DT administration. Organs were collected for cytometry, histological and immunofluorescence analysis. Following the optimization process, 6-week-old TJ2-Tg/LysM-Cre/R26^{+iDTR} mice were initially injected with 80/100 ng/g of DT, every other day, during one week, followed by two weekly administrations of 50 ng/g of DT until leukemia development. Mice were closely monitored during DT treatment to ensure IP administrations were correctly performed and to detect toxic adverse effects.

4. Mouse euthanasia and necropsy

Mice were euthanized in a CO₂ chamber that was gradually filled with CO₂ for 5 min and 1 min at a constant level. To confirm death, exsanguination or cervical dislocation was performed. Organs were collected (thymus, spleen, lymph nodes, liver, lungs, kidneys, bone marrow and heart), weighted and kept in cold phosphate-buffered saline (PBS) solution until further analysis. Lymphoid organs (thymus, spleen, lymph nodes and bone marrow) were processed for flow cytometry analysis while non-lymphoid organs (liver, lungs and kidneys) were fixed for histological characterization. In particular, whole organ or fragments of thymus and spleen, were additionally frozen in OCT solution (VWR) and kept at -80°C for immunofluorescence analysis.

5. Blood collection and processing

Blood was collected from the facial vein into a 1.5 ml microtube containing 10 µl of EDTA (50 mM) to avoid blood coagulation. Erythrocytes from blood samples were lysed before FACS analysis. For that, 1 ml of Red Blood Cells lysis solution (1 volume of 170 mM Tris-HCl (pH 7.4) plus 9 volumes of 160 mM NH_4Cl) was added to the blood samples and incubated for 5 min at RT. Next, the samples were centrifuged (Sigma 1-14) for 15 seconds (s) at 16 000 g. Supernatant was discarded and the procedure was repeated once more. Leukocytes were collected after washing with FACS buffer, followed by antibody staining.

To detect leukemia in mice, blood was collected post-mortem from the jugular vein using a 20 µL capillary (Delbrück intraEND, sodium-heparinised, BRAND) and dipped in a red blood cell lysis solution (10 mg Crystal Violet/Sigma-Aldrich; 1% acetic acid/Sigma-Aldrich; up to 100 mL dH₂O) dyeing leukocytes nuclei blue. Nuclei counting was performed using a Neubauer chamber (Marienfield), under a light microscope (Olympus CX31) and the leukocyte concentration was calculated.

6. Flow cytometry analysis

Single-cell suspensions from thymus, spleen and lymph nodes were obtained through tissue dissociation in PBS using a 70 µm cell strainer (BD Biosciences). Bone marrow was collected from femurs and tibias and suspended in cold PBS. Cells were counted and a volume containing 1×10^6 cells was added to each sample tube. For peripheral blood samples, all cells were used for analysis. Cells were washed with 1 ml of FACS buffer (PBS with 3% FBS and 10 mM NaN_3) and centrifuged (Sigma 3K15) at 350 g for 5 min, at 4°C. The supernatant was discarded, and cells were incubated with 50 µl of fluorochrome-labeled antibody mix on ice, for about 1 h in the dark. The list of antibodies (BioLegend) together with its conjugated fluorochrome and concentration used are indicated in Table 4. To remove unbound antibody, 1 mL of FACS buffer was added to the samples followed by centrifugation under the same conditions as mentioned before. This step was repeated once more. In the end, cells were resuspended in PBS with 10 mM NaN_3 and filtered in 70 µm strainers. Samples were acquired using Accuri or FACS Canto II (BD Biosciences). Cytometry data was analyzed using FlowJo 10 software.

Table 4. Antibodies used in flow cytometry analysis.

Antigen	Fluorochrome	Final Dilution
B220/CD45R	APC/Cy7	1:500
CD11b (Itgam)	PerCP	1:800
CD11c (Itgax)	APC	1:200
CD24	APC	1:200
CD25	FITC	1:200
CD4	PE; APC/Cy7	1:200
CD44	Pe/Cy5	1:400
CD45.2	PerCP-Cy5.5	1:200
CD8a	PB	1:400
CD90.2 (Thy1.2)	FITC	1:500
F4/80	PE	1:200
Gr-1	PE; PE/Cy7	1:400/1:200
Live/Dead	AmCyan	1:1000
Ly-6C	APC	1:400
Ly-6G	PE	1:200
TER-119	PE	1:200

7. Immunofluorescence Analysis

Ten µm cryosections from thymi and spleens included and frozen in OCT compound were obtained using Cryostat Leica CM 3050S. The SuperFrost glass slides with sections were kept at -80°C until further use. First, the slides were dried for 30 min at 37°C followed by fixation with pre-cooled acetone (stored at -20°C, Sigma-Aldrich) for 10 min at RT. Next, the slides were re-hydrated with 3-4 quick passages in PBS and washed three times in PBSW (PBS with 0.1% Tween 20 (VWR)) for 5 min. After washing, each section was incubated with 50 µl of blocking solution (10% bovine serum albumin (BSA) (Sigma-Aldrich) + 20% FBS in PBSW or 5% Donkey Serum in PBSW) for 1 h in a wet chamber at RT. Slides were washed three times with PBSW for 10 min before incubation with primary antibodies (50 µl per section) overnight, at 4°C. The next day, slides were washed with PBSW and incubated with secondary antibodies (50 µl per section) for 1 h at RT, in a wet chamber. After washing with PBSW, the slides were incubated with 50 µl of 0.15% DAPI (PBS) per section for 5 min and then washed twice with PBS for 5 min. Slides were mounted in 100 µl Mowiol followed by coverslip application. The slides were dried overnight at RT, sealed with nail polish and stored at 4°C. The list of primary and secondary antibodies and concentrations used are indicated in Table 5. Images were acquired with Zeiss Axio Imager Z1 fluorescence microscope and processed in ImageJ software. Assembly of sequential images was performed using the plugin Grid/collection stitching (300) in Fiji software.

Table 5. Primary and Secondary antibodies used in immunofluorescence analysis.

	Antibody	Species	Dilution
Primary Antibodies	Keratin 5	Rabbit polyclonal	1:1000
	Keratin 8	Rat	1:100
	CD11c-Biotin	—	1:500
	F4/80	Rat	1:50
	DTR	Goat	1:1000
	GFP	Rabbit	1:100
Secondary Antibodies	Anti-Rabbit Alexa 488	Goat	1:1000
	Anti-Rat Alexa 546	Goat	1:1000
	Streptavidin Alexa 546	—	1:500
	Anti-Goat Alexa 488	Goat	1:1000

8. Hematoxylin and eosin Staining

Organs were fixed in 10% formalin solution (Bio-Optica) and processed at *i3S Histology and Electron Microscopy Service (HEMS)*. Four μm sections were obtained from paraffin-embedded tissue blocks using a microtome (Microm HM335E) and left to dry on glass slides at 37°C, overnight. Then, sections were stained using hematoxylin (Merck) and eosin (Thermo Fisher), according to the protocol described below (Table 6). The slides were mounted in DPX mounting medium (Sigma-Aldrich) and after drying were analyzed in a light microscope (Olympus CX31). The images were obtained using Light microscope Olympus DP 25 Camera and Cell B software.

Table 6. H&E staining protocol.

	Solution	Time
1	Xylene	2 x 5'
2	Ethanol 100%	2 x 5'
3	Ethanol 70%	5'
4	H ₂ O	5'
5	Hematoxylin	3'
6	H ₂ O	5'
7	Ethanol 70%	5''
8	Ethanol 100%	5''
9	Eosin	30''
10	H ₂ O	30''s
11	Ethanol 70%	30''s
12	EtOH 100%	30''s
13	Xylene	2 x 5'

s – with shaking

9. Isolation and Culture of Peritoneal Macrophages

After euthanasia, mice were injected with 5 ml of ice-cold sterile PBS-EDTA (5 mM) into the peritoneal cavity and gently massaged for 15-20 s before the exudate was collected to a tube and kept in ice. Next, the samples were centrifuged (Sigma 3K15) at 350 g for 5 min, at 4°C. The supernatant was discarded, and the cell pellets resuspended in FACS buffer for flow cytometry or in DMEM (supplemented with 10% FBS, 2% glucose and 0.1% Pen-Strep (Gibco)) for culture.

Regarding murine macrophages culture, 1×10^6 cells of each genotype (LysM-Cre[+]R26^{+iDTR} and LysM-Cre[-]R26^{+iDTR}) were seeded per well in 6-well plates for 1.5-2 h, at 37°C. To ensure only macrophages bind to the wells, the non-adherent cells were removed by washing three times with PBS, and the remaining macrophages were left in culture with supplemented DMEM, at 37°C. A sterile glass coverslip was added to the wells and after macrophages adhesion to coverslips, these were washed with PBS and then fixed with 3% PFA + 2% sucrose (in PBS) for 20 min, at 4°C. After fixation, the coverslips were washed with PBS, followed by permeabilization with 0.1% Triton X-100 (VWR) in PBS, during 20 min. The samples were then washed with 0.02% Tween (in PBS), for 5 min, and the immunofluorescence protocol was performed as mentioned before (starting at the blocking step).

10. Statistical Analysis

Graphs and statistical analysis were performed using GraphPad Prism 8 software. The statistical tests applied are indicated in the figure legends. P-values < 0.05 were considered statistically significant.

RESULTS

1. Characterization of the Tumor Microenvironment in TEL-JAK2 transgenic mice

1.1. Alterations in the number and proportion of myeloid cells in lymphomas from TEL-JAK2 transgenic mice: analysis of primary and secondary lymphoid organs

ETV6-JAK2 (also called TEL-JAK2; TJ2-Tg) transgenic mice express the TEL-JAK2 fusion protein in the lymphoid lineage (driven by the E μ SR α enhancer/promoter) which promotes the constitutive activation of JAK-STAT signaling, leading to T-cell leukemia/lymphoma development. These mice usually manifest disease signs between 8 and 20 weeks of age, exhibiting several organs affected (299).

In particular, TJ2-Tg mice are characterized by an enlargement of both lymphoid (thymus, spleen, lymph nodes) and non-lymphoid organs (liver, lungs, kidneys) due to leukemic cells accumulation and expansion (299). To confirm weight/cellularity changes induced by TJ2 leukemogenesis we assessed both organs weigh and cellularity in TJ2-Tg and wild-type (WT) mice. In this case, we observed a significant increase of thymus weigh/cellularity and LN weight in diseased TJ2-Tg mice, compared to WT mice (Figure 7A and B). No significant differences were observed for the spleen or BM regarding weigh/cellularity between genotypes, nor for the non-lymphoid (liver and lungs) organs. This denotates that most leukemic cells accumulated at the thymus, suggesting that TJ2-Tg mice exhibited mostly a thymic lymphoma phenotype with low peripheral organ invasion.

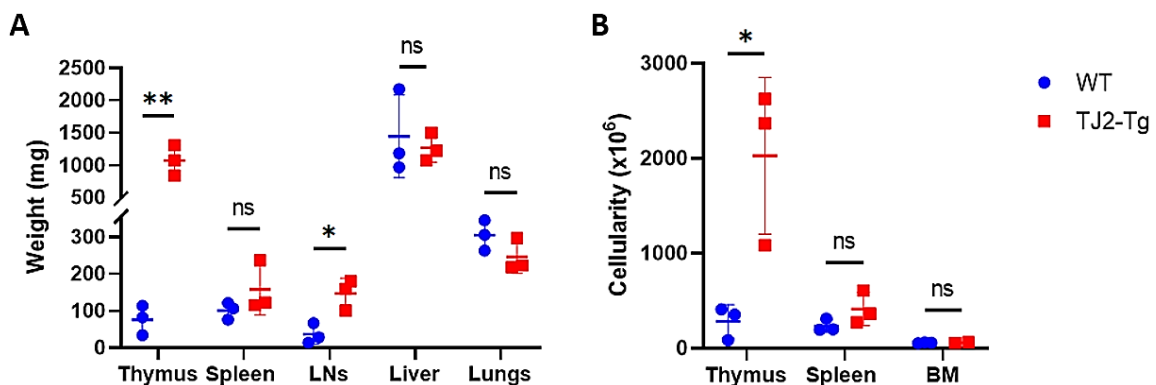


Figure 7. Organs weight and cellularity in TJ2-Tg and non-transgenic mice. (A) Graph depicts the comparison of lymphoid (thymus, spleen, LNs) and non-lymphoid (liver and lungs) organs weight between diseased TJ2-Tg and healthy WT mice. (B) Graph depicts the comparison of lymphoid organs cellularity (thymus, spleen, bone marrow) between diseased TJ2-Tg and healthy WT mice. *P* values were determined by two-tailed, unpaired *t*-test (**, *P* ≤ 0.01; *, *P* ≤ 0.05; ns (non-significant), *P* > 0.05). WT *n* = 3; TJ2-Tg *n* = 3 (Data are presented with mean ± SEM; each symbol represents an individual mouse).

We also performed an immunophenotypic analysis of TJ2-Tg thymic lymphoma, which was previously shown to exhibit an aberrant cell surface phenotype (299, 301, 302). Flow cytometry analysis revealed that healthy thymus was mainly composed by CD4, CD8 DP thymocytes, with low percentages of CD4 SP, CD8 SP and CD4, CD8 DN. On the other hand, leukemic TJ2-Tg thymus displayed an increase of CD8 SP and a tendency for CD4 CD8 DP cells to decrease, together with significant reduced percentages of CD4 SP (Figure 8A and B). These results reveal that TJ2-Tg thymic lymphomas are characterized by an enrichment in CD8⁺ T cells, in detriment of CD4⁺ T cells.

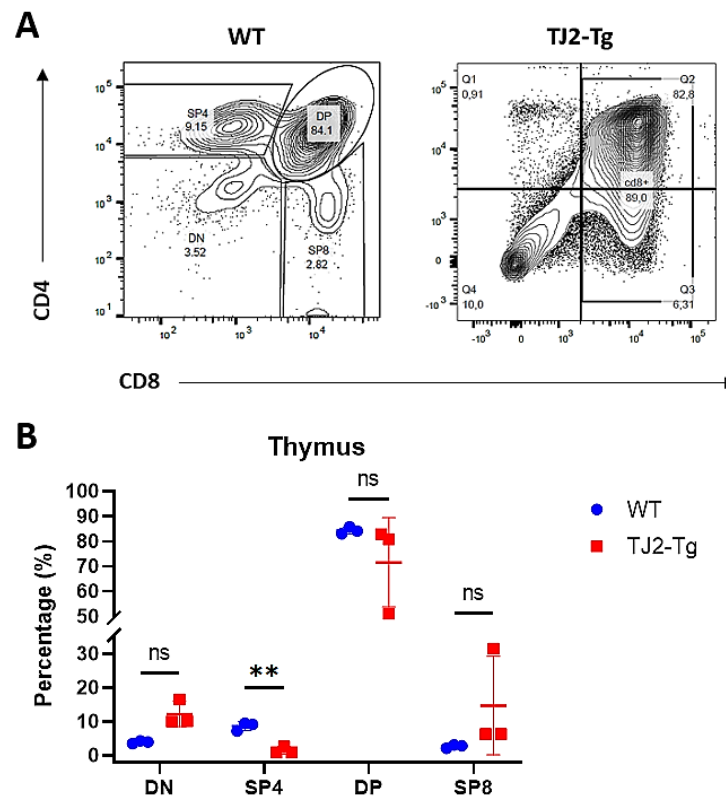


Figure 8. Thymocyte proportions in leukemic TJ2-Tg and healthy WT thymi. Cell suspensions from the thymus of TJ2-Tg and WT mice were stained for thymocyte markers (CD4, CD8, CD25, CD44 and CD24) and analyzed by flow cytometry. **(A)** Representative gating strategy to identify thymocyte populations (DN, SP4, DP, SP8) in diseased TJ2-Tg and healthy WT mice. **(B)** Graph depicts the proportion of thymocyte populations in the thymus of TJ2-Tg and non-transgenic mice (WT). *P* values were determined by two-tailed, unpaired *t*-test (**, *P* ≤ 0.01; *, *P* ≤ 0.05; ns (non-significant), *P* > 0.05). WT *n* = 3; TJ2-Tg *n* = 3 (Data are presented with mean ± SEM; each symbol represents an individual mouse).

Regarding the myeloid compartment, our group has previously demonstrated that TJ2-Tg mice exhibit an increased proportion of dendritic cells in the thymic lymphoma microenvironment (150). Likewise, Triplett *et al.* revealed a significant increase in CD11c⁺ DC and CD11c⁻CD11b⁺ myeloid cell content in both thymus and spleen of lymphoma-bearing LN3 transgenic mice (156). Hence, we hypothesized that in addition to DCs, CD11b⁺ myeloid cells, including monocytes, macrophages and granulocytes, could be

disturbed in the stroma of thymus and secondary lymphoid organs of TJ2-Tg mice. To confirm our hypothesis, cell suspensions from the thymus, spleen and bone marrow of TJ2-Tg mice and WT controls were analyzed by flow cytometry using myeloid-specific surface markers (CD11b, Gr1, Ly6C, CD11c and F4/80). The gating strategy used to identify organ-specific myeloid subsets is shown in Figure 9 and Annex 1. Both total cell number and proportion of myeloid cell populations in lymphoid organs were obtained (Figures 10-14).

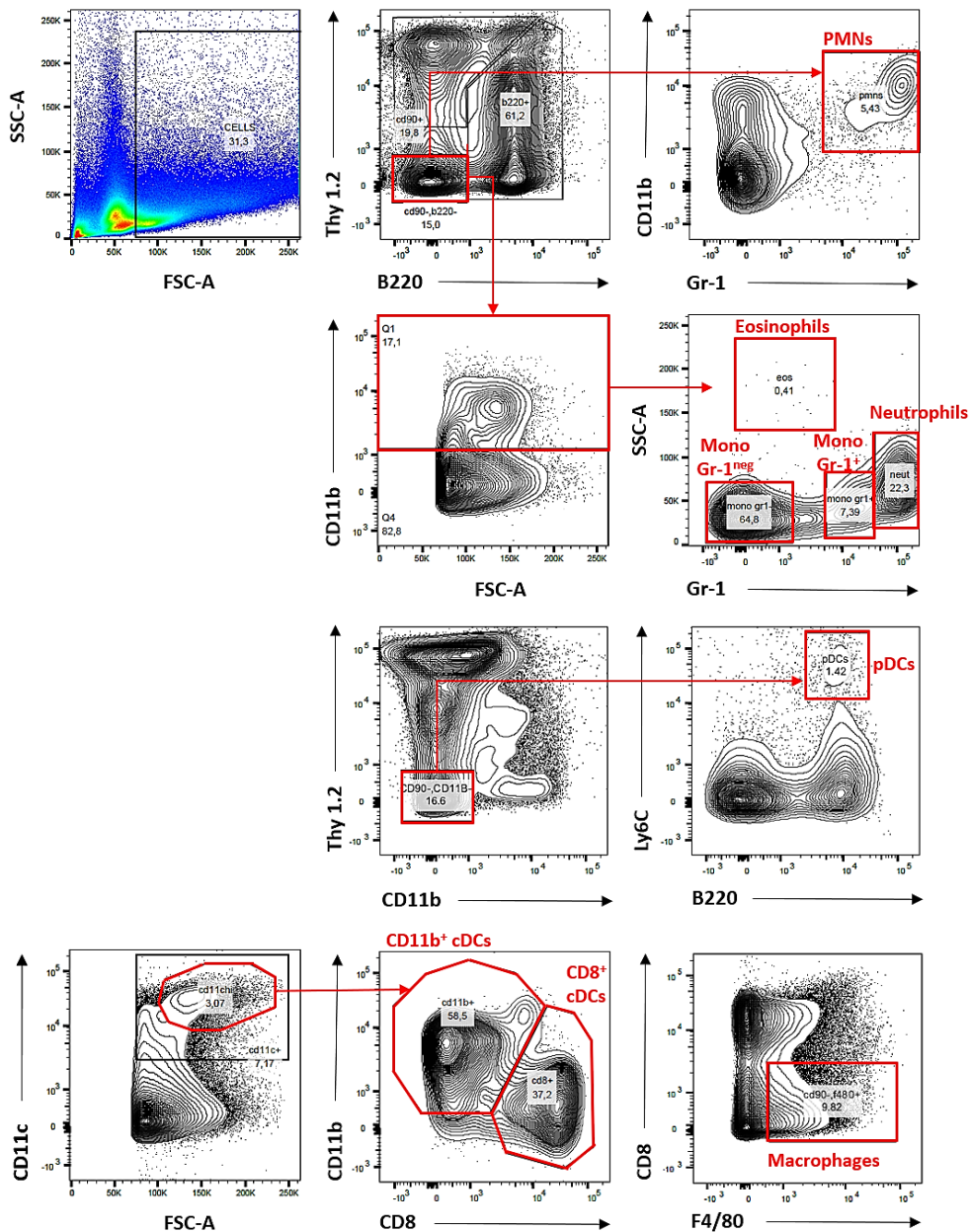


Figure 9. Representative gating strategy to identify myeloid subsets in lymphoid organs. Cell-suspensions from thymus, spleen, and bone marrow of TJ2-Tg and WT mice were analyzed by flow cytometry. Viable cells were selected based on Forward (FSC) versus Side scatter (SSC) gating. In all organs, lineage negative cells (Thy 1.2^{neg} and B220^{neg}) were analyzed to identify polymorphonuclear cells (PMNs) (CD11b⁺Gr-1⁺) and other myeloid CD11b-expressing cells (excepting for the thymus), including neutrophils (CD11b⁺SSC^{int}Gr-1^{hi}), eosinophils (CD11b⁺SSC^{hi}Gr-1^{int}), and both monocyte subsets, Gr-1^{low/neg} (CD11b⁺SSC^{low}Gr-1^{low/neg}) and Gr-1^{hi} (CD11b⁺SSC^{low}Gr-1⁺). In the thymus and spleen, macrophages (F4/80⁺CD8^{neg}), pDCs (CD11b^{neg}B220⁺Ly6C^{hi}) and cDCs (CD11c^{hi}) were assessed. The latter were divided into cDC1/CD8⁺ cDCs (CD11c^{hi}CD8⁺) and cDC2/CD11b⁺ cDCs (CD11c^{hi}CD11b⁺).

FACs analysis revealed a trend towards a substantial increase in cDC (both CD11b⁺ and CD8⁺ subsets) total number in the thymus of TJ2-Tg mice, compared to healthy controls (Figure 10A). The same pattern was verified for spleens of transgenic mice, which presented an increase in both cDC subsets, namely CD11b⁺ cDCs (statistically significant, $P = 0.0295$), compared to healthy spleens (Figure 10C). In addition to increased numbers, spleens of diseased mice also tended to exhibit higher proportions of splenic cDCs, while in the thymus these percentages were conserved (Figure 10B and D). This may be due to the fact that TJ2-Tg leukemogenesis arises in the thymus (301, 302), and, therefore, the majority of leukemic cells accumulates in this organ as shown in Figure 7. This suggests that the observed increase in thymic cDCs is proportional to the growth of lymphoma cells in the thymus. On the other hand, splenic pDCs but not thymic pDCs, appeared to be lower in both total number and proportion in TJ2-Tg mice, in comparison with healthy control mice (Figure 10A-D).

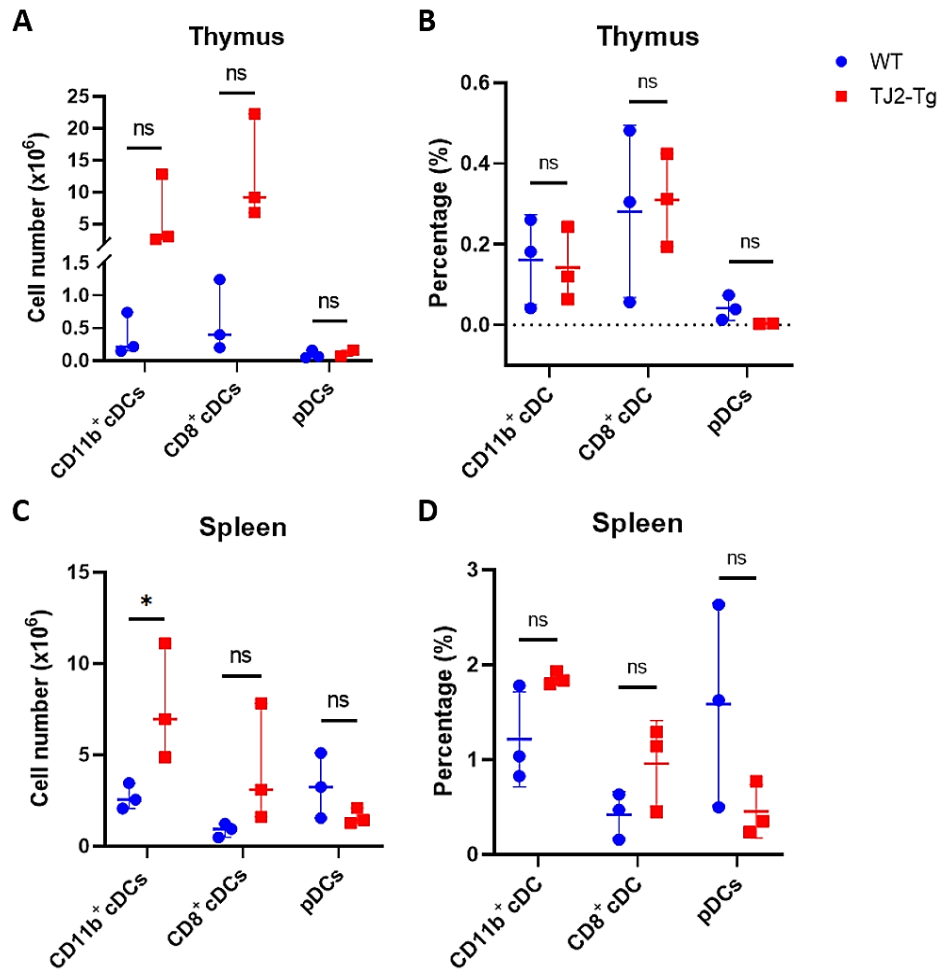


Figure 10. Total cell number and percentage of classical (CD11b⁺ and CD8⁺ cDCs) and plasmacytoid DCs in the thymus and spleen of TJ2-Tg and non-transgenic mice. Cell-suspensions from the thymus and spleen of transgenic and non-transgenic mice were stained for dendritic cell markers (CD11c, CD11b, CD8, B220, Ly6C) and analyzed by flow cytometry. Graphs depict the number (**A, C**) and frequency (**B, D**) of CD8⁺ cDCs, CD11b⁺ cDCs, and pDCs (identified as in Figure 9) in the thymus and spleen of TJ2-Tg and WT mice. *P* values were determined by two-tailed, unpaired *t*-test with Welch's correction when variances were unequal (*, *P* ≤ 0.05; ns (non-significant), *P* > 0.05). Non-transgenic *n* = 3; Transgenic *n* = 3. (Data are presented with mean ± SEM; each symbol represents an individual mouse).

Both mouse thymus (303) and spleen (304) harbor different tissue-resident macrophages, characterized by variable expression of F4/80 and CD11b staining. For the sake of simplicity, we identified macrophage subsets based on F4/80 labeling, which is common to all. Using this strategy, we were able to observe a clear tendency for thymic macrophage number to increase in TJ2-Tg mice, comparing to WT mice (Figure 11A). However, this increase was not proportional, in extent, to lymphoma cell growth, since the proportion of thymic macrophages in diseased mice was decreased compared to controls (Figures 7, 11B). In the spleen, the macrophage number appeared to vary considerably, depending on if TJ2-Tg leukemogenesis manifested more in the form of thymic lymphoma or leukemia (Figure 11C, 11F). In the case of behaving as thymic lymphoma, we could not observe any

differences regarding splenic macrophage number between TJ2-Tg and healthy mice (Figure 11E and F). However, when TJ2-Tg mice displayed a more scattered disease, and consequently enlarged spleen due to leukemic cell infiltration, they appeared to undergo an increase in splenic macrophage content.

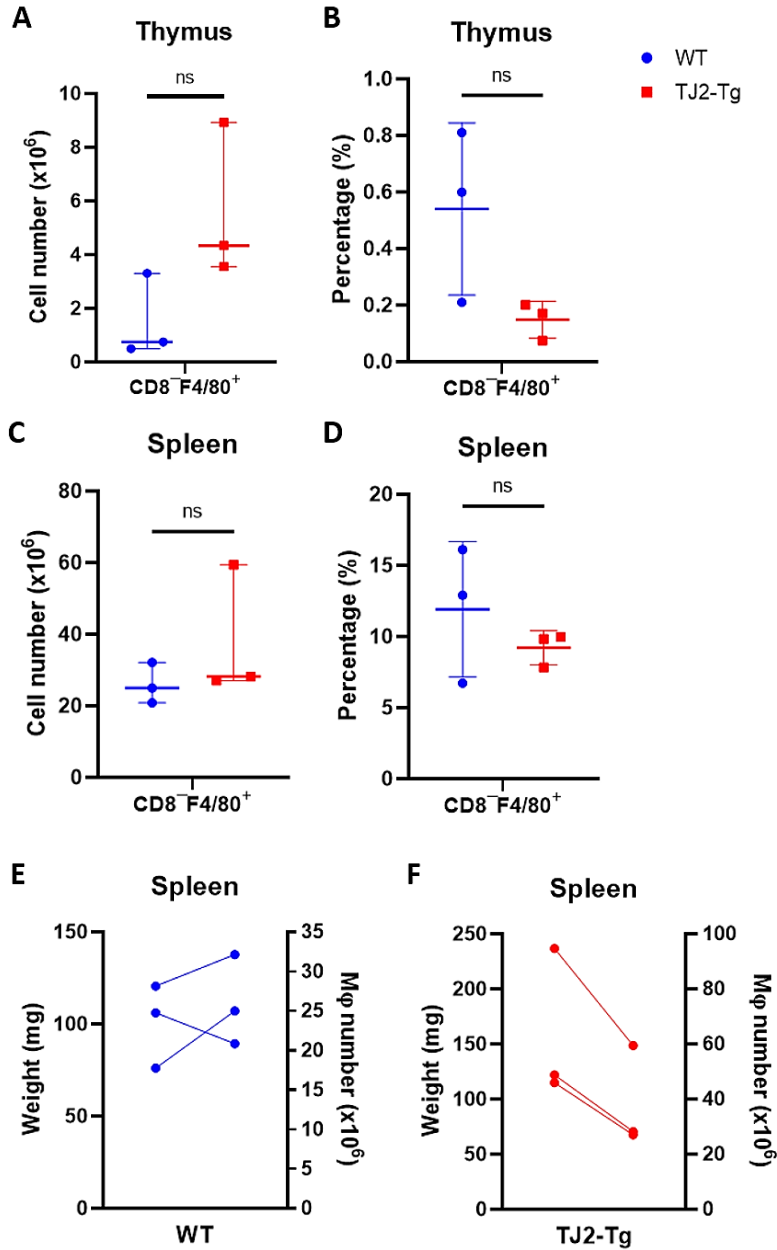


Figure 11. Total cell number and percentage of macrophage populations in the thymus and spleen of TJ2-Tg and non-transgenic mice. Thymic and splenic cell suspensions from transgenic and non-transgenic mice were stained for macrophage marker, F4/80, and analyzed by flow cytometry. Graphs represent the number (**A, C**) and frequency (**B, D**) of macrophages (identified as in Figure 9) in the thymus and spleen of TJ2-Tg and WT mice. (**E, F**) Variation of splenic macrophage number in WT (**E**) and TJ2-Tg mice (**F**) according to spleen weight. *P* values were determined by two-tailed, unpaired *t*-test (*ns*, *P* > 0.05). Non-transgenic *n* = 3; Transgenic *n* = 3. (Data are presented with mean ± SEM; each symbol represents an individual mouse).

Besides the resident macrophages, we also analyzed the monocytes and monocyte-derived macrophages (MOMF) in the spleen of transgenic mice to evaluate if their number or proportion could be altered in the leukemia context, as recently suggested (293). To do so, we applied the Rose *et al.* gating strategy to identify both inflammatory and patrolling monocytes, characterized by $CD11b^+Gr-1^+SSC^{low}$ / $CD11b^+Ly6C^+SSC^{low}$ and $CD11b^+Gr-1^{low/neg}SSC^{low}$ / $CD11b^+Ly6C^{low/neg}SSC^{low}$ expression, respectively (Figure 9) (305). The flow cytometry results revealed that $Gr-1^{lo/neg}$ splenic monocyte/macrophage numbers tended to oscillate considerably in TJ2-Tg mice, whereas no differences were observed for $Gr-1^+$ monocyte/macrophage content between leukemic and healthy mice (Figure 12A). However, the proportion of $Gr-1$ -expressing monocytes tended to be low in TJ2-Tg mice, compared to WT mice (Figure 12B).

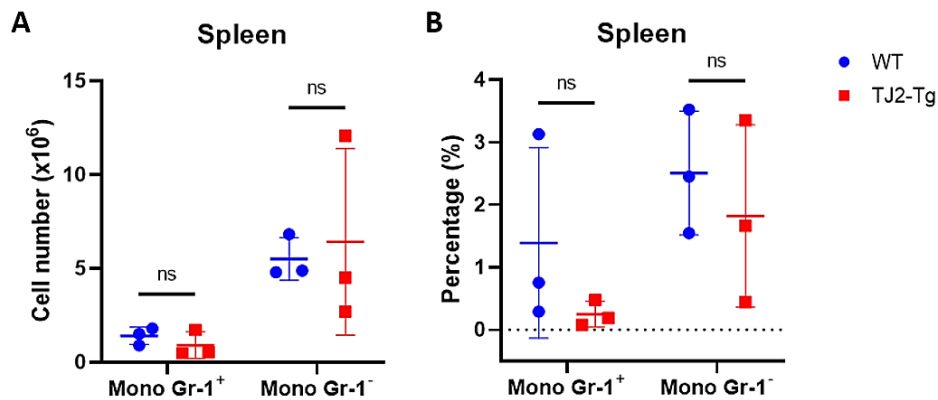


Figure 12. Total cell number and percentage of inflammatory and patrolling monocytes/macrophages in the spleen of TJ2-Tg and non-transgenic mice. Cell-suspensions from the spleen of transgenic and WT mice were stained for monocyte markers (CD11b, Gr-1) and analyzed by flow cytometry. Both number and percentage of splenic $Gr-1^+$ and $Gr-1^{lo/neg}$ monocytes (identified as in Figure 9) are represented in **A** and **B**, respectively. *P* values were determined by two-tailed, unpaired *t*-test with Welch's correction when variances were unequal (*ns*, *P* > 0.05). Non-transgenic *n* = 3; Transgenic *n* = 3. (Data are presented with mean ± SEM; each symbol represents an individual mouse).

Yang *et al.* have shown that circulating monocytes contribute to LAM increase in extramedullary organs of Notch1-driven T-ALL mice (293). Since we observed great variability for splenic $Gr-1^{neg}$ monocyte/macrophage numbers in TJ2-Tg leukemic mice, we evaluated monocyte (classical $Ly6C^+/Gr-1^+$ and non-classical $Ly6C^{neg}/Gr-1^{neg}$ subsets) production in the BM of transgenic mice. Comparing with WT mice, we found no significant differences regarding $Ly6C^+/Gr-1^+$ nor $Ly6C^-/Gr-1^-$ monocyte populations (Figure 13A and B). We further assessed polymorphonuclear cell (PMN) production and found that diseased mice showed no significant differences in BM PMN total number or proportion (Figure 13C and D).

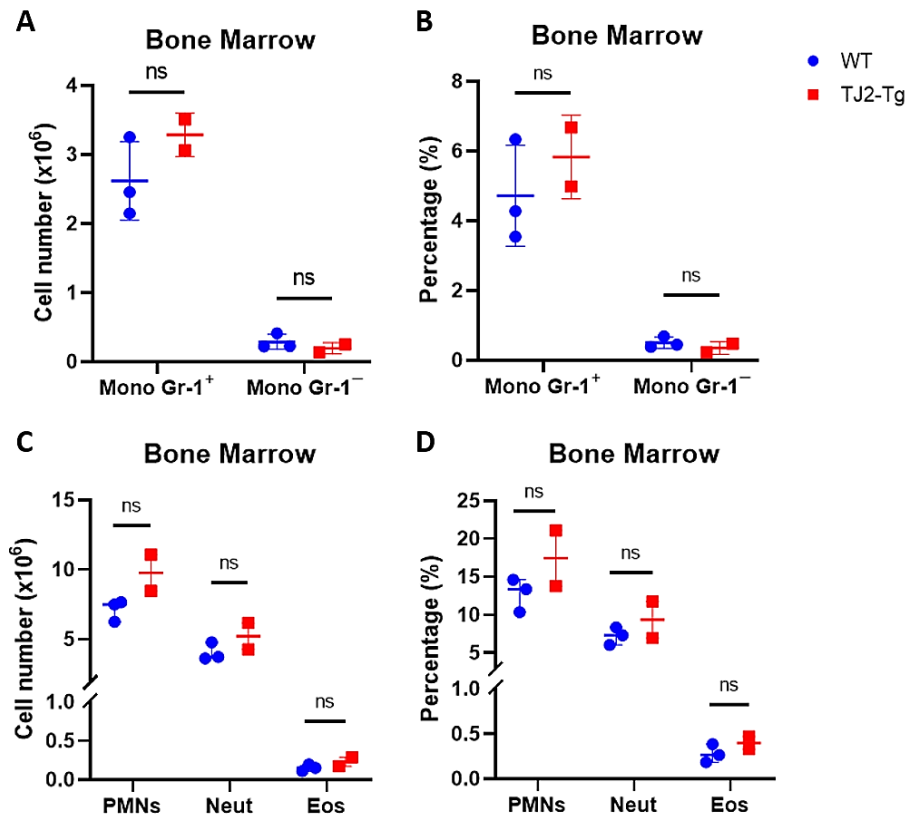


Figure 13. Total cell number and percentage of blood monocytes (classical Ly6C⁺/Gr-1⁺ and non-classical Ly6C^{neg}/Gr-1^{neg}) and PMNs in the BM of TJ2-Tg and non-transgenic mice. Medullary cell-suspensions from leukemic and healthy mice were stained for blood monocyte and PMN (CD11b, Gr-1) markers followed by flow cytometry analysis. Graphs depict the number (**A, C**) and frequency (**B, D**) of monocytes and PMNs (identified as in Figure 9) in the BM of TJ2-Tg and WT mice. *P* values were determined by two-tailed, unpaired *t*-test (*ns*, *P* > 0.05). Non-transgenic *n* = 3; Transgenic *n* = 2. (Data are presented with mean $\pm$ SEM; each symbol represents an individual mouse).

We also explored PMN content in the spleen and thymus of TJ2-Tg mice to verify if any of these lymphoid organs could be recruiting granulocytes from the BM to support leukemia progression. No major differences were observed regarding PMN total numbers in both thymus and spleen of TJ2-Tg mice, compared to WT mice (Figure 14A and C). However, PMN proportions in these organs were substantially reduced, namely in the thymus of transgenic mice (statistically significant *P* = 0.0388), probably due to increased accumulation or growth of TJ2⁺ leukemic cells without concomitant increase in PMNs (Figure 7, 14B, 14D).

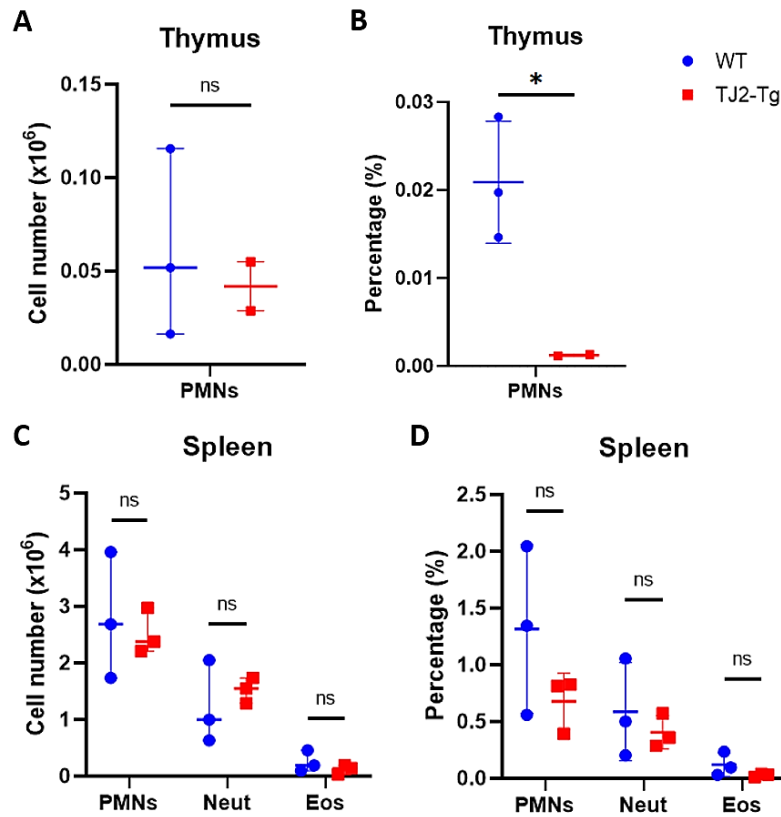


Figure 14. Total cell number and percentage of PMNs in the thymus and spleen of transgenic and non-transgenic mice. Thymic and splenic cell-suspensions from TJ2-Tg and WT mice were stained for blood PMN (CD11b, GR-1) markers and analyzed by flow cytometry. Graphs display the number (**A, C**) and proportion (**B, D**) of PMNs (identified as in Figure 9) in the thymus and spleen of TJ2-Tg and WT mice. *P* values were determined by two-tailed, unpaired *t*-test (*, $P \leq 0.05$; ns, $P > 0.05$). Non-transgenic $n = 3$; Transgenic $n = 3$. (Data are presented with mean $\pm$ SEM; each symbol represents an individual mouse).

Together, these results demonstrate that lymphoid organs from diseased TJ2-Tg mice tend to display an increase in different myeloid cell subsets, namely cDCs, supporting the possibility that TEL-JAK2-induced leukemia requires myeloid stromal support for its progression and survival.

1.2. Histological alterations driven by TJ2 leukemogenesis in thymic and splenic niches from transgenic mice

Ghezzo *et al.* have previously shown that TJ2-Tg mice display major alterations in the thymic stroma, including increased DC proportions and loss of clear spatial segregation between cortex and medulla (resulting in keratin negative areas (Krt⁻)) (150). Normally, the thymic medulla is marked by keratin 5 (Krt5) expression while the cortex expresses keratin 8 (Krt8). In contrast, TJ2-Tg thymic lymphomas present Krt5⁺Krt8⁺ epithelial areas adjacent to keratin-negative areas (150). We performed an immunofluorescence analysis to validate these alterations and, most importantly, to identify and assess myeloid cell (DCs and

macrophages) spatial distribution in both thymic and splenic microenvironments of TJ2-Tg mice.

First, we confirmed that thymi from TJ2-Tg mice displayed impairment of medulla and cortex division, as previously observed (150, 299). To do so, cryopreserved thymic sections from transgenic and non-transgenic mice were stained with keratin 5 and keratin 8 antibodies to identify medullary and cortical epithelial cells, respectively. While in WT thymus, we could observe a clear spatial segregation between the Krt5⁺ medullary and Krt8⁺ cortical regions (Figure 15A), in thymic lymphomas these appear to be intermingled or overlapped, as evidenced by the areas exhibiting Krt5 and Krt8 co-expression (Figure 15B; arrows).

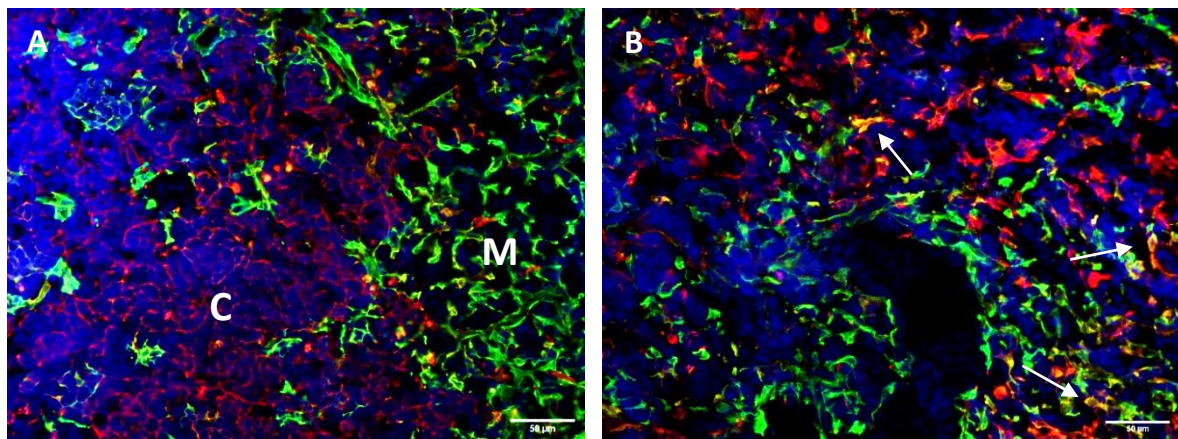


Figure 15. Compartmentalization of cortical and medullary regions in thymi from TJ2-Tg and WT mice. Thymic sections from healthy (A) and transgenic mice (B) were immunostained with Keratin 5 (in green) and Keratin 8 (in red) to identify the medullary and cortical epithelium, respectively. DAPI staining (blue) was additionally performed to reveal cell nuclei. Both thymic medulla (M) and cortex (C) are indicated in the images. Scale bars: 50 µm (Original magnification 20x)

Then, we assessed myeloid cell distribution in the thymic lymphoma microenvironment. So, thymic sections from transgenic and WT mice were immunostained with antibodies against Krt5 and CD11c, to identify mTECs and DCs, respectively. As previously reported (150, 156), in non-transgenic mice, CD11c⁺ DCs were mainly localized in the Krt5⁺ medullary region, exhibiting very little CD11c staining in the cortex (Figure 16A). Similarly, in TJ2-Tg mice the majority of DCs accumulated in Krt5-positive areas, forming dense aggregates, despite being found throughout the thymus, including Krt5-negative non-epithelial regions (Figure 16B).

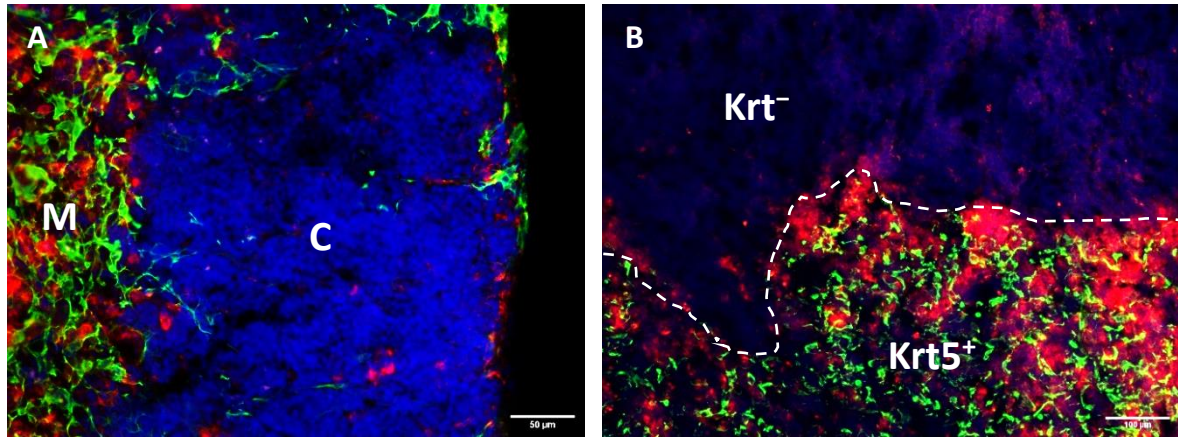


Figure 16. Dendritic cell distribution in the thymic microenvironment of TJ2-Tg and WT mice. Thymic sections from healthy (A) and transgenic mice (B) were immunostained using anti-Keratin 5 (in green) to identify the medullary zone, and anti-CD11c (in red) to assess DC localization. DAPI staining (blue) was additionally performed to reveal cell nuclei. Medullary (M), cortical (C), keratin 5-positive (Krt5⁺) and keratin negative (Krt⁻) areas are indicated in the images. (A) Scale bar: 50 µm (Original magnification 20x). (B) Dashed-line delimitates the Krt5⁺ region. Scale bar: 100 µm (Original magnification 10x).

To assess macrophage presence, we also performed double-immunostaining of thymic lymphomas and non-transgenic thymus with Krt5 and the F4/80 macrophage-specific antibody. In both cases, macrophages appeared to be scattered throughout the thymus (Figure 17A and B). However, in non-transgenic thymus, macrophages were more abundant in the medullary region. Similarly, macrophages in thymic lymphomas were preferentially associated with Krt5-expressing areas, which is in accordance with the data previously reported by our group (150).

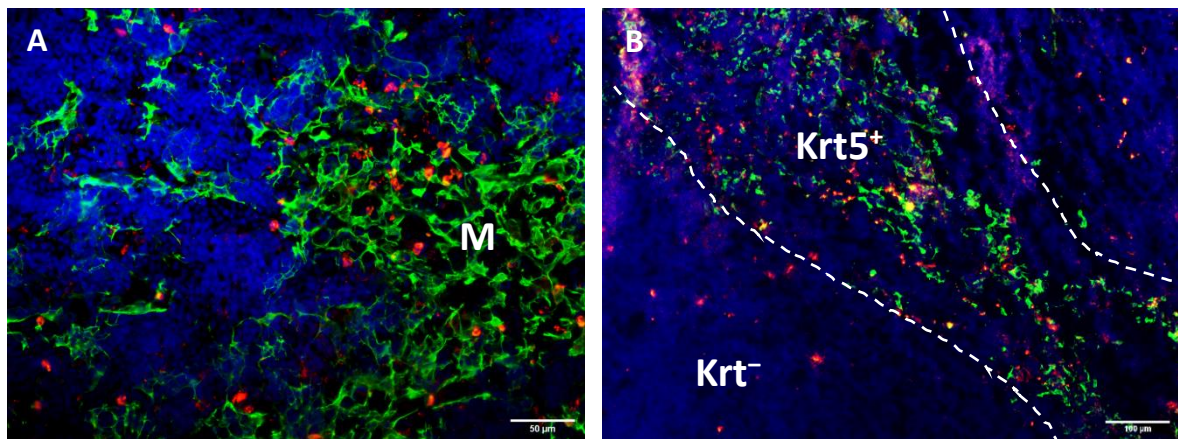


Figure 17. Macrophage spatial distribution in the thymic microenvironment of TJ2-Tg and WT mice. Thymic sections from healthy (A) and transgenic mice (B) were immunostained with Krt5 (in green) to identify the medulla, and F4/80 (in red) to detect macrophages. DAPI staining (blue) was additionally performed to reveal cell nuclei. Medullary (M), Krt5 positive (Krt5⁺) and keratin negative (Krt⁻) regions are indicated in the images. (A) was obtained by combining 4 sequential images (grid stitching, ImageJ). Scale bar: 50 µm (Original magnification 20x) (B) Dashed line delimitates the Krt5⁺ region. Scale bar: 100 µm (Original magnification 10x)

To characterize the myeloid compartment in spleens of TJ2-Tg mice we performed double-staining of splenic sections with antibodies against laminin, an extracellular matrix marker, and CD11c or F4/80, to identify DCs or macrophages.

Microanatomically, the mouse spleen is composed of red pulp (RP) (connective tissue and sinusoids) and white pulp (WP) (lymphoid tissue), separated by the marginal zone (MZ) (306). Laminins, in particular, are a major component of basement membranes found in several extracellular matrix elements of the spleen, including vascular walls, venous sinus and reticular fiber networks. Laminin staining allows the distinction of spleen compartments, based on the structural organization of the reticular fiber network (Figure 18) (307).

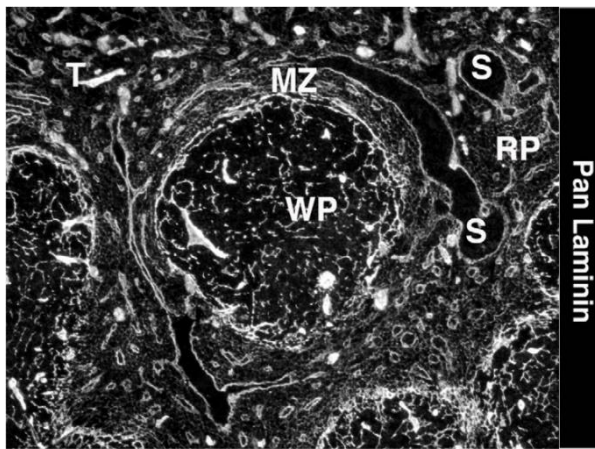


Figure 18. Pan-laminin immunofluorescence (Pan LM) staining of adult murine spleen. The WP is delineated by the marginal sinus-lining basement membrane, containing a network of larger and more spatially distributed reticular fibers. The MZ and RP, on the other hand, present thinner fibers but more closely packed reticular networks. (From Sorokin L. et al. *Semin Immunol.* 2008;20:4-13)

In TJ2-Tg mice, laminin immunolabeling revealed loss of normal spleen parenchyma compartmentalization (Figure 19B), with the exception of limited regions wherein the structure was preserved to some extent, as shown in Figure 19A.

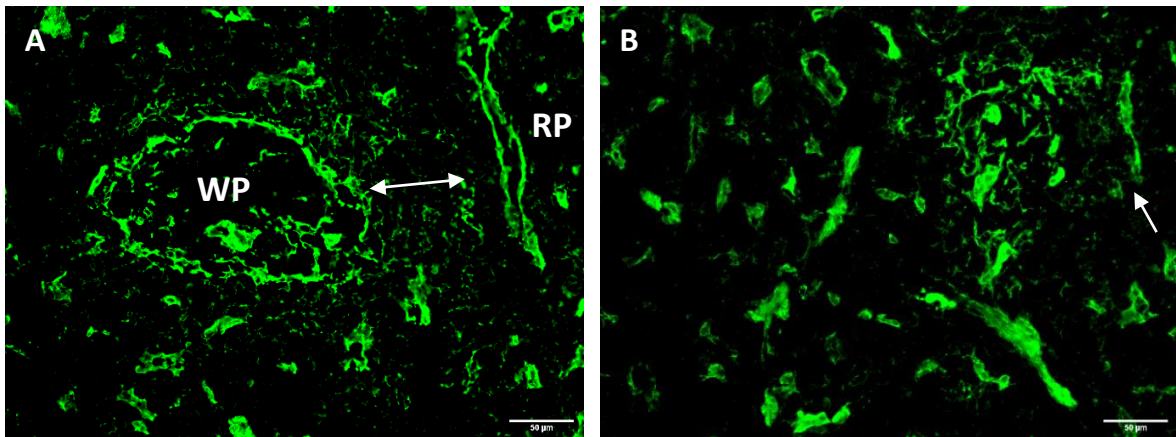


Figure 19. Loss of splenic compartmentalization in TJ2-Tg mice. (A, B) Splenic sections from a diseased TJ2-Tg mouse were immunostained with laminin (in green) to identify the basement membrane of the reticular fiber networks. (A) Section in which spleen compartmentalization was preserved to a certain degree (rarely found), while (B) represents an area with no clear separation (commonly found) between the white pulp (WP) and red pulp (RP), due to disruption of the marginal sinus-lining basement membrane (indicated by the arrow). Double arrows correspond to marginal zone. Scale bar: 50 μ m (Original magnification 20x)

Next, we immunostained splenic sections from WT and TJ2-Tg mice with laminin and F4/80 antibodies to assess macrophage distribution. In WT mice, F4/80⁺ splenic macrophages accumulated predominantly at the MZ and RP (Figure 20A). In contrast, TJ2-Tg mice exhibited complex macrophage networks throughout the spleen, together with abundant laminin extracellular matrix support (Figure 20B). We also observed a clear disruption of WP and RP segregation in transgenic mice, as evidenced by the discontinuous marginal sinus-lining basement membrane (arrows in Figure 20C). Areas exhibiting weak or no staining for F4/80 in TJ2-Tg spleen may correspond to sections of the WP that remained intact or to areas of exclusive leukemic cell infiltration (Figure 20B).

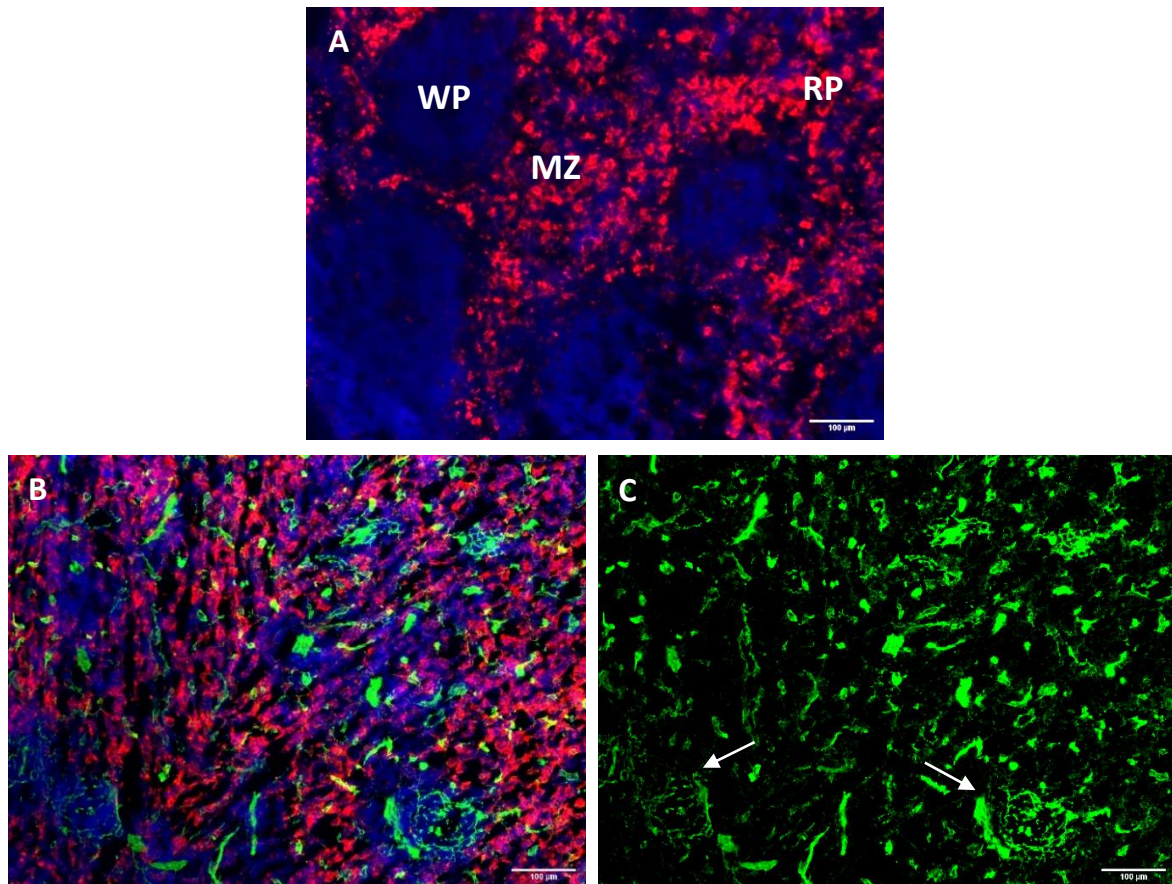


Figure 20. Macrophage distribution in the splenic microenvironment of TJ2-Tg and WT mice. (A) Splenic sections from WT mice were stained with F4/80 (in red) to assess macrophages normal distribution. (B) Splenic sections from transgenic mice were double-stained with laminin (in green) to identify the extracellular matrix and F4/80 (in red) to assess macrophages presence. DAPI staining (blue) was additionally performed to reveal cell nuclei. (C) Mono-labeling with laminin identifies the structural alterations driven by TJ2 leukemogenesis; arrows point to the disruption of the marginal sinus-lining basement membrane. White pulp (WP), marginal zone (MZ) and red pulp (RP) areas are indicated in the images. Scale bar: 100 µm (Original magnification 10x)

Likewise, to assess DC localization, co-staining of splenic sections from TJ2-Tg mice with laminin and CD11c were performed. In normal mice, splenic CD11c⁺ DCs are mostly found at the MZ and RP (Figure 21A). On the other hand, in TJ2-Tg leukemogenesis, spleens

displayed large DC networks throughout the organ, along with strong extracellular matrix laminin staining (Figure 21B). Besides, we observed a disruption of the marginal sinus-lining basement membrane surrounding the WP in TJ2-Tg mice, revealing no clear separation between compartments (arrow in Figure 21C). Despite this, CD11c⁺ DCs appear to be scattered all over the TJ2-Tg splenic parenchyma, and not specifically accumulated as shown in Figure 21A, which may reflect an increase in DC number or proportion due to leukemic cell invasion, as suggested by flow cytometry results (Figure 10C and D).

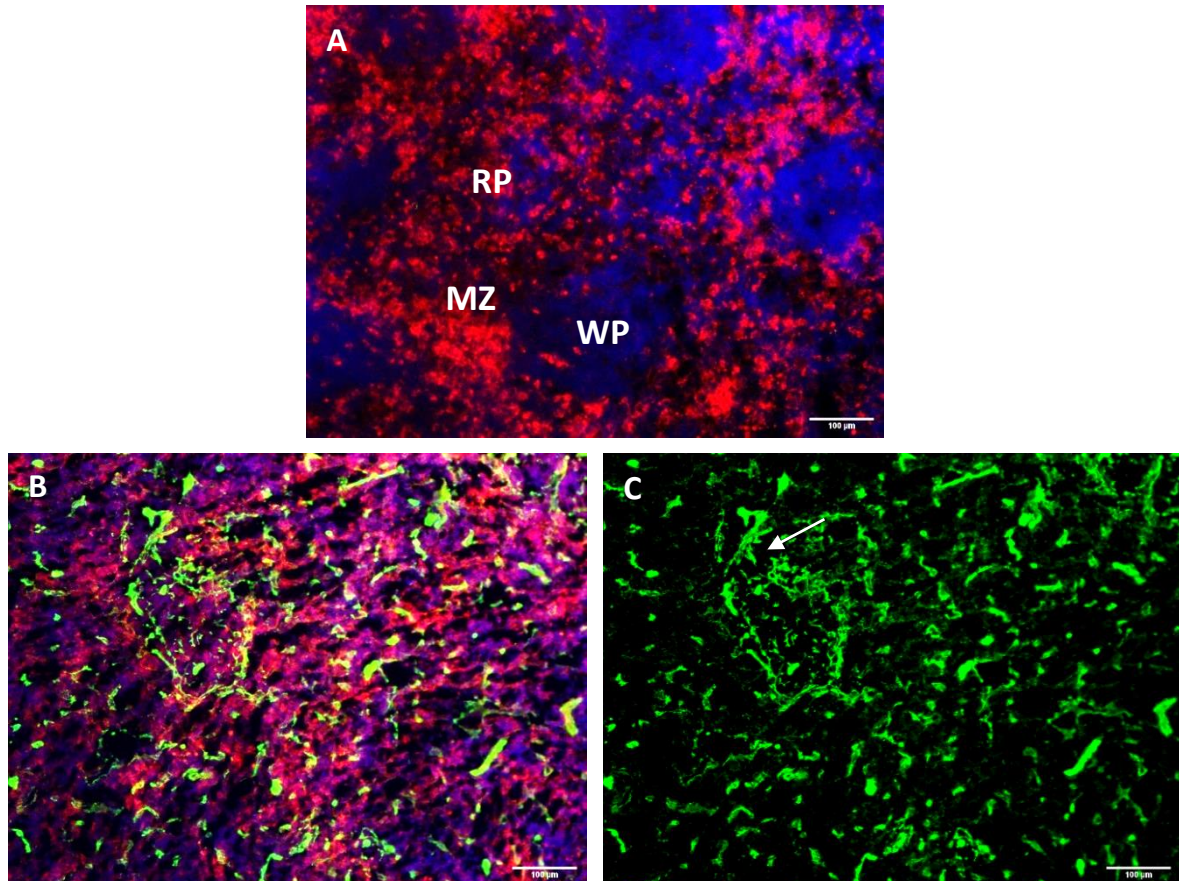


Figure 21. DC distribution within the splenic stroma of TJ2-Tg and WT mice. (A) Splenic sections from WT mice were stained with CD11c (in red) to assess DC normal distribution. (B) Splenic sections from transgenic mice were double-stained with laminin (shown in green) to identify the extracellular matrix and CD11c (in red) to assess dendritic cell presence. DAPI staining (blue) was additionally performed to reveal cell nuclei. (C) Mono-labeling with laminin identifies the reticular fiber network in transgenic mice; arrow points to the disruption of the marginal sinus-lining basement membrane. White pulp (WP), marginal zone (MZ) and red pulp (RP) areas are indicated in the images. Scale bar: 100 µm (Original magnification 10x)

Together, these results suggest that TJ2-Tg leukemogenesis induces structural alterations in both thymus and spleen, associated with enhanced myeloid cell and extracellular matrix support.

2. Generation of a conditional myeloid cell ablation system *in vivo*

2.1. Generation of Lysozyme M-Cre/R26iDTR transgenic mice: confirming specificity and efficiency of DTR expression in myeloid cells

Having shown that TJ2-Tg mice present an increase in several myeloid cell subsets within lymphoid organs, we hypothesized that these cells could play a fundamental role in leukemia progression and survival, as suggested by *in vitro* (156, 285), and more recently, *in vivo* (293, 294) studies. To test this hypothesis, we aimed to deplete lymphoma-associated myeloid cells and evaluate the impact on disease development. Therefore, we generated an inducible myeloid cell lineage-specific ablation system based on the transgenic expression of the simian diphtheria toxin (DT) receptor (DTR) to transform naturally DT-resistant murine cells to DT-sensitive (308, 309). To do so, we used two transgenic mouse strains: one expressing the Cre recombinase transgene under the control of the myeloid cell-specific Lysozyme M (LysM) promoter (LysM-Cre) (310, 311), and the other encoding the simian/human DTR (also called heparin-binding EGF-like growth factor (HB-EGF)) gene under control of a loxP-flanked STOP cassette in the ROSA26 (R26) locus (R26iDTR). The breeding of these two strains resulted in the LysM-Cre/R26iDTR double transgenic mice, which should express the DTR in LysM-positive myeloid cells after Cre-mediated excision of the STOP sequence (Figure 22).

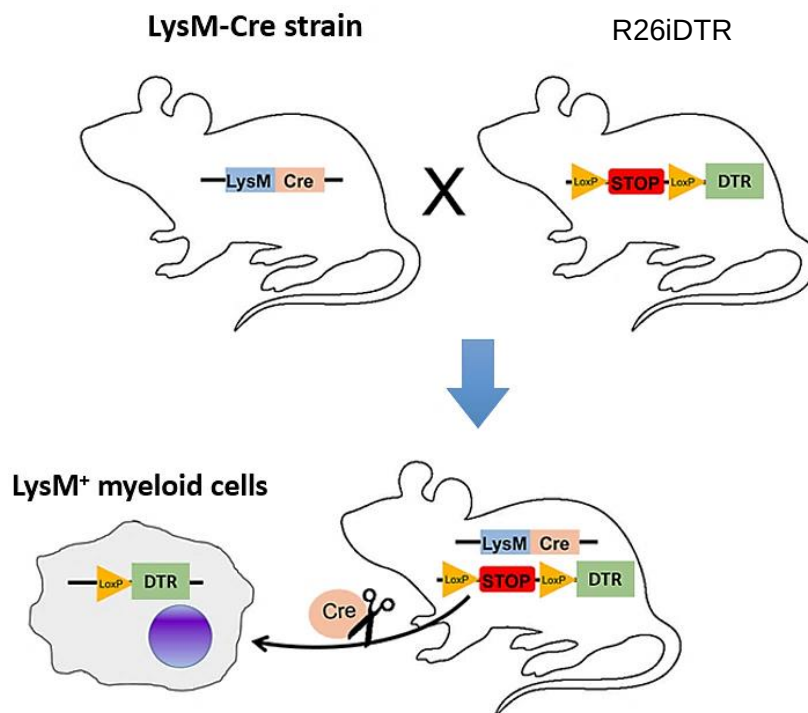


Figure 22. Schematic of the inducible DTR mouse strain. Cre/loxP system was used to generate a conditional myeloid cell-specific expression of the diphtheria toxin receptor (DTR) by crossing C57BL/6 Lysozyme M (LysM)-Cre transgenic mice with C57BL/6 ROSA26STOP*DTR(R26iDTR) mice. LysM⁺ myeloid cells express Cre recombinase, which mediates removal of the STOP sequence and drives expression of DTR. When DT is administered to LysM-Cre[+]/R26iDTR mice, myeloid lineage cells are depleted. (Adapted from Shi et al. Methods Mol Bio, 2018).

Upon DT administration, the DTR-expressing myeloid cells can be eliminated via apoptosis. Briefly, DT is a bifunctional A–B toxin, whose B subunit binds to DTR on expressing cells, inducing DT endocytosis. Upon acidification of the endosome, the A subunit translocates to the cytosol where it catalyzes the inactivation of elongation factor 2 (EF-2), fundamental for protein synthesis, resulting in cell apoptosis (312) (Figure 23). In the absence of Cre recombinase in LysM-Cre[–]/R26iDTR control mice, no DTR expression on myeloid cells will be induced and, therefore, DT administration should have no deleterious effect.

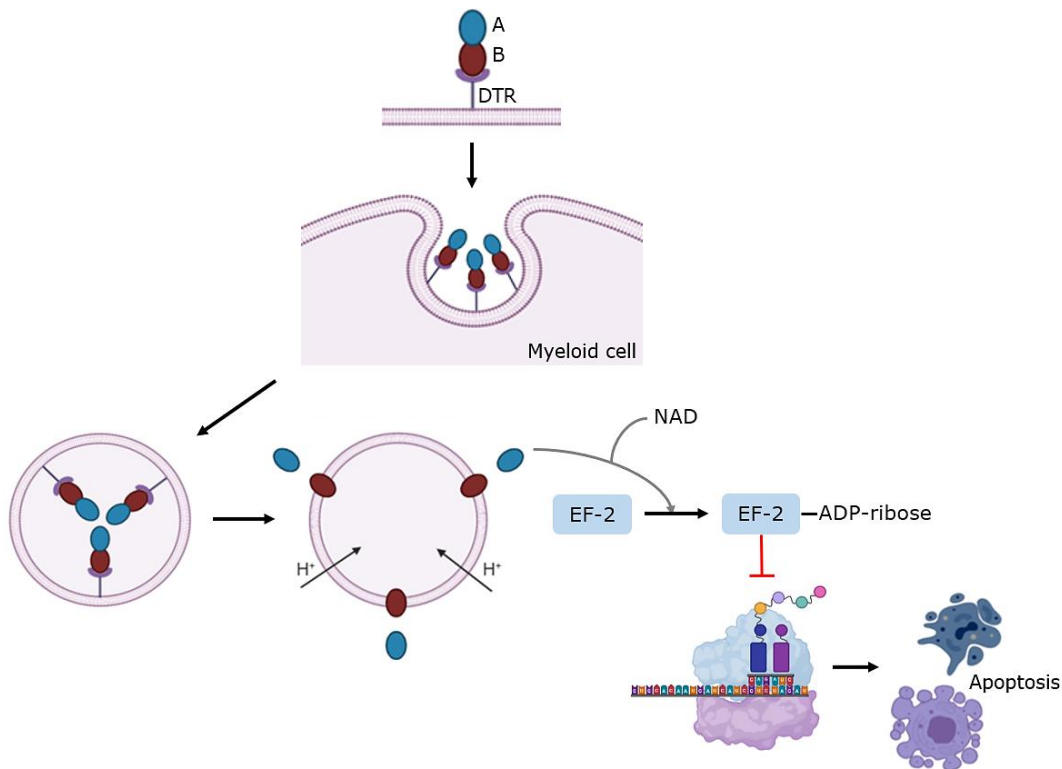


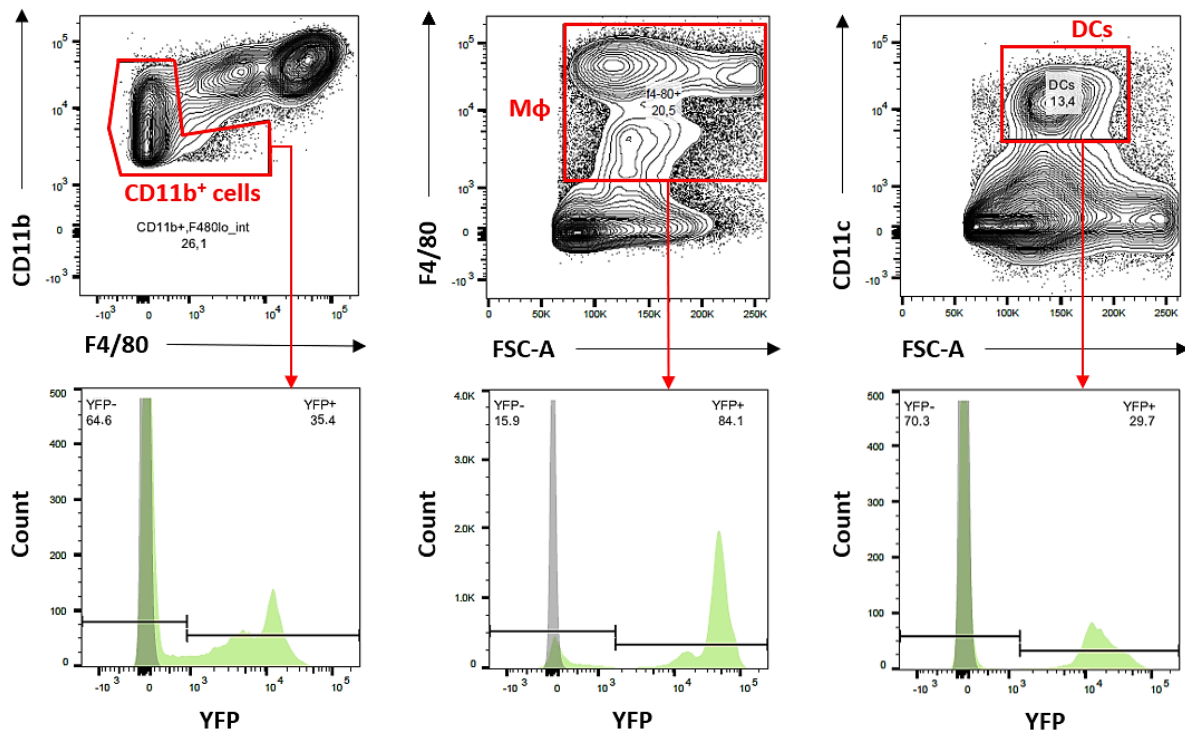
Figure 23. Diphtheria toxin mode of action. The binding of the diphtheria toxin B subunit to DTR on myeloid cells induces receptor-mediated endocytosis of DT. After acidification of the endosome, the B chain is inserted into the endosomal membrane and translocates subunit A into the cytosol, where it catalyzes the ADP ribosylation of EF-2. As a result, protein synthesis is inhibited, and the myeloid-targeted cell dies. Figure created with BioRender.com.

To evaluate both specificity and efficiency of Cre-mediated DTR expression, we crossed LysM-Cre[+] mice with a reporter strain encoding the Enhanced Yellow Fluorescent Protein (EYFP) gene under control of the loxP-flanked STOP sequence in the ROSA26 locus

(R26EYFP). The LysM-Cre[+]/R26^{+EYFP} double transgenic mice obtained should express the EYFP in LysM-positive cells after Cre-mediated removal of the STOP cassette. So, we aimed to quantify the percentage of YFP⁺ cells and, thereby, assess the proportion of Cre-mediated loxP recombination occurring in different myeloid cell populations within different organs.

To do so, cell suspensions from the peritoneal cavity, spleen, and thymus from LysM-Cre[+]/R26^{+EYFP} mice were analyzed by flow cytometry using myeloid-specific surface markers (CD11b, CD11c, F4/80). Following the gating strategy shown in Figure 24, the proportions of YFP⁺ cells in each myeloid subset within the organs analyzed were obtained and are represented in Figure 25.

A) Peritoneal cavity



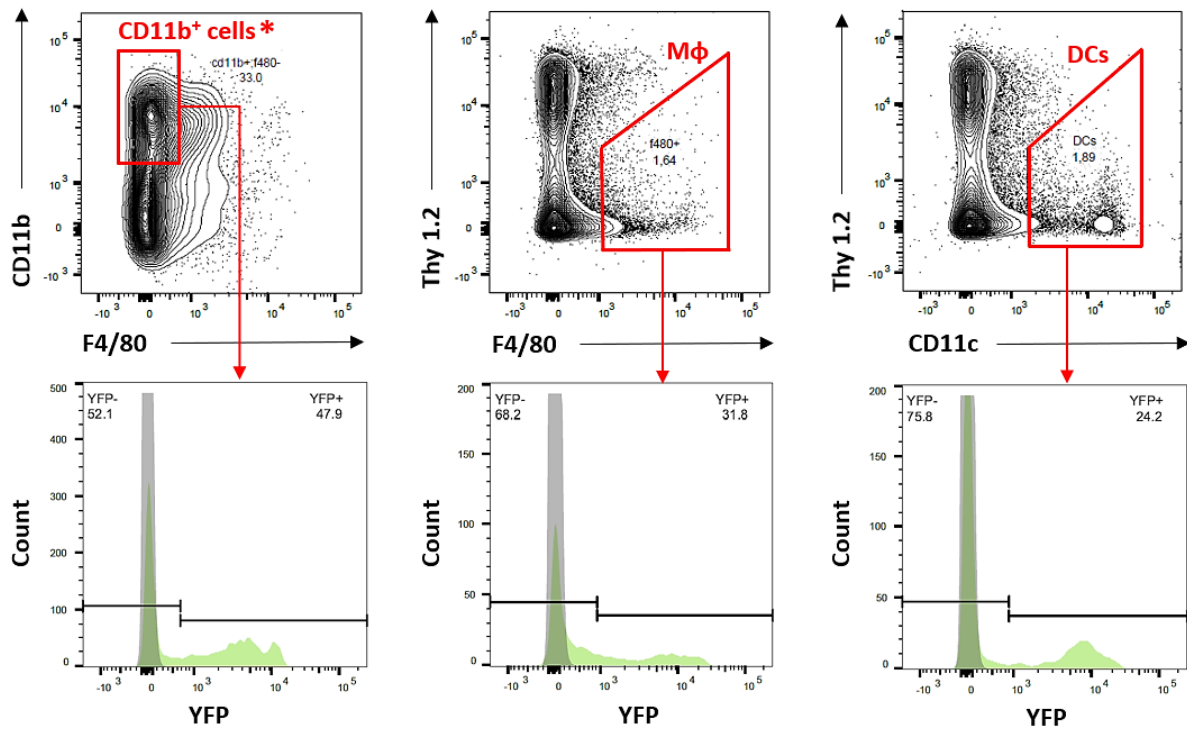
B) Lymphoid organs (thymus and spleen)

Figure 24. Representative gating strategy to assess YFP expression in myeloid populations within the peritoneal cavity and lymphoid organs (spleen and thymus) of *LysM-Cre*[+]/*R26*^{+/EYFP} mice. Cell suspensions from the peritoneal cavity, spleen, and thymus of *LysM-Cre*[+]/*R26*^{+/EYFP} mice were stained with myeloid markers (CD11b, F4/80, CD11c) and analyzed by flow cytometry. The gating strategy for cell viability and myeloid subsets characterization by flow cytometry is shown in Annex 2. **(A)** In the peritoneal cavity, the percentage of YFP-positive cells in CD11b⁺ cells (including granulocytes and monocytes – CD11b⁺F4/80^{low/int}), macrophages (F4/80⁺) and DCs (CD11c^{hi}) was assayed. **(B)** In both spleen and thymus, macrophages (F4/80⁺Thy1.2⁻) and DCs (CD11c⁺Thy1.2⁻) were analyzed for YFP expression. * In particular, in the spleen, CD11b⁺ cells (CD11b⁺F4/80⁻), including splenic monocytes/MOMF and granulocytes, were additionally assessed for YFP transcription.

Several myeloid subsets, including CD11b⁺ cells (monocytes and granulocytes), macrophages and dendritic cells, exhibited *LysM-Cre* activity, as revealed by the expression of YFP (Figures 24 and 25). Nevertheless, this activity appeared to vary depending on the myeloid lineage, being higher in macrophages, monocytes and granulocytes, and lower in dendritic cells (Figure 25). Indeed, within the *LysM-Cre*[+]/*R26*^{+/EYFP} organs assayed, approximately 30-70% of macrophages and 50-60% of CD11b⁺ cells expressed YFP, while in DCs this percentage was around 30% or lower (Figure 25A-C). We also observed large variability in *LysM-Cre* activity between littermates, affecting more commonly CD11b⁺ cells and macrophages, but also thymic DCs, as demonstrated by the high standard deviation observed (Figure 25A-C).

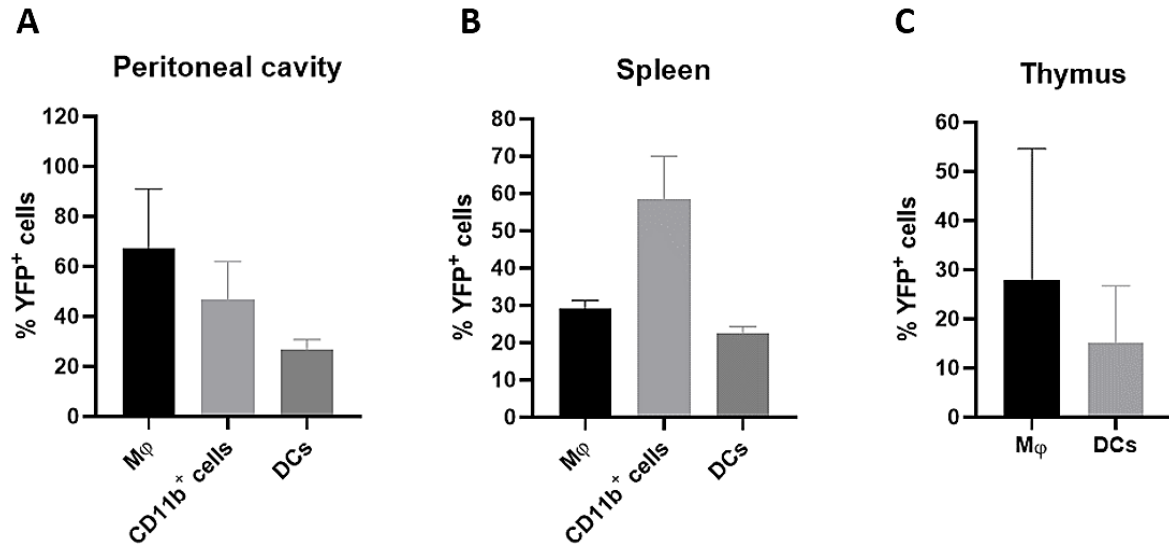


Figure 25. Proportion of YFP⁺ cells from myeloid populations (macrophages, CD11b⁺ cells and DCs) within the peritoneal cavity and lymphoid organs (spleen and thymus) of LysM-Cre⁺/R26^{+/EYFP} mice. Peritoneal, splenic and thymic cell-suspensions from LysM-Cre⁺/R26^{+/EYFP} transgenic mice were analyzed by flow cytometry to assess YFP expression (macrophages, CD11b⁺ cells and DCs were defined as in Figure 24 and Annex 2). Graphs depict the percentage of YFP⁺ cells from myeloid subsets in the **(A)** peritoneal cavity, **(B)** spleen and **(C)** thymus. Bars represent the mean ± SD from 2 mice analyzed. Mφ, macrophages; CD11b⁺, CD11b-positive myeloid cells; DCs, dendritic cells

In particular, the peritoneal macrophages exhibited the highest proportion of YFP⁺ cells (≈70%), among the myeloid subsets (Figure 25A). The peritoneal cavity is known to comprise two populations of macrophages, the large (LPMs) and the small (SPMs) peritoneal macrophages, mainly differentiated by size and CD11b and F4/80 expression levels (313). So, we additionally gated for these subsets within peritoneal macrophages and evaluated their LysM-Cre activity (Figure 26).

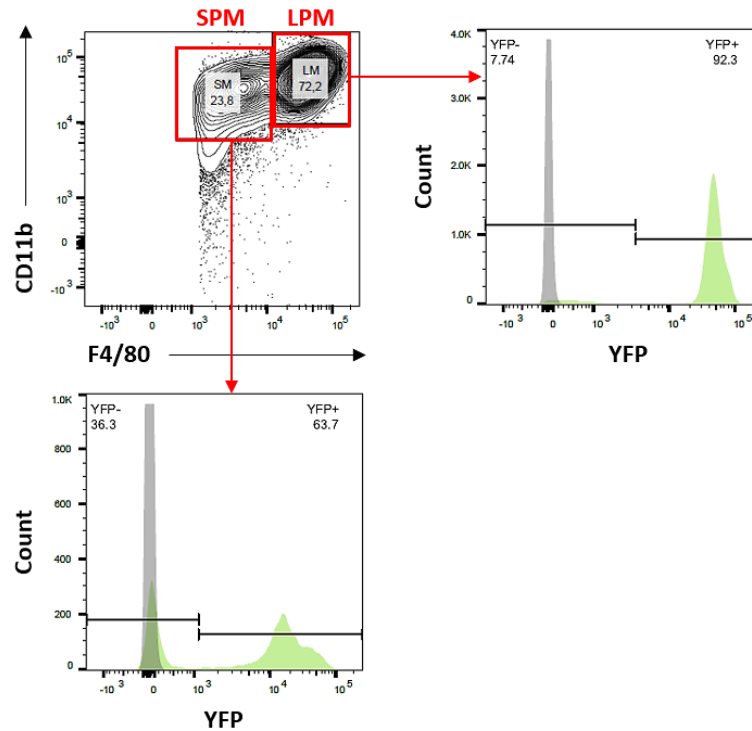


Figure 26. Representative gating strategy to assess YFP expression in peritoneal macrophage subsets of LysM-Cre⁺/R26^{+/EYFP} mice. Cell suspensions from the peritoneal cavity of LysM-Cre⁺/R26^{+/EYFP} mice were stained for prototypical M ϕ markers (F4/80 and CD11b) and analyzed by flow cytometry. SPM (CD11b^{hi}F4/80⁺) and LPM (CD11b^{hi}F4/80^{hi}) were identified and assessed for YFP expression.

The analysis revealed that within the peritoneal cavity of LysM-Cre⁺/R26^{+/EYFP} mice, LPMs represented the majority of macrophages ($\approx 70\%$ of M ϕ – Figure 26) and exhibited the highest recombination rate ($\approx 90\%$, compared to 50% in SPMs) (Figure 27), as well.

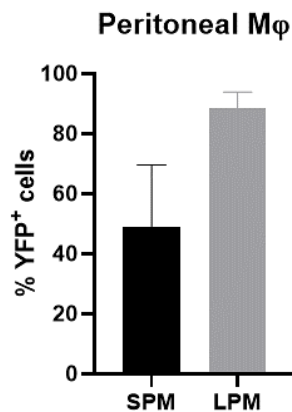


Figure 27. Proportion of YFP⁺ cells from peritoneal macrophage subsets of LysM-Cre⁺/R26^{+/EYFP} mice. Peritoneal cell-suspensions from LysM-Cre⁺/R26^{+/EYFP} transgenic mice were analyzed by flow cytometry to assess YFP expression (SPMs and LPMs were defined as in Figure 26 and Annex 2). Graph depicts the percentage of YFP⁺ cells from SPMs and LPMs in the peritoneal cavity. Bars represent the mean $\pm$ SD from 2 mice analyzed.

Likewise, in lymphoid organs, we further assessed if Cre-mediated YFP transcription could differ between conventional and plasmacytoid DC subsets (Figure 28).

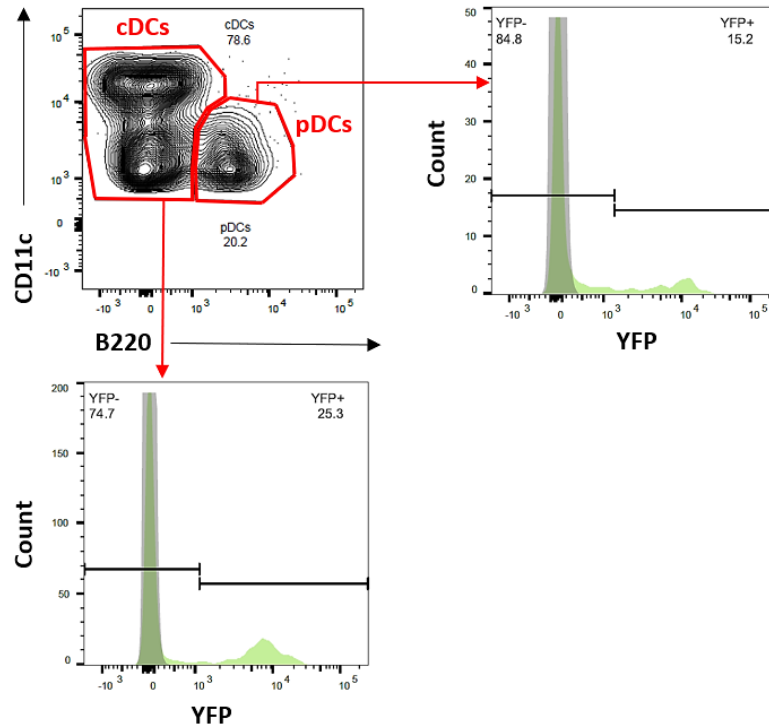


Figure 28. Representative gating strategy to assess YFP expression in DC subsets within the lymphoid organs (spleen and thymus) of *LysM-Cre[+]/R26^{EYFP}* mice. Cell suspensions from the spleen (sample represented) and thymus of *LysM-Cre[+]/R26^{EYFP}* mice were stained for DC specific marker, CD11c, together with B220 to distinguish cDCs (CD11c⁺B220⁻) from pDCs (CD11c^{low}B220⁺), and analyzed by flow cytometry.

In both thymus and spleen of reporter mice, cDCs (≈15-30%) displayed a higher proportion of YFP-expressing cells in comparison with pDCs (≈5-10%) (Figure 29A and B).

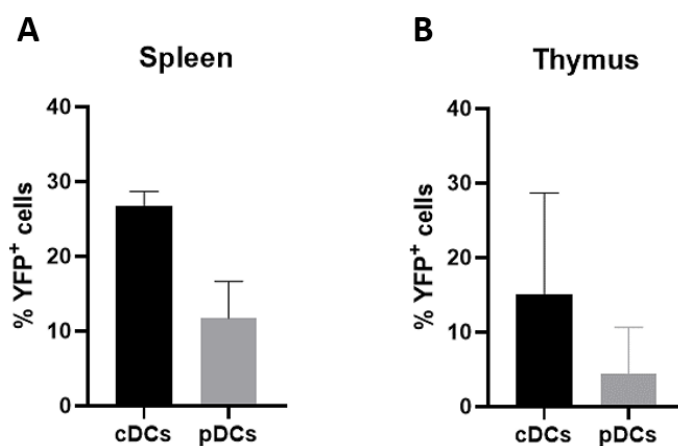


Figure 29. Proportion of YFP⁺ cells from DC subsets within the lymphoid organs (spleen and thymus) of *LysM-Cre[+]/R26^{EYFP}* mice. Splenic and thymic cell-suspensions from *LysM-Cre[+]/R26^{EYFP}* transgenic mice were analyzed by flow cytometry to assess YFP expression (cDCs and pDCs were defined as in Figure 28 and Annex 2). Graphs depict the percentage of YFP⁺ cells from cDCs and pDCs in the (A) spleen and (B) thymus. Bars represent the mean ± SD from 2 mice analyzed.

Even though LysM is known to be expressed in several myeloid cells (myeloblasts, macrophages, granulocytes (mainly neutrophils), and DCs) (310, 311, 314), it was previously shown that both lymphoid and erythroid cells can also exhibit lysozyme M expression at low levels (315). Using the reporter system, we investigated if LysM promoter activity was found in lymphoid subsets (T and B cells) within the peritoneal cavity and lymphoid organs of LysM-Cre⁺/R26^{+EYFP} mice (Figure 30). To do so, cell suspensions from these organs were analyzed by flow cytometry using surface markers specific for T (Thy1.2) and B (B220) cells. The gating strategy used to identify the lymphoid populations is shown in Figure 30 and the corresponding proportions of YFP⁺ cells obtained by FACS are represented in Figure 31.

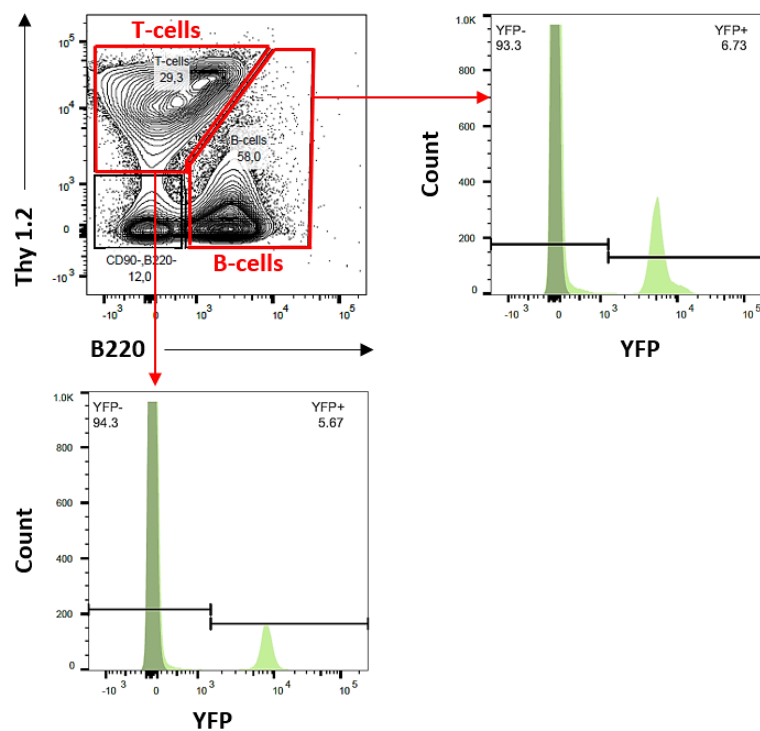


Figure 30. Representative gating strategy to assess YFP expression in lymphoid subsets within the peritoneal cavity and lymphoid organs (spleen and thymus) of LysM-Cre⁺/R26^{+EYFP} mice. Cell suspensions from the peritoneal cavity (sample represented), spleen, and thymus of LysM-Cre⁺/R26^{+EYFP} mice were stained for T- and B-lymphoid markers (Thy1.2 and B220, respectively) and analyzed by flow cytometry. In all organs, T-cells (Thy1.2⁺B220⁻) and B-cells (B220⁺Thy1.2⁻) were assessed for YFP expression.

Lysozyme M-mediated Cre activity was detected in low percentages (5-10%) in both T and B cells within the organs addressed (Figure 31A-C), which supports the idea of multipotent progenitors expressing the Lysozyme M gene, as previously suggested (315).

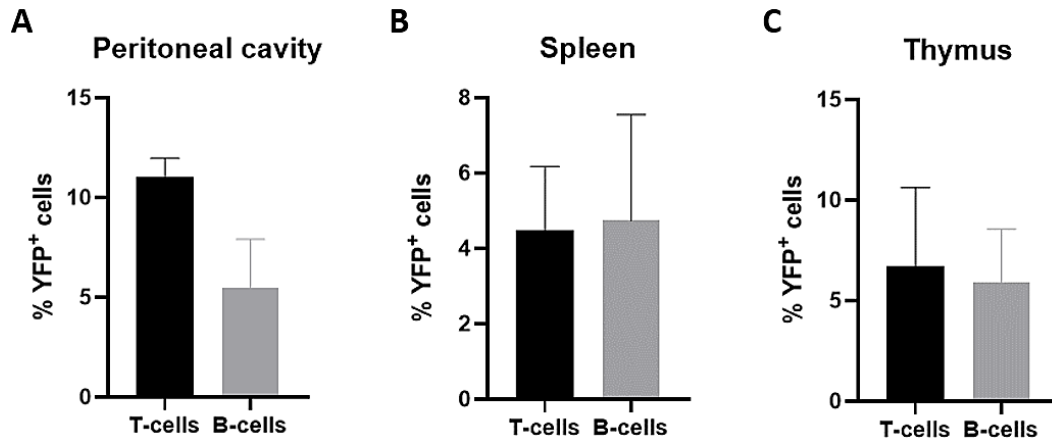


Figure 31. Proportion of YFP⁺ cells from lymphoid subsets within the peritoneal cavity and lymphoid organs (spleen and thymus) of LysM-Cre⁺/R26^{+/EYFP} mice. Peritoneal, splenic and thymic cell-suspensions from LysM-Cre⁺/R26^{+/EYFP} transgenic mice were analyzed by flow cytometry to assess YFP expression (T cells and B cells were defined as in Figure 30 and Annex 2). Graphs depict the percentage of YFP⁺ cells from T and B cells in the **(A)** peritoneal cavity, **(B)** spleen and **(C)** thymus. Bars represent the mean $\pm$ SD from 2 mice analyzed.

Additionally, we performed an immunofluorescence analysis of splenic and thymic sections from LysM-Cre⁺/R26^{+/EYFP} mice to confirm Cre-mediated YFP expression on myeloid cells. To do so, tissue samples were stained with a myeloid marker, F4/80 or CD11c, together with an antibody against GFP (which recognizes the related YFP protein), to detect YFP (Figure 32). In both organs, we could observe co-expression of YFP and F4/80 (Figure 32A and B) or CD11c (Figure 32C and D), confirming that Cre-mediated loxP recombination took place in macrophages and dendritic cells, respectively. Supporting the flow cytometry results (Figure 25), we observed a larger proportion of F4/80⁺ macrophages (Figure 32A and B) expressing YFP in comparison to CD11c⁺ DCs (Figure 32C and D). These results underline the differential activity of the Lysozyme M promoter according to myeloid sub-lineage.

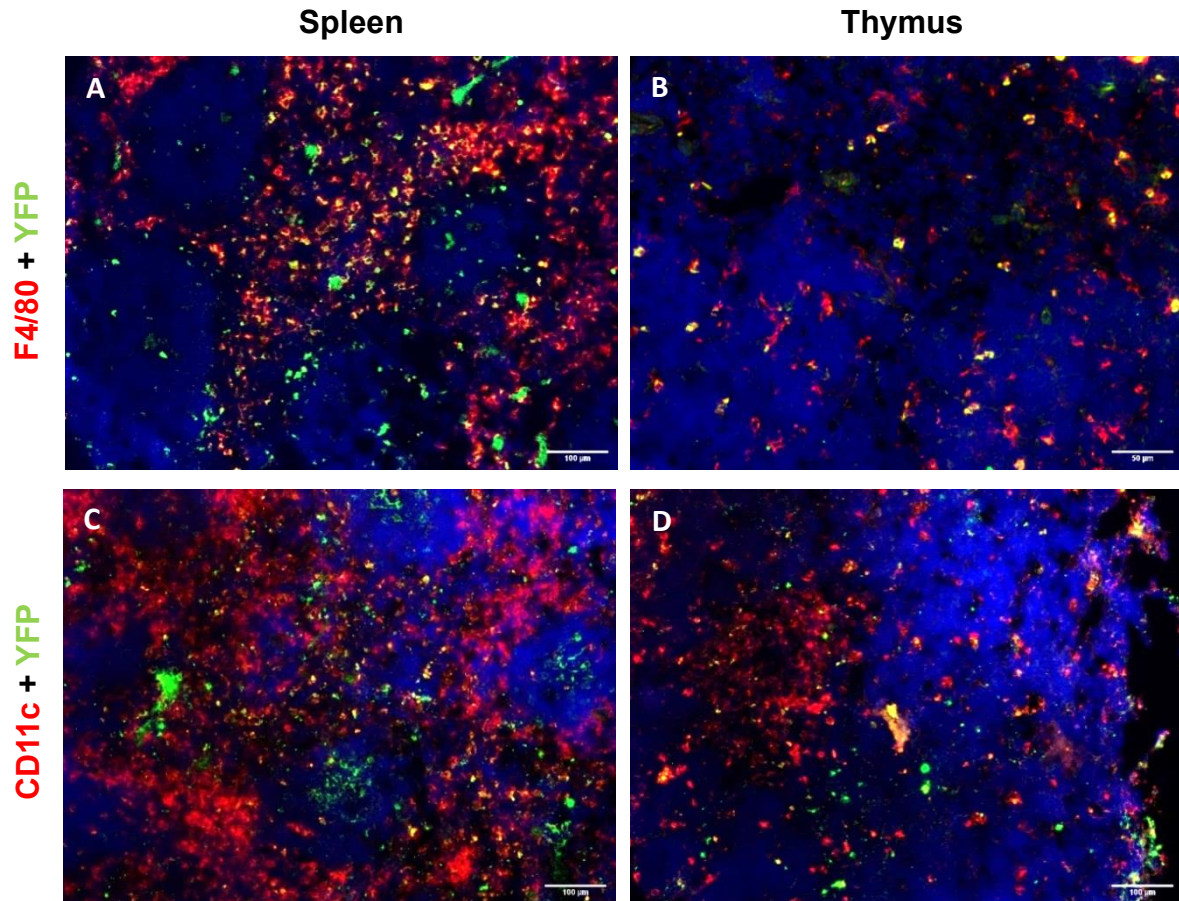


Figure 32. Assessing Cre-mediated YFP expression in macrophages and DCs from lymphoid organs (spleen and thymus) of LysM-Cre/R26EYFP mice. Splenic (A, C) and thymic (B, D) sections from LysM-Cre[+]/R26^{+EYFP} mice were immunolabeled for GFP (in green) and F4/80 or CD11c (in red), to assess YFP expression on macrophages or DCs, respectively. DAPI staining (blue) was additionally performed to reveal cell nuclei. (A) Scale bar: 100 μ m (Original magnification 10x). (b) was obtained by combining 4 sequential images (grid stitching, ImageJ); Scale bar: 50 μ m (Original magnification 20x). (C, D) Scale bar: 100 μ m (Original magnification 10x).

Next, to assess the specificity of our model, we set out to confirm that DTR was expressed in LysM-Cre/R26iDTR mice. To do so, we performed immunofluorescence analysis of peritoneal macrophages isolated from LysM-Cre/R26iDTR mice. The mouse peritoneal cavity is enriched in macrophages that can be easily collected for analysis (313, 316, 317). Besides, we observed that peritoneal macrophages displayed high activity of LysM promoter (Figure 25 and 27). Thus, we stained macrophages isolated from the peritoneal cavity of LysM-Cre[+]/R26^{+iDTR} and LysM-Cre[-]/R26^{+iDTR} mice with antibodies against F4/80 and simian/human DTR to identify macrophages and assess DTR expression, respectively (Figure 33). As expected, we could observe co-expression of F4/80 and DTR proteins in LysM-Cre[+]/R26^{+iDTR} mice, confirming that Cre-mediated loxP recombination took place in myeloid cells from these mice. In particular, DTR staining was detected on macrophages membrane and cytoplasm (Figure 33B). In contrast, no DTR expression was

detected in F4/80⁺ macrophages from LysM-Cre[-]/R26^{+/iDTR} mice, corroborating the absence of Cre recombinase expression (Figure 33A).

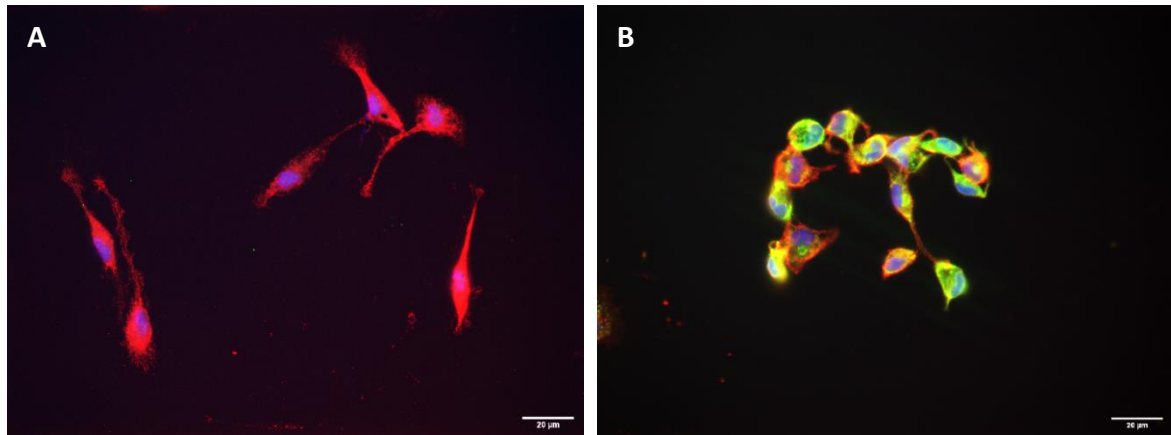


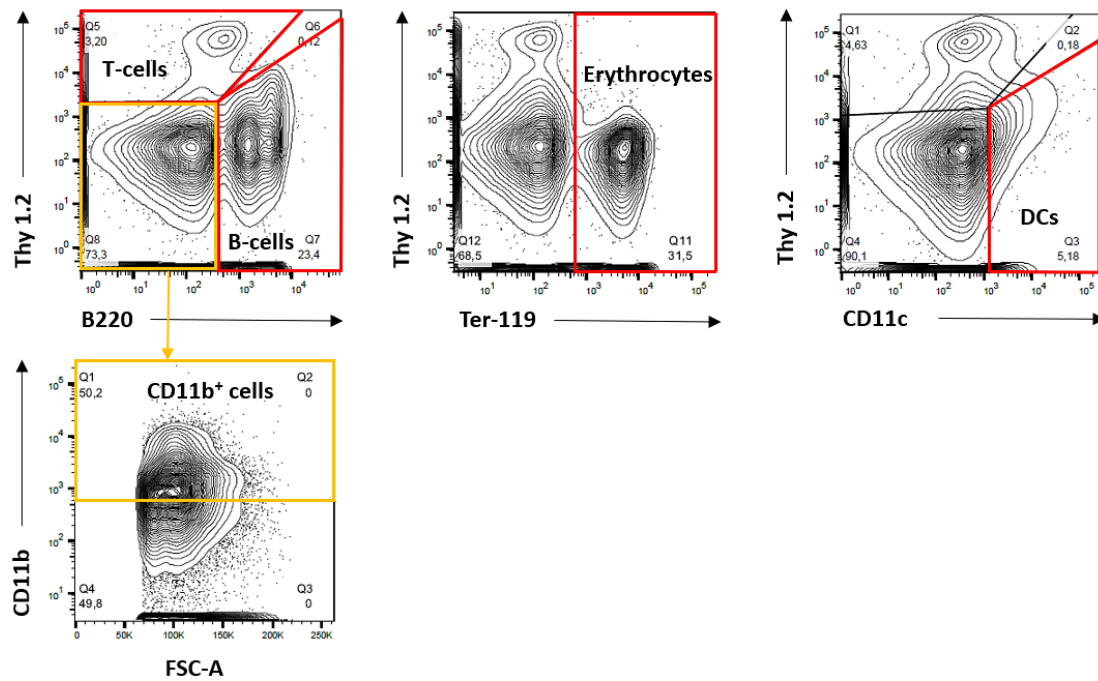
Figure 33. Assessing Cre-mediated DTR expression in peritoneal macrophages from LysM-Cre/R26iDTR mice. Macrophages isolated from the peritoneal cavity of LysM-Cre[-]/R26^{+/iDTR} (A) and LysM-Cre[+]/R26^{+/iDTR} (B) mice were immunolabeled for F4/80 (in red) and huDTR (in green) to identify macrophages and assess DTR expression, respectively. DAPI staining (blue) was additionally performed to reveal cell nuclei. Scale bar: 25 µm (Original magnification 40x).

Taken together, these results demonstrate that the LysM-Cre strain targets multiple myeloid populations in different organs, allowing for an efficient Cre-mediated gene expression in macrophages and CD11b⁺ cells and only partly in dendritic cells. Moreover, R26iDTR was able to efficiently induce DTR expression on myeloid cells mediated by LysM-Cre recombinase activity.

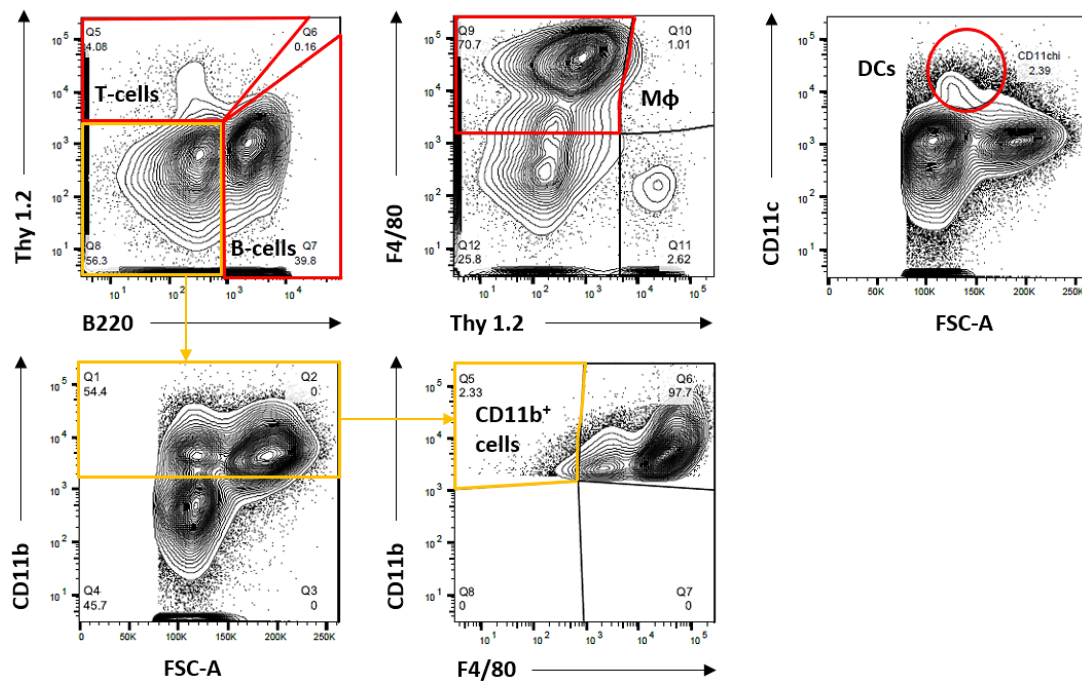
2.2. Optimization of Diphtheria Toxin-driven myeloid cell ablation *in vivo*

After confirming both specificity and efficiency of LysM-Cre strain, we proceeded to deplete myeloid cells via DT administration to LysM-Cre/R26iDTR mice. Thus, LysM-Cre[+]/R26^{+/iDTR} and LysM-Cre[-]/R26^{+/iDTR} (control) mice were treated intraperitoneally with variable doses of DT, following different administration regimens in order to achieve an optimized protocol for DT-mediated myeloid cell ablation. Peripheral blood, lymphoid organs (thymus, spleen, bone marrow) and peritoneal exudate were analyzed by flow cytometry to monitor myeloid cell depletion, as well as any alteration in the proportion of other cell types, after DT treatment. Following the gating strategy shown in Figure 34, the frequency of myeloid, lymphoid and erythroid subsets within the organs assayed were obtained and are represented in Figure 36. In addition, euthanized mice were re-genotyped by PCR to confirm Cre transgene presence and validate the results.

A) Bone Marrow



B) Peritoneal Cavity



C) Spleen and Thymus

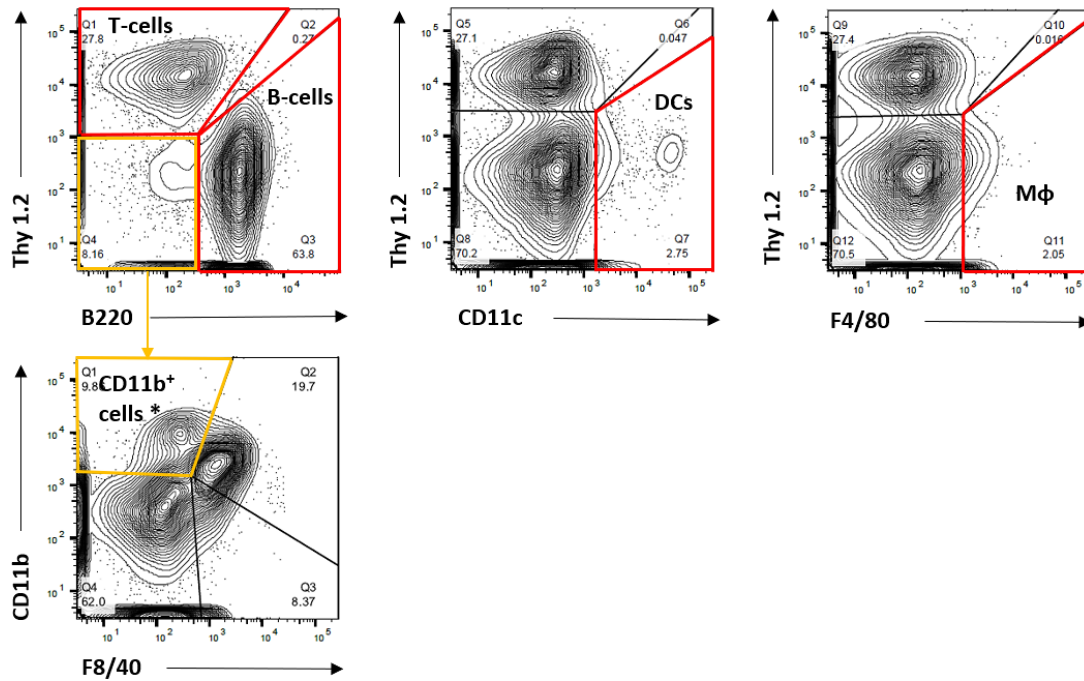


Figure 34. Representative gating strategy to identify myeloid, lymphoid, and erythroid subsets in the bone marrow, peritoneal cavity, and lymphoid organs (spleen and thymus). Cell suspensions from the bone marrow, peritoneal cavity, spleen, and thymus of LysM-Cre⁻/R26^{+/iDTR} and LysM-Cre⁺/R26^{+/iDTR} mice were analyzed by flow cytometry. Cell viability was assessed using Live/Dead staining. **(A)** In the bone marrow, T cells (Thy1.2⁺), B cells (B220⁺), erythrocytes (Ter-119⁺Thy1.2⁻), monocytes/granulocytes (CD11b⁺) and DCs (CD11c⁺Thy1.2⁻) were examined. **(B)** In the peritoneal cavity, macrophages (F4/80⁺Thy1.2⁻), CD11b⁺ cells (CD11b⁺F4/80^{lo/neg}), and DCs (CD11c^{hi}) were assayed. T- and B-cells were defined as for BM. **(C)** In the spleen and thymus, macrophages (F4/80⁺Thy1.2⁻) and DCs (CD11c⁺Thy1.2⁻) were analyzed. T and B cells were defined as for the BM. * In particular, in the spleen, CD11b⁺ cells (CD11b⁺F4/80^{lo/neg}), including splenic monocytes and granulocytes, were additionally assessed.

We started by injecting LysM-Cre/R26iDTR mice with a single dose of 50 ng/g of DT ($\approx 1 \mu\text{g}$ per mice), and then euthanized the animals 1, 2, 4 or 6 days after DT injection to evaluate if this amount of toxin was sufficient to induce ablation of DTR-expressing cells and if so, at which timepoint ablated cell types begin to recover (Figure 35).

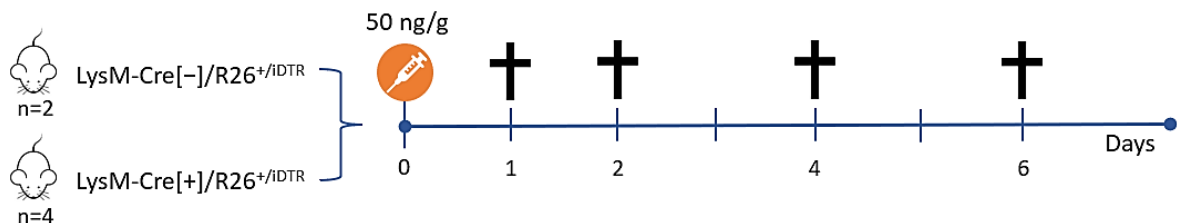
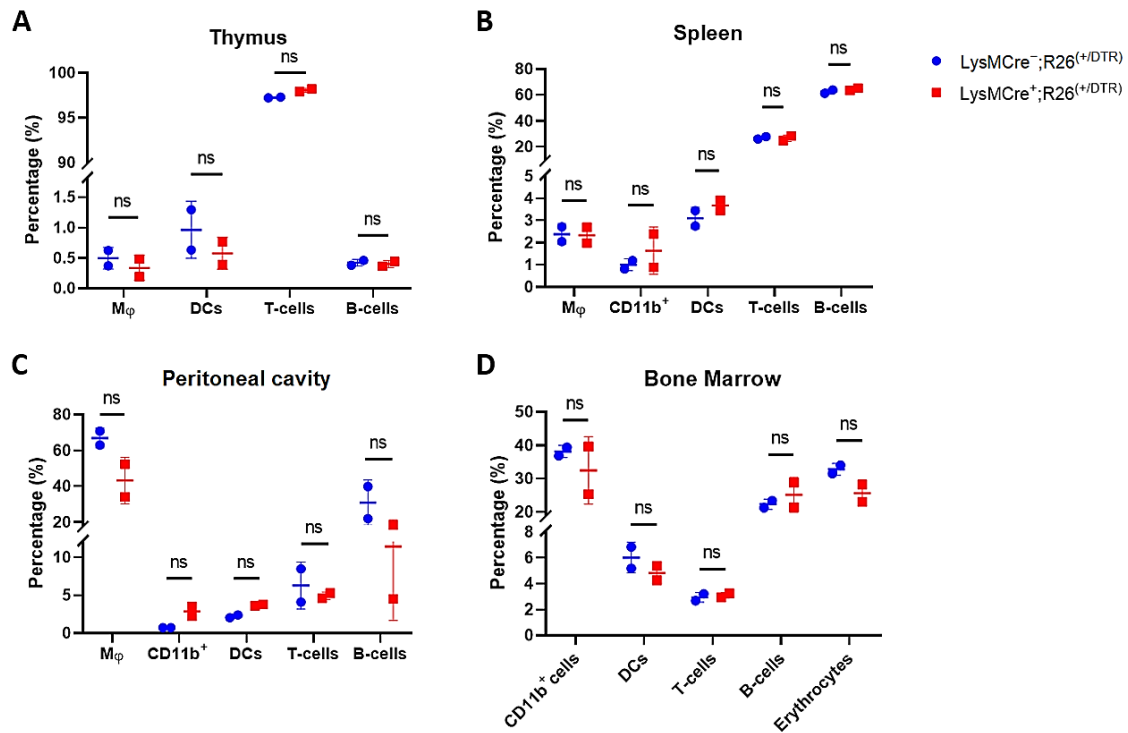


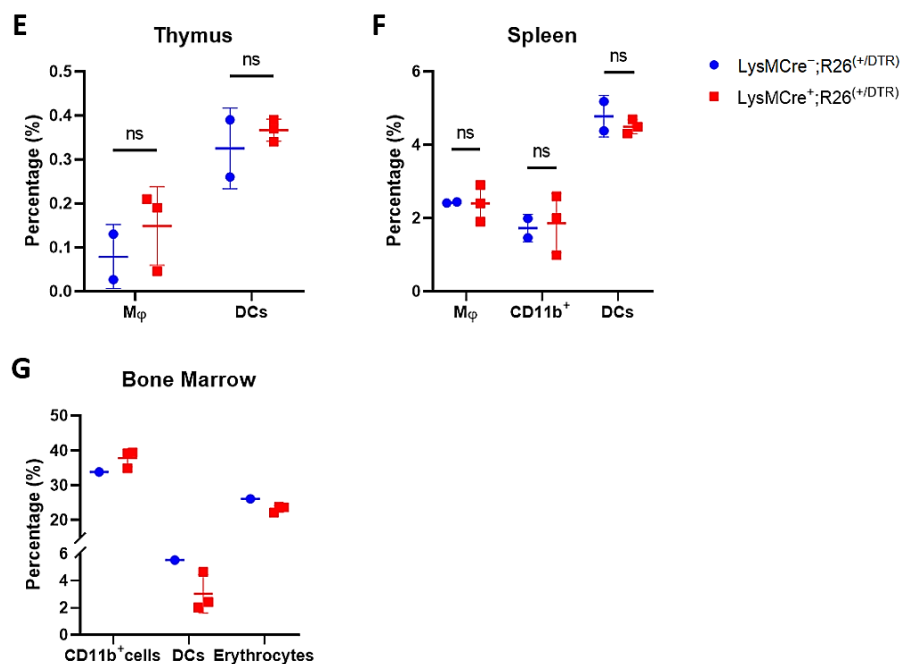
Figure 35. Schematic representation of Diphtheria Toxin treatment. LysM-Cre⁻/R26^{+/iDTR} and LysM-Cre⁺/R26^{+/iDTR} mice were injected intraperitoneally with 50 ng of DT per gram of body weight, and then euthanized 1, 2, 4 or 6 days post-DT treatment for flow cytometry analysis.

We used 50 ng/g of DT as our starting concentration, based on previous experiments performed by our group involving another DTR-expressing mouse strain (Krt5-Cre/R26iDTR - data not published) in which this amount of toxin efficiently depleted DTR-expressing epithelial cells.

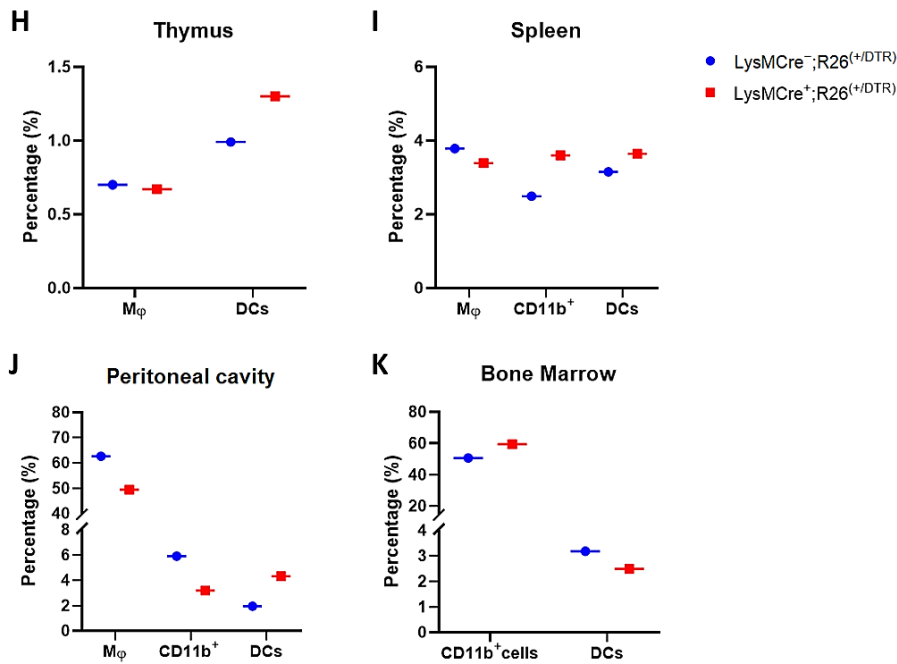
I) 1 day post-DT treatment



II) 2 days post-DT treatment



III) 4 days post DT-treatment



IV) 6 days post-DT treatment

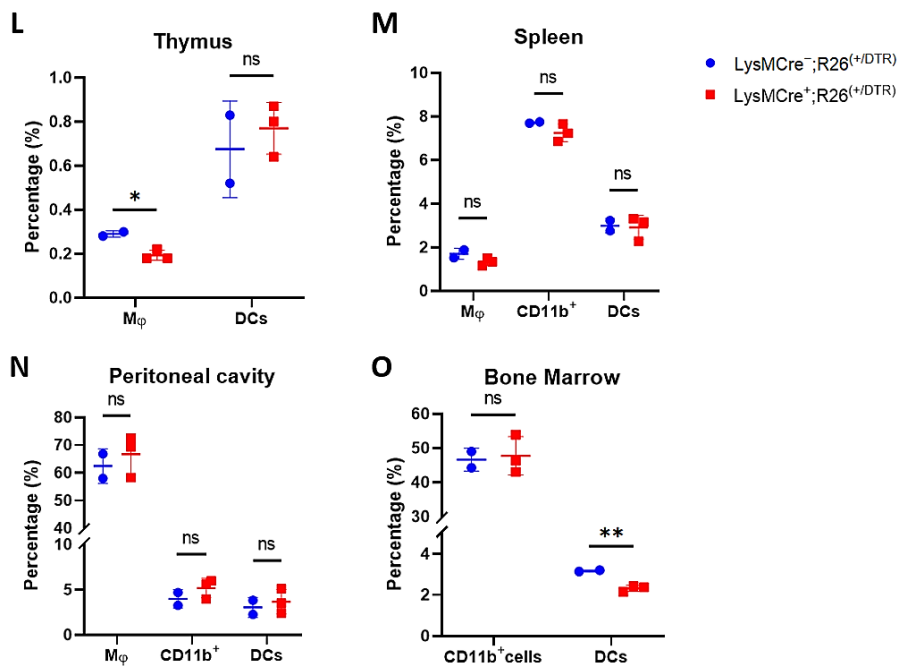


Figure 36. Monitoring DT-driven cell ablation in LysM-Cre/R26^{+/IDTR} mice. LysM-Cre⁻/R26^{+/IDTR} and LysM-Cre⁺/R26^{+/IDTR} mice were treated as shown in Figure 35 and euthanized at different timepoints, having their organs (thymus, spleen, bone marrow and peritoneal exudate) collected for flow cytometry analysis. Graphs depict the percentage of myeloid (CD11b⁺ cells, Mφ and DCs), lymphoid (T- and B- cells) and erythroid populations (defined as in Figure 34) within the thymus, spleen, bone marrow and peritoneal cavity of LysM-Cre⁻/R26^{+/IDTR} and LysM-Cre⁺/R26^{+/IDTR} mice, (A) 1, (B) 2, (C) 4, and (D) 6 days post-treatment (DT 50 ng/g). *P* values were determined by two-tailed, unpaired *t*-test (**, *P* ≤ 0.01; *, *P* ≤ 0.05; ns (non-significant), *P* > 0.05). (Data are presented with mean ± SEM; each symbol represents an individual mouse).

Flow cytometry showed a decrease only in macrophages ($\approx 35\%$ reduction) from the peritoneal cavity of LysM-Cre[+]/R26^{+iDTR} mice 24h after treatment with a single i.p. injection of DT (50 ng/g) (Figure 36C). No depletion was detected on splenic myeloid populations from DTR-expressing mice (Figure 36B), but in both thymus and BM we could observe a tendency for DCs to decrease, one day after injection (Figure 36A and D). Data from the second day following DT treatment revealed a more evident depletion of BM DCs ($\approx 45\%$ reduction) in LysM-Cre[+]/R26^{+iDTR} mice (Figure 36G), suggesting that 50 ng/g of DT appears to be capable to reduce CD11c⁺ DCs in the BM. No alterations were detected for neither thymic nor splenic myeloid subsets, 2 days after DT administration (Figure 36E and F). However, at day 6 post-DT treatment, thymic macrophages were significantly ($P = 0,0142$) reduced in LysM-Cre[+]/R26^{+iDTR} mice, which suggests that 50 ng/g may be sufficient to induce M ϕ depletion in thymus, as well (Figure 36L). Moreover, BM DCs from DTR-expressing mice remained significantly ($P = 0,0047$) reduced (Figure 36O) 6 days after DT injection, which suggests that DC lifespan in the BM is superior to 6 days. On the other hand, peritoneal macrophages depletion in LysM-Cre[+]/R26^{+iDTR} mice persisted for 4 days ($\approx 20\%$ reduction) (Figure 36J), but exhibited total recovery at day 6 (Figure 36N), which means that the lifespan of these macrophages is around 5-6 days.

As mentioned before, we additionally analyzed lymphoid and erythroid subsets to assess the possibility of non-myeloid cell ablation. No alterations were observed for T and B cells one day after DT treatment in LysM-Cre[+]/R26^{+iDTR} mice, except for the decrease in B lymphocytes in the peritoneal cavity of one mouse (Figure 36C). Besides, we have also observed a decrease in erythrocytes from the BM of DT-treated LysM-Cre[+]/R26^{+iDTR} mice in comparison with control ($\approx 20\%$ decrease) (Figure 36D). Nevertheless, this reduction appears to be irrelevant since 48h-post DT treatment no differences were observed in erythrocytes proportion between the two groups (Figure 36C). Thus, we can discard the possibility of non-myeloid specific ablation.

Given that the cellular impacts of 50 ng/g of DT were mild, to say the least, we can conclude this dosage seems to be insufficient to induce a systemic myeloid cell depletion and fulfill our objectives. Thus, we tested if administrating an initial higher concentration, followed by lower maintenance doses could result in more effective and broader cell depletion. We started by injecting 100 ng/g of DT to LysM-Cre/R26iDTR mice and then collected blood the following day to quantify myeloid cell depletion. Next, these mice were daily injected with 50 ng/g of DT for 3 days ($\approx 5 \mu\text{g}$ of DT per mouse, in total), and euthanized one day after the last treatment, collecting the blood as well, to monitor the dynamics of myeloid cell depletion (Figure 37).

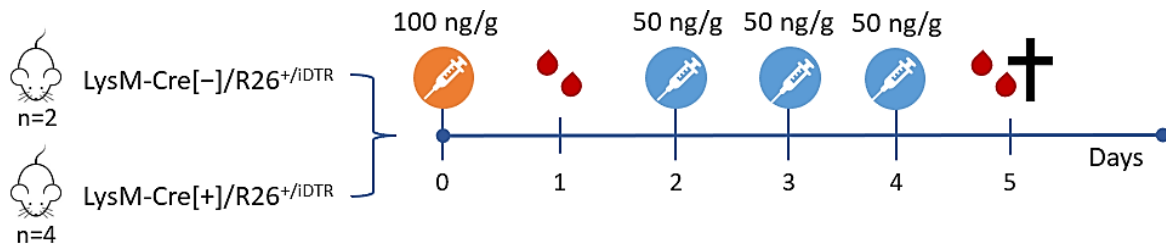


Figure 37. Schematic representation of Diphtheria Toxin treatment. LysM-Cre⁻/R26^{+/iDTR} and LysM-Cre⁺/R26^{+/iDTR} mice were injected intraperitoneally with 100 ng of DT per gram of body weight, followed by blood collection one day after. Then, for three consecutive days, DT at 50 ng/g was administrated to mice (i.p) and the blood collected after the treatment for flow cytometry analysis. n = number of animals

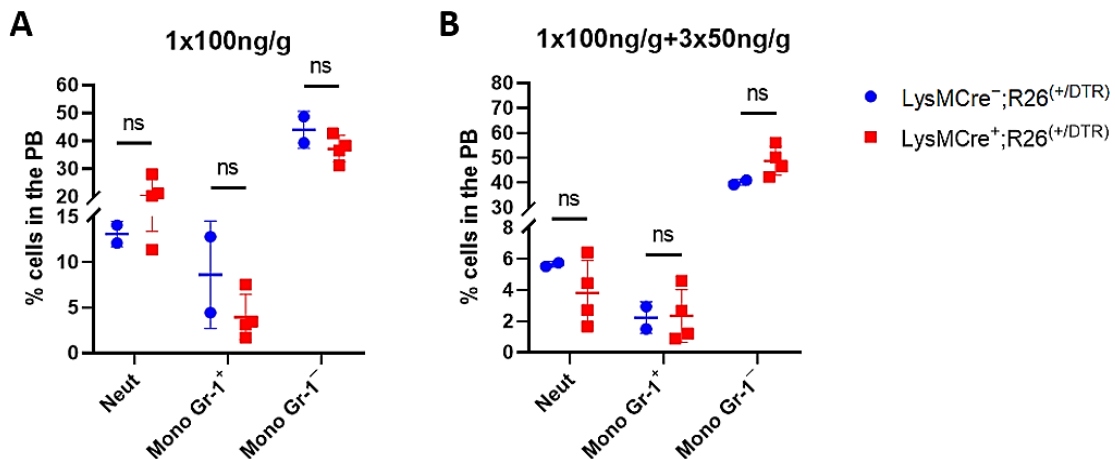


Figure 38. Monitoring DT-driven peripheral blood myeloid cell ablation in LysM-Cre/R26^{+/iDTR} mice. LysM-Cre⁻/R26^{+/iDTR} and LysM-Cre⁺/R26^{+/iDTR} mice were treated as shown in Figure 37, having their blood collected at different timepoints for flow cytometry analysis. Graphs depict the percentage of neutrophils, monocytes Gr-1⁺, and monocytes Gr-1⁻ (defined as in Figure 9 for BM or spleen) within the PB of LysM-Cre⁻/R26^{+/iDTR} and LysM-Cre⁺/R26^{+/iDTR} mice, **(A)** 1 (1x100 ng/g) or **(B)** 5 days (1x100 ng/g + 3x50 ng/g) after starting the treatment. *P* values were determined by two-tailed, unpaired *t*-test with Welch's correction when variances were unequal (*ns* (non-significant), *P* > 0.05). LysM-Cre⁻/R26^{+/iDTR} n = 2; LysM-Cre⁺/R26^{+/iDTR} = 4. (Data are presented with mean ± SEM; each symbol represents an individual mouse).

As flow cytometry analysis revealed, after a single injection of 100 ng/g of DT, LysM-Cre⁺/R26^{+/iDTR} mice exhibited a tendency for decreased Gr-1⁺ and Gr-1⁻ monocytes in circulation (~55% and ~15% reduction, respectively), while neutrophil proportion was somewhat enhanced in comparison with control mice (Figure 38A). After receiving the following 3 doses of 50 ng/g DT, LysM-Cre⁺/R26^{+/iDTR} mice showed instead a reduction in neutrophils (~30% reduction), while PB monocytes were similar to those of control mice (Figure 38B). These results suggest that 100 ng/g of DT could induce monocyte depletion, accompanied by an increase in circulating neutrophils, 24 h later. On the other hand, daily doses of DT at lower concentration (50 ng/g) enabled neutrophil depletion, while not having an effect on monocytes. Thus, to improve myeloid cell depletion we thought higher and more frequent doses of DT were need.

Based on the previous experiment, we tested an even higher concentration of DT to try to maximize myeloid cell depletion. In particular, we used LysM-Cre/R26^{EYFP/iDTR} mice, resulting from crossing LysM-Cre mice with R26EYFP/iDTR strain, harboring R26^{EYFP} reporter and R26^{iDTR} alleles at the ROSA26 locus. Myeloid cells in LysM-Cre[+]/R26^{EYFP/iDTR} mice should express both YFP and DTR, allowing us to validate both specificity and efficiency of DT-driven myeloid cell depletion. LysM-Cre[+]/R26^{+EYFP} mice, which lacked the R26iDTR gene, were used as control. Thus, LysM-Cre[+]/R26^{+EYFP} and LysM-Cre[+]/R26^{EYFP/iDTR} mice were injected i.p. with 200 ng/g DT for one ($\approx 4 \mu\text{g}$ per mice) or two days ($\approx 8 \mu\text{g}$ per mice), and euthanized 24 h later (3rd day) for spleen and peritoneal exudate flow cytometry analysis (Figure 39).

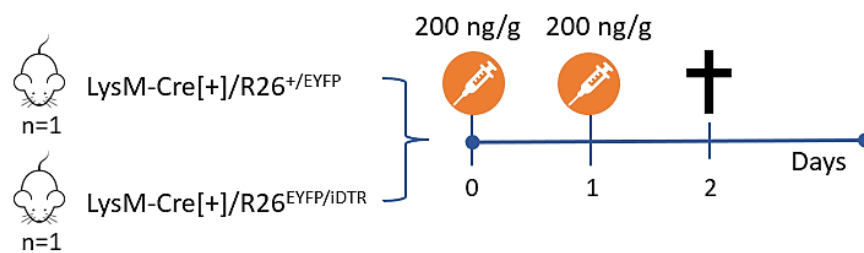


Figure 39. Schematic representation of Diphtheria Toxin treatment. LysM-Cre[+]/R26^{+EYFP} and LysM-Cre[+]/R26^{EYFP/iDTR} mice were injected intraperitoneally with 200 ng of DT per gram of body weight, for one or two days, and euthanized the following day for FACS analysis. n = number of animals, per each timepoint

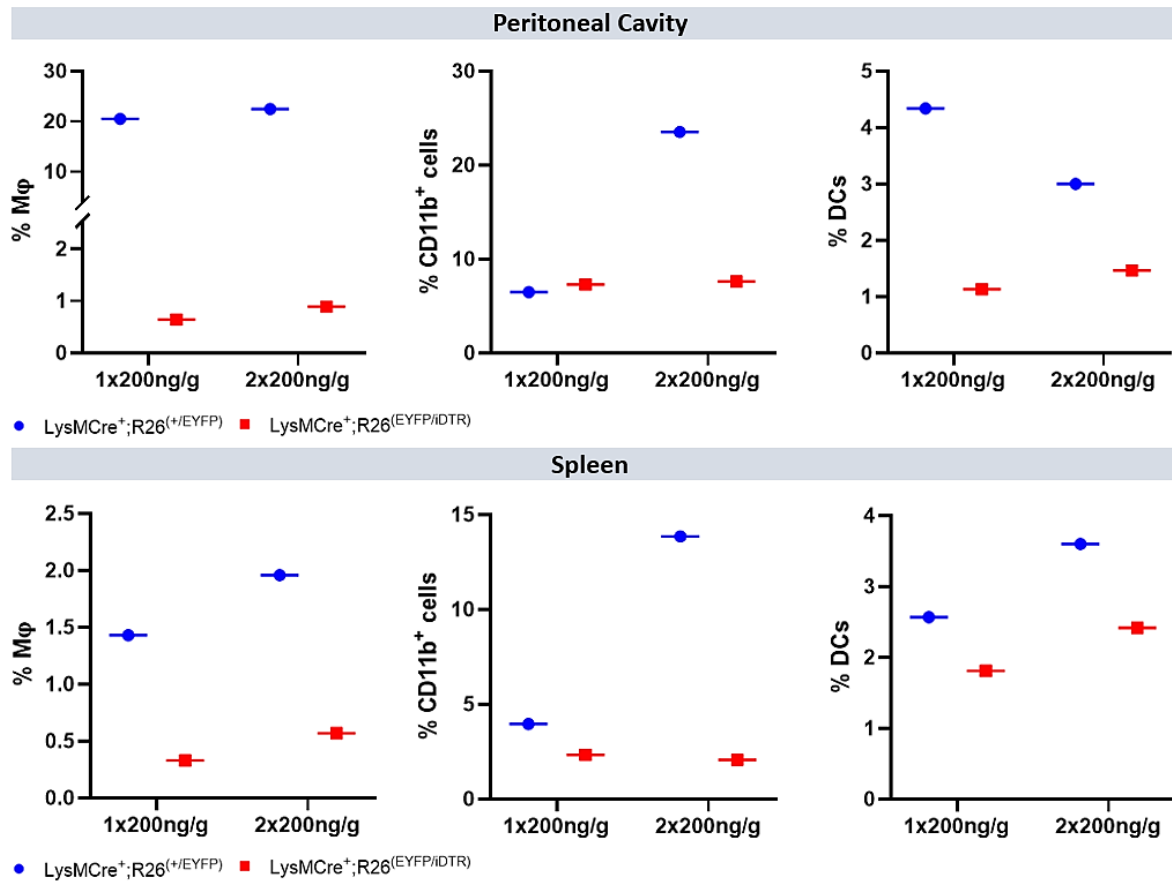


Figure 40. Monitoring DT-driven cell ablation in LysM-Cre[+]/R26^{EYFP/iDTR} mice. LysM-Cre[+]/R26^{+/EYFP} and LysM-Cre[+]/R26^{EYFP/iDTR} mice were treated as shown in Figure 39, having their organs (spleen and peritoneal exudate) collected for flow cytometry analysis. Graphs depict the percentage of macrophages, CD11b⁺ cells and DCs (defined as in Figure 34) within the peritoneal cavity and spleen of LysM-Cre[+]/R26^{+/EYFP} and LysM-Cre[+]/R26^{EYFP/iDTR} mice 48 h (1x100 ng/g)/24 h (2x200 ng/g) after the treatment. LysM-Cre[+]/R26^{+/EYFP} n = 1; LysM-Cre[+]/R26^{EYFP/iDTR} n = 1, per each timepoint (Data are presented with mean ± SEM; each symbol represents an individual mouse).

FACS analysis revealed that a single i.p. injection of DT at 200 ng/g in LysM-Cre[+]/R26^{EYFP/iDTR} mice resulted in complete depletion of peritoneal and splenic macrophages 48 h later, while having no effect in CD11b⁺ populations (Figure 40). Peritoneal DCs from DTR-expressing mice also suffered a striking decrease (≈74% reduction) after one dose of DT (200 ng/g), while splenic DCs were mildly reduced (≈30%). On the other hand, a second injection of DT did not enhance myeloid cell depletion in both organs. These data suggest that one dose of DT at 200 ng/g is sufficient to completely deplete macrophages and partially DCs, without affecting CD11b⁺ cells.

To confirm that the depleted cells were those that recombined the R26iDTR locus in LysM-Cre[+]/R26^{EYFP/iDTR} mice, we detected YFP⁺ cells within the CD11b⁻, F4/80⁻ and CD11c⁻ positive myeloid populations (Figure 41). Data shown in Figure 41 is related to peritoneal populations, but splenic subsets followed the same trend (data not shown).

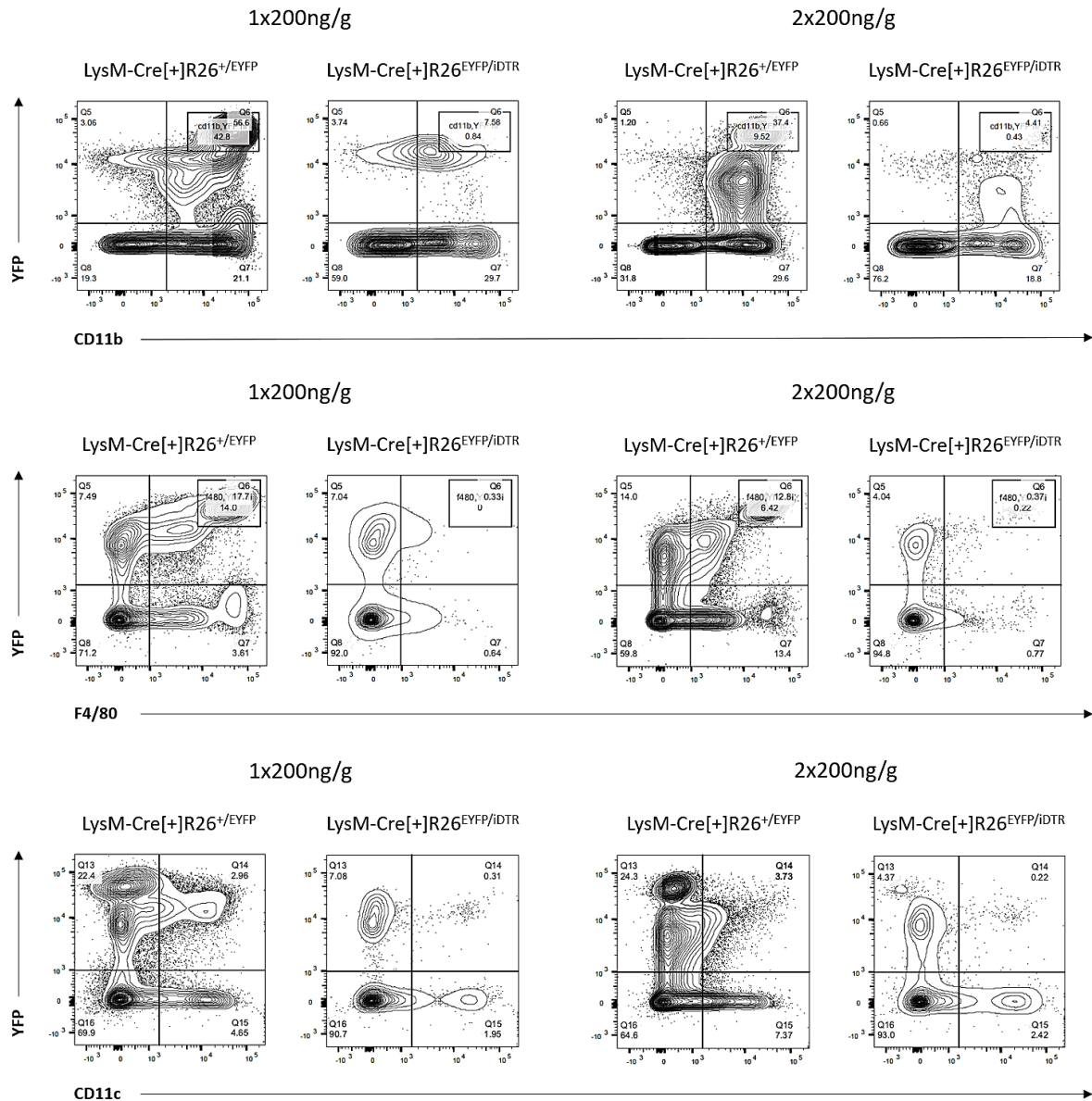


Figure 41. Assessing Cre recombinase efficiency in LysM-Cre[+]/R26^{EYFP/IDTR} mice. LysM-Cre[+]/R26^{+/EYFP} and LysM-Cre[+]/R26^{EYFP/IDTR} mice were injected i.p. with 200 ng/g of DT, during 1 or 2 days and euthanized the following day for flow cytometry analysis. Graphs depict the percentage of YFP⁺ and YFP⁻ cells within CD11b⁺, F4/80⁺ and CD11c⁺ populations from the peritoneal cavity of LysM-Cre[+]/R26^{+/EYFP} and LysM-Cre[+]/R26^{EYFP/IDTR} mice 48h (1x100 ng/g)/24h (2x200 ng/g) after the treatment. LysM-Cre[+]/R26^{+/EYFP} n = 1; LysM-Cre[+]/R26^{EYFP/IDTR} n = 1, per each timepoint (Data are presented with mean ± SEM; each symbol represents an individual mouse).

Flow cytometry showed that half or more than half of CD11b⁺ and F4/80⁺ myeloid cells in LysM-Cre[+]/R26^{EYFP/IDTR} mice expressed YFP. These cells also likely expressed DTR because most CD11b^{hi} and F4/80⁺ YFP-expressing cells were strongly reduced after DT treatment (Figure 41). On the contrary, most of the CD11c⁺ cells in LysM-Cre[+]/R26^{+/EYFP}/LysM-Cre[+]/R26^{EYFP/IDTR} mice were YFP-negative, corresponding YFP⁺ cells to one third of the total cells. Nevertheless, the majority of double positive YFP⁺CD11c⁺ cells most probably expressed DTR as confirmed by the great depletion observed in this

population after DT administration. Yet, there is still a considerable percentage of myeloid cells that were YFP-negative in both genotypes, mainly within CD11b⁺ and CD11c⁺ populations. Moreover, most F4/80⁺YFP⁻ and CD11c⁺YFP⁻ cells appeared to be DTR-expressing cells because they were efficiently depleted upon DT injection, contrary to CD11b⁺YFP⁻ cells. These results suggest that high LysM promoter activity in CD11b^{hi} and F4/80⁺ cells, which include peritoneal macrophages, induces high levels of Cre recombinase expression. The Cre recombinase consequently recombines both floxed alleles in the ROSA26 locus, resulting apparently in simultaneous YFP and DTR expression in most cells. In turn, reduced LysM promoter activity in CD11c⁺ cells, that includes DCs, likely resulted in lower expression of Cre recombinase and subsequently decreased recombination capacity, as demonstrated by the small percentage of CD11c⁺ cells that seems to be expressing both YFP and DTR. On the other hand, within cells in which Cre likely recombined only the DTR allele, the F4/80⁺YFP⁻ (macrophages) and CD11c⁺YFP⁻ (DCs) populations were efficiently depleted after a single injection of DT.

Nevertheless, 24 h after one injection with DT at 200 ng/g, half of the animals, including control mice, showed hypomotility and apparent breathing difficulties. Macroscopic observation revealed that lungs from both genotypes had a hemorrhagic appearance. This phenotype was more pronounced in LysM-Cre[+]/R26^{EYFP/DTR} mice, which also exhibited a darker liver. Thus, we performed a histology analysis to assess any DT-driven tissue alterations. H&E staining revealed diminished alveoli and congested blood vessels in the lungs of LysM-Cre[+]/R26^{+EYFP} and LysM-Cre[+]/R26^{EYFP/DTR} mice (Figure 42A and B). Moreover, abundant erythrocytes were found in the alveolar spaces and interstitial mesenchyme of DTR-expressing mice, indicating severe pulmonary congestion (Figure 42B), while control mice appeared to show signs of mild congestion (Figure 42A). Similarly, livers from LysM-Cre[+]/R26^{EYFP/DTR} animals exhibited vessels engorged with blood, suggesting that color alteration resulted from hepatic congestion (Figure 42D). These results indicate that 200 ng/g of DT is toxic and causes severe lung and liver injuries in LysM-Cre[+]/R26^{EYFP/DTR} and LysM-Cre[+]/R26^{+EYFP} mice, which suggests that murine natural DT resistance was compromised.

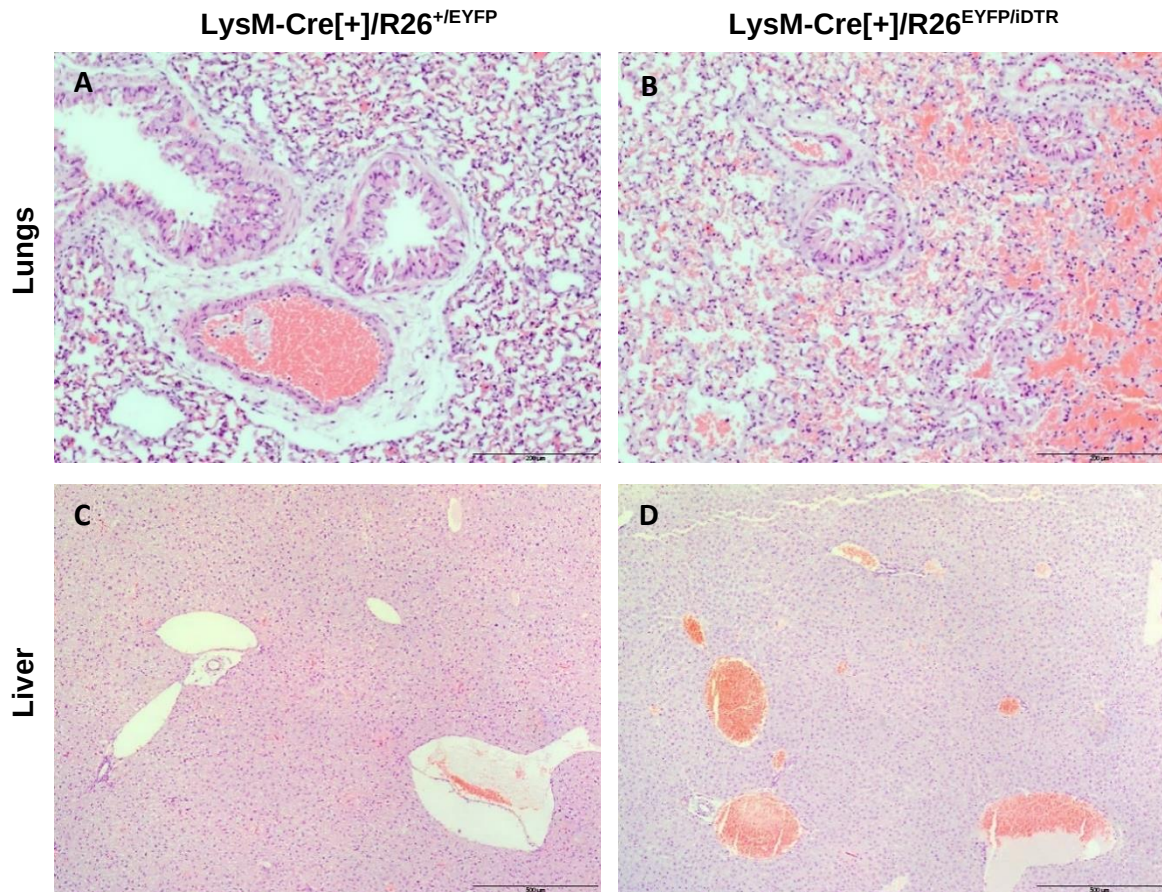


Figure 42. Microscopy characterization of lungs and livers from LysM-Cre[+]/R26^{EYFP} and LysM-Cre[+]/R26^{EYFP/iDTR} mice after treatment with 200 ng/g of DT. H&E staining of lungs and livers from LysM-Cre[+]/R26^{EYFP} (A and C, respectively) and LysM-Cre[+]/R26^{EYFP/iDTR} (B and D, respectively) mice after one injection with DT at 200 ng/g. (Lungs - original magnification x10; Liver - original magnification x4)

Given all the information collected so far and since we aimed to obtain a long-term depletion to assess its impact on leukemogenesis, we decided to administrate at first higher doses of DT at 100 ng/g, which was shown to be well-tolerated before, followed by lower doses at 50 ng/g for maintenance. For this experiment, we used TJ2-Tg/LysM-Cre/R26iDTR mice, that resulted from crossing TJ2-Tg mice with LysM-Cre/R26iDTR strain. Thus, we planned a two-phase DT regimen for young (6 week-old) healthy TJ2-Tg/LysM-Cre[-]/R26^{+iDTR} and TJ2-Tg/LysM-Cre[+]/R26^{+iDTR} mice: first, 100 ng/g of DT every other day during one week, followed by 50 ng/g DT every other two days per week until leukemia onset. However, some animals from both genotypes, revealed toxicity symptoms after the first phase of treatment, including hunched posture and hypomotility, being eventually euthanized based on human endpoints. Thus, to reduce toxicity, and still ensure efficient myeloid cell depletion, we tested injecting initial doses of 80 ng/g instead of 100 ng/g of DT on the following experimental groups.

We started by determining the extent of myeloid cell depletion after the first phase of treatment. To this end, we treated TJ2-Tg/LysM-Cre/R26^{+/iDTR} mice following the regimen shown in Figure 43 and collected the blood on the following day after last treatment for flow cytometry analysis.

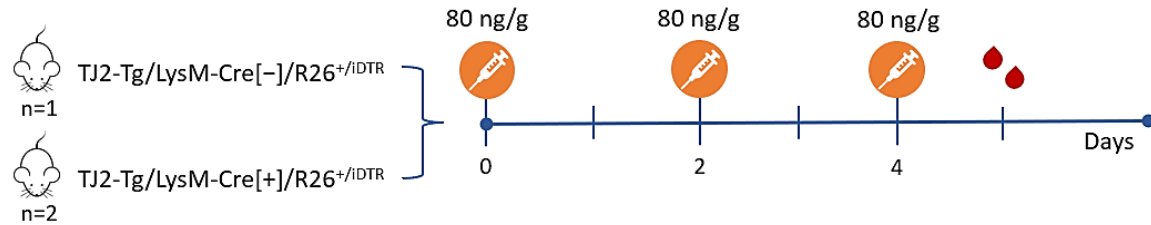


Figure 43. Schematic representation of Diphtheria Toxin treatment. TJ2-Tg/LysM-Cre[-]/R26^{+/iDTR} and TJ2-Tg/LysM-Cre[+]/R26^{+/iDTR} mice were injected intraperitoneally with 80 ng of DT per gram of body weight, every other day during one week, followed by blood collection one day after for FACS analysis. n = number of animals

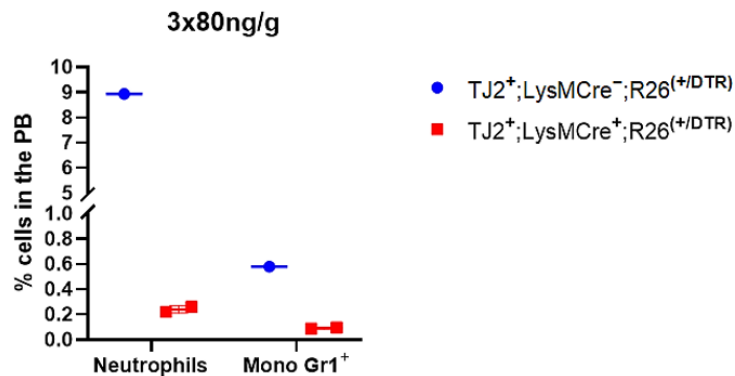


Figure 44. Monitoring DT-driven cell ablation in TJ2-Tg/LysM-Cre/R26^{+/iDTR} mice. TJ2-Tg/LysM-Cre[-]/R26^{+/iDTR} and TJ2-Tg/LysM-Cre[+]/R26^{+/iDTR} mice were treated as shown in Figure 43, having their blood collected after last treatment for flow cytometry analysis. Graph depicts the percentage of neutrophils and Gr-1⁺ monocytes (defined as in Figure 9) within the PB of TJ2-Tg/LysM-Cre[-]/R26^{+/iDTR} and TJ2-Tg/LysM-Cre[+]/R26^{+/iDTR} mice post-DT treatment. TJ2-Tg/LysM-Cre[-]/R26^{+/iDTR} n = 1; TJ2-Tg/LysM-Cre[+]/R26^{+/iDTR} n = 2 (Data are presented with mean ± SEM; each symbol represents an individual mouse).

Results showed a complete depletion of both circulating neutrophils and Gr-1⁺ monocytes in TJ2-Tg/LysM-Cre[+]/R26^{+/iDTR} mice, after 3 doses of DT at 80 ng/g. This suggests that 80 ng/g of DT efficiently induced systemic myeloid cell depletion (Figure 44).

Thus, we carried on with TJ2-Tg/LysM-Cre/R26^{+/iDTR} mice treatments adapting the strategy mentioned above to use 80 ng/g instead of 100 ng/g of DT in the first phase of treatment (Figure 45). We submitted TJ2-Tg/LysM-Cre[-]/R26^{+/iDTR} and TJ2-Tg/LysM-Cre[+]/R26^{+/iDTR} mice to the full DT regimen until mice developed leukemia and were euthanized following human endpoints. Lymphoid organs were collected to evaluate myeloid cell depletion by flow cytometry. We specifically looked for the myeloid subsets that were shown to be disturbed under TJ2 leukemia, as assessed in chapter 1.

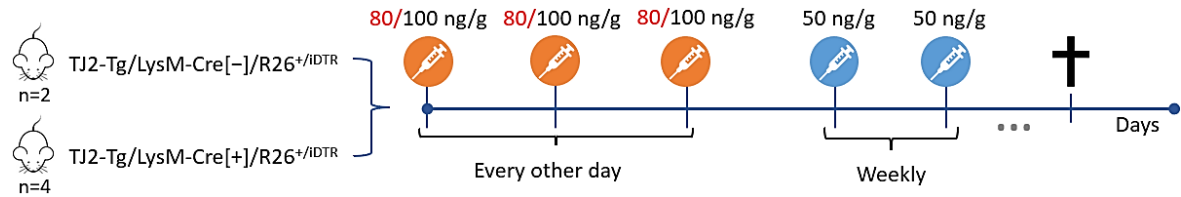
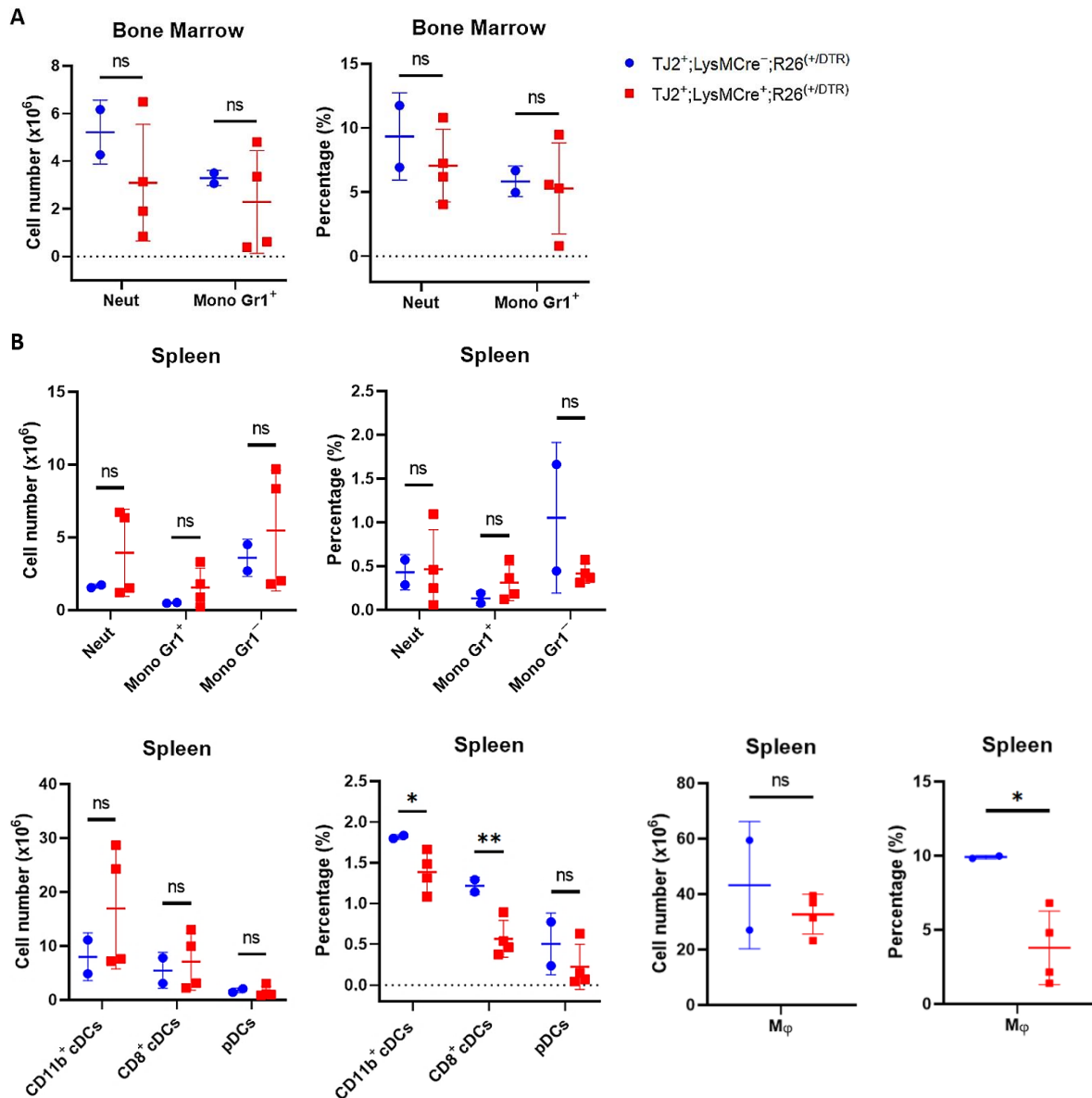


Figure 45. Schematic representation of Diphtheria Toxin treatment. TJ2-Tg/LysM-Cre[-]/R26^{+/iDTR} and TJ2-Tg/LysM-Cre[+]/R26^{+/iDTR} mice were initially injected intraperitoneally with 80/100 ng of DT per gram of body weight, every other day during one week, followed by two 50 ng/g DT administrations per week until leukemia development. n = number of animals



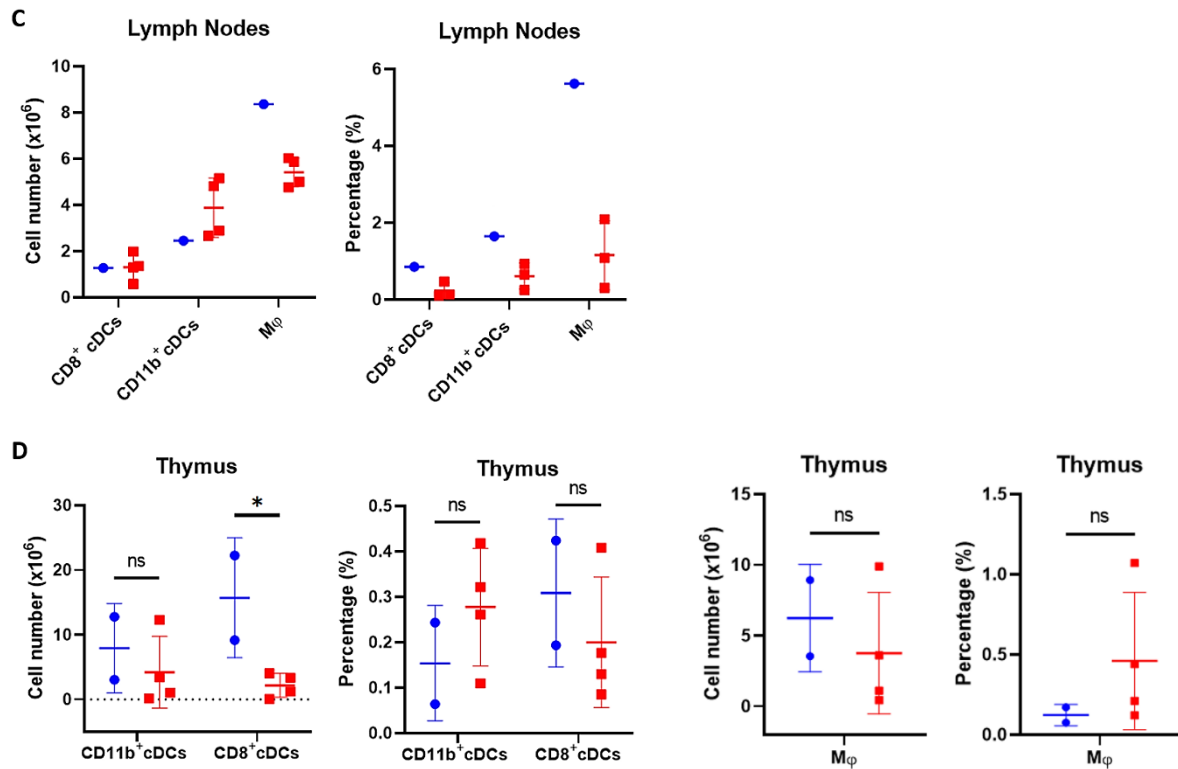


Figure 46. Monitoring DT-driven cell ablation in TJ2-Tg/LysM-Cre/R26^{+/iDTR} mice. TJ2-Tg/LysM-Cre⁻/R26^{+/iDTR} and TJ2-Tg/LysM-Cre⁺/R26^{+/iDTR} mice were treated as in Figure 45 until developing leukemia and being euthanized following human endpoints. Lymphoid organs were collected for flow cytometry analysis. Graphs depict the total number and percentage of myeloid populations (defined as in Fig. 9) within the (A) BM, (B) spleen, (C) lymph nodes and (D) thymus of TJ2-Tg/LysM-Cre⁻/R26^{+/iDTR} and TJ2-Tg/LysM-Cre⁺/R26^{+/iDTR} mice post-treatment. *P* values were determined by two-tailed, unpaired *t*-test with Welch's correction when variances were unequal (**, *P* ≤ 0.01; *, *P* ≤ 0.05; ns (non-significant), *P* > 0.05). TJ2-Tg/LysM-Cre⁻/R26^{+/iDTR} *n* = 2; TJ2-Tg/LysM-Cre⁺/R26^{+/iDTR} *n* = 4 (Data are presented with mean ± SEM; each symbol represents an individual mouse).

Flow cytometry results revealed that long-term DT treatment promoted a significant decrease in both CD11b⁺ (*P* = 0.0387) and CD8⁺ (*P* = 0.009) cDC, as well as macrophage (*P* = 0.0159) proportions in the spleen of TJ2-Tg/LysM-Cre⁺/R26^{+/iDTR} mice (Figure 46B). Similarly, thymic CD8⁺ cDC total number was significantly lower (*P* = 0.0269) in TEL-JAK2 DTR-expressing mice, compared to their TJ2-Tg/LysM-Cre⁻/R26^{+/iDTR} counterparts (Figure 46D). Moreover, we observed low LNs cDC and macrophage percentages (Figure 46C), and a tendency for reduced proportions of splenic Gr-1⁺ monocytes in TJ2-Tg/LysM-Cre⁺/R26^{+/iDTR} mice (Figure 46B), after DT administration. These results indicate that prolonged DT treatment promoted the long-term depletion of both splenic/LNs macrophages and cDCs, besides thymic CD8⁺ cDCs in TJ2-Tg/LysM-Cre⁺/R26^{+/iDTR} mice.

3. Impact of myeloid cell depletion on TEL-JAK2 leukemogenesis *in vivo*

3.1. Impact of myeloid cell depletion in TJ2-Tg/LysM-Cre/R26^{+/iDTR} mouse survival and leukemia progression

To evaluate whether DT-mediated myeloid cell ablation in TJ2-Tg/LysM-Cre/R26^{+/iDTR} mice affected leukemia development, we started by performing a mouse survival analysis. Comparing the survival curves, no significant differences in leukemia onset were observed between TJ2-Tg/LysM-Cre[-]/R26^{+/iDTR} and TJ2-Tg/LysM-Cre[+]/R26^{+/iDTR} mice (Figure 47), suggesting that the presence of myeloid cells does not confer a survival advantage to TJ2-Tg mice. Indeed, while median survival in TJ2-Tg/LysM-Cre[-]/R26^{+/iDTR} mice was 17.95 weeks, in TJ2-Tg/LysM-Cre[+]/R26^{+/iDTR} animals it was 15.40 weeks.

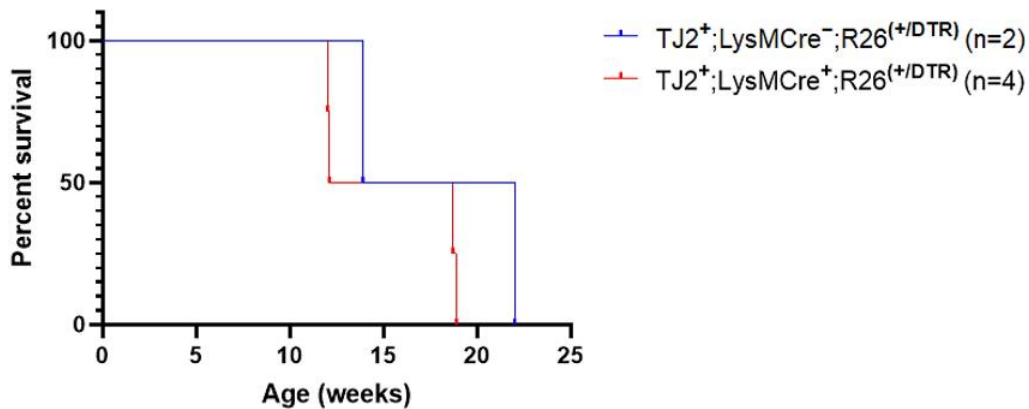


Figure 47. Kaplan-Meier survival curves for cohorts of TJ2-Tg/LysM-Cre[-]/R26^{+/iDTR} and TJ2-Tg/LysM-Cre[+]/R26^{+/iDTR} mice. Graph depicts the percentage of mice surviving in weeks in each time-point. Statistical analysis obtained with Log-rank (Mantel-Cox) test (*ns* (non-significant), *P* value = 0.3270)

Even though no differences regarding leukemia onset were observed upon DT treatment of TJ2-Tg/LysM-Cre/R26^{+/iDTR} mice, further analyses were performed to assess possible alterations in the pathological features of TEL-JAK2-induced leukemia/lymphoma due to myeloid cell ablative DT treatment.

3.2. Assessment of peripheral blood leukemia in TJ2-Tg/LysM-Cre/R26^{+/iDTR} mice

To detect leukemia in diseased TJ2-Tg/LysM-Cre/R26^{+/iDTR} mice, we assessed the concentration of white blood cells (WBC) in the peripheral blood. As shown in Figure 48, diseased TJ2-Tg/LysM-Cre[+]/R26^{+/iDTR} mice exhibited increased white blood cell counts compared to TJ2-Tg/LysM-Cre[-]/R26^{+/iDTR} mice, suggesting that DT treatment influenced TJ2⁺ leukemic cell dissemination.

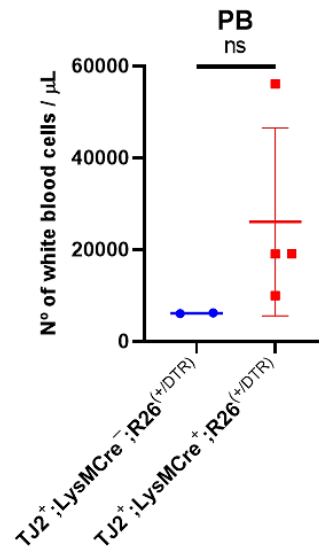


Figure 48. White blood cell counts for TJ2-Tg/LysM-Cre⁻/R26^{+/iDTR} and TJ2-Tg/LysM-Cre⁺/R26^{+/iDTR} mice. Graph depicts the number of white blood cells per mL within the PB of TJ2-Tg/LysM-Cre⁻/R26^{+/iDTR} and TJ2-Tg/LysM-Cre⁺/R26^{+/iDTR} mice. *P* values were determined by two-tailed, unpaired *t*-test with Welch's correction (*ns* (non-significant), *P* > 0.05). TJ2-Tg/LysM-Cre⁻/R26^{+/iDTR} *n* = 2; TJ2-Tg/LysM-Cre⁺/R26^{+/iDTR} *n* = 4 (Data are presented with mean $\pm$ SEM; each symbol represents an individual mouse).

3.3. Assessment of tumor load in lymphoid and non-lymphoid organs from TJ2-Tg/LysM-Cre/R26^{+/iDTR} mice

As mentioned before, diseased TEL-JAK2 transgenic mice are characterized by an increase in both lymphoid and non-lymphoid organs, due to leukemic cell infiltration and growth (299).

To evaluate if myeloid cell ablative DT treatment in TJ2-Tg/LysM-Cre/R26^{+/iDTR} mice affected the weight of hematopoietic (thymus, spleen and LNs (including maxillary, cervical, axillary, inguinal, mesenteric LNs)) and non-hematopoietic organs (liver, lungs), these were collected for analysis. We observed a greater tendency for thymus weight/size to be lower in TJ2-Tg/LysM-Cre⁺/R26^{+/iDTR} mice, while spleen and lymph node weight were substantially increased, compared to TJ2-Tg/LysM-Cre⁻/R26^{+/iDTR} mice (Figure 49A). Similarly, thymus cellularity was significantly (*P* = 0.0452) lower in TJ2-Tg/LysM-Cre⁺/R26^{+/iDTR} mice, whereas spleen and LNs showed increased cell numbers, compared to non DTR-expressing mice (Figure 49B). No differences were detected for liver and lung weight (Figure 49A), as well as for bone marrow cellularity (Figure 49B), between the two groups. These data indicate that the myeloid ablative DT treatment in TJ2-Tg/LysM-Cre⁺/R26^{+/iDTR} mice modulated leukemic cell distribution in lymphoid organs, without affecting non-lymphoid organs.

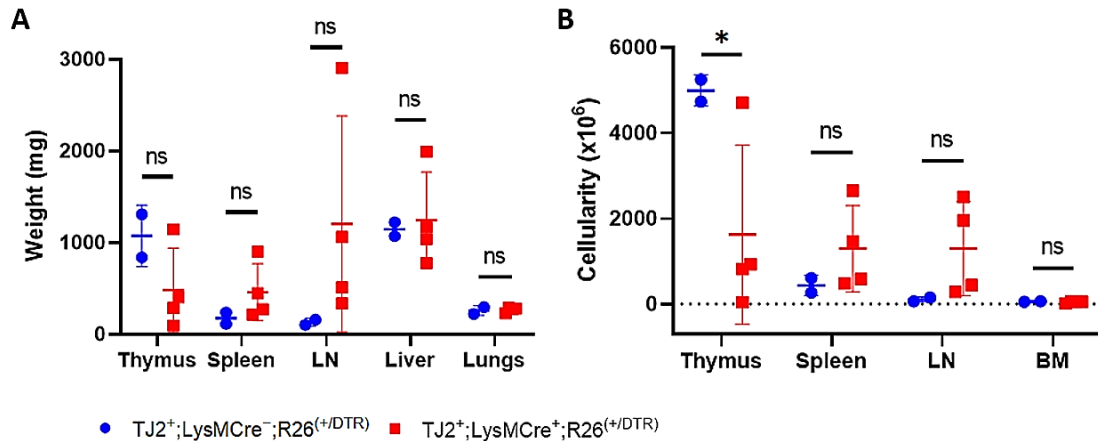


Figure 49. Organs weight and cellularity in TJ2-Tg/LysM-Cre⁻/R26^{+/iDTR} and TJ2-Tg/LysM-Cre⁺/R26^{+/iDTR} mice. (A) Graph depicts the comparison of lymphoid (thymus, spleen, LNs) and non-lymphoid (liver and lungs) organs weight between TJ2-Tg/LysM-Cre⁻/R26^{+/iDTR} and TJ2-Tg/LysM-Cre⁺/R26^{+/iDTR} mice post-DT treatment. (B) Graph depicts the comparison of lymphoid organs cellularity (thymus, spleen, LNs, bone marrow) between TJ2-Tg/LysM-Cre⁻/R26^{+/iDTR} and TJ2-Tg/LysM-Cre⁺/R26^{+/iDTR} mice after DT treatment. *P* values were determined by two-tailed, unpaired *t*-test with Welch's correction when variances were unequal (*, *P* ≤ 0.05; ns (non-significant), *P* > 0.05). TJ2-Tg/LysM-Cre⁻/R26^{+/iDTR} *n* = 2; TJ2-Tg/LysM-Cre⁺/R26^{+/iDTR} *n* = 4 (Data are presented with mean ± SEM; each symbol represents an individual mouse).

As previously described, increased organ size in TEL-JAK2 transgenic mice was associated with leukemic cell invasion and expansion (299). Further, TJ2 leukemogenesis is thought to be originated in the thymus, where leukemic cells accumulate and then migrate to secondary lymphoid organs and non-lymphoid organs (301, 302). Thus, we hypothesized that the changes observed in weight and cellularity of lymphoid organs of TJ2-Tg/LysM-Cre⁺/R26^{+/iDTR} mice result from alterations in the leukemia infiltration pattern, driven by myeloid cells deficiency.

To detect leukemic cells in different organs, cell suspensions from lymphoid organs (thymus, spleen and LNs) were stained for specific surface markers, including T-cell markers, CD8, CD4, CD24, and immature thymocyte markers, CD25 and CD44, and analyzed by flow cytometry. Previous reports have shown that TJ2⁺ leukemic cells are characterized by aberrant co-expression of CD8 and CD25, besides other markers (CD3ε, CD4, CD24, TCRβ, CD5) (301, 302). So, we quantified leukemic double-positive CD8⁺CD25⁺ cells (including CD8⁺CD4⁺ cells) within the thymus, spleen and LNs from TJ2-Tg/LysM-Cre/R26iDTR mice (gating strategy in Figure 50). Also, BM suspensions were stained for the Thy1.2 pan-T cell marker, to evaluate BM T-cell content.

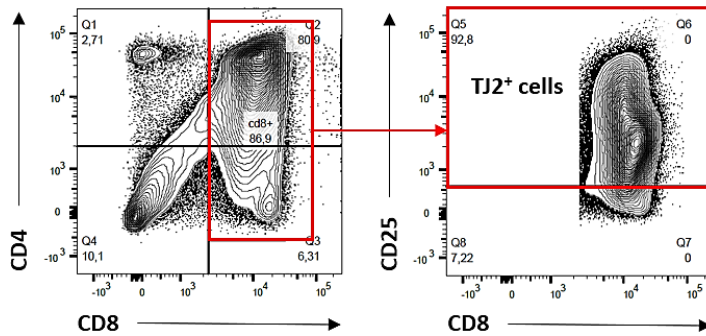
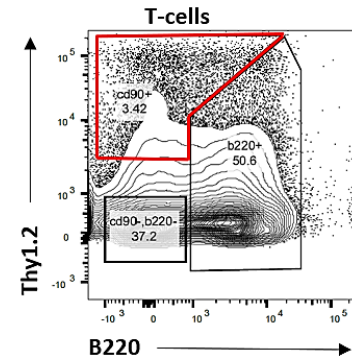
A) Thymus, Spleen and LNs**B) Bone Marrow**

Figure 50. Representative gating strategy to identify leukemic cells and T cells within the lymphoid organs of TJ2-Tg/LysM-Cre/R26^{iDTR} mice. Cell suspensions from the thymus, spleen, LNs and bone marrow of TJ2-Tg/LysM-Cre⁻/R26^{iDTR} and TJ2-Tg/LysM-Cre⁺/R26^{iDTR} mice were analyzed by flow cytometry. **(A)** In the thymus, spleen, and LNs, TJ2⁺ cells (CD8⁺CD25⁺) were assessed. **(B)** In the bone marrow, T cells (Thy1.2⁺B220^{neg}), were analyzed.

Thus, the thymus from DT-treated TJ2-Tg/LysM-Cre⁺/R26^{iDTR} mice presented significantly ($P = 0.0325$) reduced numbers of CD8⁺CD25⁺ cells (Figure 51A) while these cells were increased in both spleen and LNs (Figure 51B and C). Moreover, BM T-cell density was increased in DTR-expressing mice (Figure 51D), suggesting higher percentage of infiltrating leukemic cells. Overall, these results indicate that DT treatment of TJ2-Tg/LysM-Cre⁺/R26^{iDTR} mice was associated with lower thymic tumor burden, and higher dissemination to the spleen, LNs and BM. Conversely, the myeloid cell ablative DT treatment of DTR-expressing mice did not seem to impair leukemic cell accumulation in spleen and LNs.

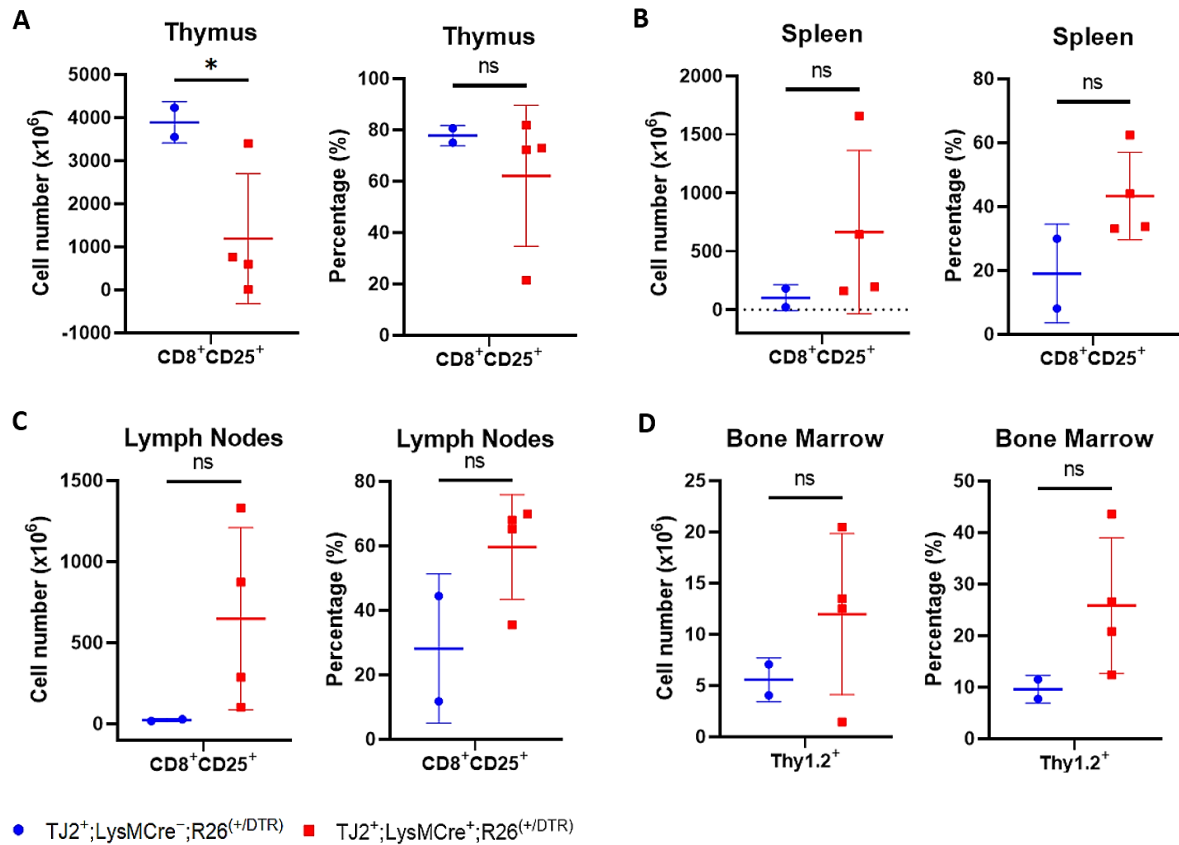


Figure 51. Assessment of the lymphoid organ and BM tumor load in TJ2-Tg/LysM-Cre/R26^{+/iDTR} mice. TJ2-Tg/LysM-Cre^[-]/R26^{+/iDTR} and TJ2-Tg/LysM-Cre^[+]/R26^{+/iDTR} mice were treated as in Figure 45 until leukemia development. Lymphoid organs were collected for flow cytometry analysis. Graphs depict the total number and percentage of leukemic TJ2⁺ cells (as defined in Fig. 50) within the **(A)** thymus, **(B)** spleen, **(C)** LNs and **(D)** BM of TJ2-Tg/LysM-Cre^[-]/R26^{+/iDTR} and TJ2-Tg/LysM-Cre^[+]/R26^{+/iDTR} mice post-treatment. *P* values were determined by two-tailed, unpaired *t*-test with Welch's correction when variances were unequal (*, *P* ≤ 0.05; *ns* (non-significant), *P* > 0.05). TJ2-Tg/LysM-Cre^[-]/R26^{+/iDTR} *n* = 2; TJ2-Tg/LysM-Cre^[+]/R26^{+/iDTR} *n* = 4 (Data are presented with mean ± SEM; each symbol represents an individual mouse).

DISCUSSION

1. Myeloid cell populations are disturbed in lymphoid organs from TEL-JAK2 transgenic mice

With the present work, we aimed to test the hypothesis that myeloid cells within the tumor microenvironment might play a role in TJ2-Tg leukemogenesis. We started by performing a characterization of the tumor-associated myeloid cells present in lymphoid organs of TJ2-Tg mice. Regarding DCs, flow cytometry analysis revealed a substantial increase in cDC (both CD8⁺ and CD11b⁺ subsets) total numbers in the thymus of diseased TJ2-Tg mice, as compared to healthy controls. These findings reinforce data previously reported by our group (150). Even though not statistically significant due to low sample numbers and great variability within groups, cDC numbers in thymic lymphomas were close to 15 to 20-fold higher than healthy thymi, demonstrating that thymic cDCs were clearly modulated by the disease. Similarly, spleens of TJ2-Tg mice exhibited a statistically significant increase in cDC content, namely CD11b⁺ cDCs, which is in accordance with Triplett *et al.* that have shown in the LN3 murine model of T-ALL that lymphoma-bearing mice displayed an increment of CD11c⁺ DCs in both thymus and secondary lymphoid organs, including spleen (156). Likewise, Lyu *et al.* have recently shown a significant increase of both splenic cDC subsets, namely CD11b⁺ cDCs, in mice transplanted with T-ALL (294). Besides increased numbers, spleens of transgenic diseased mice also revealed higher proportions of cDCs than in healthy spleens, whereas cDC percentages did not change in TJ2-Tg thymic lymphomas, as compared to WT thymi. This may be due to the fact that TJ2-Tg leukemogenesis arises in the thymus (301, 302) and so often the majority of leukemic cells accumulates in this organ, generating large thymic lymphomas. This suggests that the increase verified in thymic cDCs is proportional to the growth of malignant T cells in the thymic lymphomas, underlining the potential leukemogenic role of these cells. On the other hand, pDCs in spleens of transgenic diseased mice, were less frequent in number and proportion than in healthy spleens. pDC accumulation in the TME has been associated with poor outcome in several solid cancers (254, 256, 257, 318). However, in T-ALL, the role of pDCs is poorly understood. But since pDCs are highly efficient in inducing pro-inflammatory responses (193, 198), the low frequency of these cells in TJ2-Tg spleens may eventually contribute for leukemia progression.

Besides DCs, FACS analysis revealed a clear tendency for thymic macrophage total numbers to increase in TJ2-Tg leukemic mice. Despite this increase, the proportion of macrophages in transgenic thymi was decreased (compared to WT mice), which suggests that macrophage expansion is not proportional to leukemic cell growth in the thymus of diseased mice, as observed for thymic cDCs. Even though macrophage total numbers did not increase as strongly as cDCs in thymic lymphomas (5-fold versus 20-fold), it is possible

that macrophages participate in TJ2-Tg leukemogenesis. Interestingly, in TJ2-Tg spleens, macrophage numbers appear to vary according to disease phenotype: thymic lymphoma, with little leukemic cell dissemination, versus leukemia, with smaller thymic lymphoma, enlarged peripheral lymphoid organs and high white blood cell counts. In the case of thymic lymphoma, no macrophage quantitative differences were observed between TJ2-Tg leukemic mice and healthy controls. However, in one transgenic animal exhibiting more scattered disease, splenic macrophages were substantially increased (3-fold) compared to healthy controls. This suggests that upon the accumulation of leukemic cells in the spleen, macrophages are recruited to support T-ALL. This notion is in accordance with the reported increase in splenic macrophage numbers in mice transplanted with LN3 T-ALL cells (294).

Since Yang *et al.* recently suggested that monocytes and monocyte-derived macrophages modulate T-ALL extramedullary infiltration (293), we also determined the frequency of these cells in the spleens of TJ2-Tg leukemic mice, in addition to resident macrophages. Flow cytometry revealed that while splenic Gr-1⁻ monocytes/MOMF numbers oscillated substantially among TJ2-Tg leukemic mice, the proportion of Gr-1⁺ monocytes/MOMF in these mice was apparently reduced compared to healthy controls. Yang *et al.* showed that splenic Ly6C⁺ and Ly6C⁻ monocytes expand significantly at the early stages of Notch1-driven T-ALL mice but decreasing and reaching normal levels at later stage (293). Our observation of no significant changes in monocytes/MOMF numbers is not irreconcilable with this report since we only assessed monocytes at late stage of TJ2-induced leukemia. Furthermore, the Notch1-driven T-ALL model, where disease originates in the BM, exhibits distinct disease latency and leukemic cell anatomic distribution than the TJ2-Tg model. So, these T-ALL models may have different stromal requirements. Nevertheless, it would be interesting to determine if monocyte numbers and frequency are altered at an earlier stage of TJ2 leukemia development. In particular, Yang *et al.* have shown that the increase in MOMF numbers in extramedullary organs of T-ALL mice was dependent on circulating monocytes (293). Thus, we assessed BM monocyte production in TJ2-Tg leukemic mice but found no significant quantitative alterations. In addition to monocytes, we also assessed polymorphonuclear cells (neutrophils, eosinophils, basophils) numbers, which have been linked to several steps in the tumorigenic process (158, 161), but their function in T-ALL remains elusive. TJ2-Tg mice exhibited no major changes in PMN numbers in BM, thymus or spleen. However, PMN proportions were substantially reduced in these lymphoid organs, most notably in the thymus, probably due to increased expansion of TJ2⁺ leukemic cells without expansion of PMNs. In contrast to these results, Lyu *et al.* showed a significant increase of splenic granulocytes in T-ALL transplanted mice (294).

2. Structural alterations were observed in both thymic and splenic TMEs of TEL-JAK2 transgenic mice

We performed immunofluorescence analysis of TJ2-Tg thymic and splenic microenvironments to assess the tissue architecture and spatial distribution of myeloid cells. Contrary to WT mice, thymus from TJ2-Tg diseased mice exhibited enlarged size due to leukemic cell accumulation, and loss of clear spatial segregation between medulla and cortex regions, based on the epithelial expression of keratin-5 and -8 analysis, as previously reported (150). Regarding the myeloid compartment, thymic DCs were distributed throughout the thymic lymphoma area, including non-epithelial regions, that Ghezzi *et al.* designated as keratin-negative areas (150). Nonetheless, they preferentially accumulated in keratin 5-positive areas, forming dense aggregates. Given that thymic DCs in WT mice are restricted to the medulla (marked by keratin 5) and in TJ2-Tg mice were also found in keratin-negative areas, confirms that these cells are increased and likely support the growth of TJ2⁺ leukemic cells in the thymic TME. Likewise, Triplett *et al.* described that thymic DCs accumulated at peripheral epithelial-free regions in lymphoma-bearing mice (156). On the other hand, macrophages were distributed throughout the thymus of diseased mice, but eventually more abundantly in keratin 5-expressing areas, similar to what is observed for WT thymi. The results here presented are in accordance with the data previously reported by our group (150), where several structural alterations in the thymic lymphoma microenvironment of TJ2-Tg mice were found, including: an expansion of mTECs in detriment of cTECs; increased fibroblasts, endothelial cells and DCs.

To characterize splenic microenvironmental changes associated with TJ2 leukemogenesis, we performed immunolabeling with laminin, an extracellular matrix marker, that allows to distinguish spleen compartments (white pulp, marginal zone and red pulp) (307). The experiments revealed loss of normal splenic compartmentalization in leukemic mice, as manifested by the clear disruption of the marginal sinus-lining basement membrane (which delineates WP). These structural alterations probably resulted from leukemic cell infiltration coming from the WP central arterioles and flowing into the marginal sinus and RP. To confirm this, we could triple-stain splenic sections with laminin, CD25, and CD8 to map leukemic cells in the tissue and infer possible infiltration routes. Moreover, TJ2-Tg mice exhibited complex macrophage and DC networks throughout the spleen associated with abundant extracellular matrix support, instead of being confined to the MZ and RP, as observed in WT conditions. Notwithstanding the loss of normal compartmentalization, which may contribute for myeloid cell scattered distribution, we observed a clear enrichment of CD11c staining throughout the spleen, which denotes an increase in cDCs. This finding

corroborates the increase detected by flow cytometry and underlines the potential leukemogenic role of these cells in the splenic TME of TJ2-Tg mice.

3. Specificity and efficiency of LysM-Cre/R26iDTR mouse model

We demonstrated that TJ2-Tg mice exhibit an increase in different myeloid cell populations in lymphoid organs. While the leukemogenic role of these cells was previously investigated *in vitro* (156, 285), their functions *in vivo* remain elusive. Thus, we aimed to deplete tumor-associated myeloid cells and study the impact on leukemia/lymphoma development in the TJ2-Tg mouse model. For that, we generated LysM-Cre/R26iDTR mice, by crossing myeloid cell specific LysM-Cre strain with Rosa26-Stop^{flox}iDTR encoding the simian diphtheria toxin receptor gene. LysM was shown to be expressed in distinct myeloid subsets, including macrophages, granulocytes (mainly neutrophils), DCs and myeloblasts (310, 311, 314). In accordance, we found that myeloid cells from these mice expressed DTR after Cre-mediated removal of the STOP sequence.

To assess both specificity and efficiency of Cre activity in LysM-Cre mice, we crossed this strain with R26EYFP reporter strain. Like the R26iDTR strain, the R26EYFP strain carries a floxed STOP cassette upstream the yellow fluorescent protein gene, so we expect that the LysM-Cre transgene will recombine similarly the R26EYFP and R26iDTR strains and induce EYFP expression in the same cells as DTR. Nevertheless, it is known that deletion efficiency can vary depending on the floxed alleles (304, 319). Thus, we evaluated EYFP expression in myeloid subsets from different organs (peritoneal cavity, spleen and thymus) from LysM-Cre/R26EYFP mice. Flow cytometry showed that several myeloid populations, including macrophages, CD11b⁺ cells (monocytes and PMNs) and DCs expressed EYFP, indicating the LysM promoter was active in these subsets. As expected from previous reports (304, 310, 314), this activity was specific to the myeloid lineage, being more frequent in macrophages and CD11b⁺ cells than in DCs. In particular, peritoneal macrophages exhibited the highest recombination rate among the myeloid populations (~70%), followed by peritoneal and splenic CD11b⁺ cells (~50-60%), splenic and thymic macrophages (~30%), and splenic and thymic DCs (~15-20%). We further assessed if Cre-mediated EYFP expression differed between DC subsets and found that cDCs (~15-30%) expressed higher levels of EYFP than pDCs (~5-10%). Using the same Cre-strain, Abram and colleagues demonstrated higher LysM promoter activity in peritoneal macrophages (~90%), similar recombination rates in splenic macrophages (~40%) and lower EYFP expression in splenic cDCs (~10%) (304). Several variables could explain these discrepancies. Our flow cytometry gating strategy was similar or equal to those used by Abram *et al.* The genetic background of our and their mice was the same C57BL/6. The parental inheritance of the

Cre allele can lead to differences in Cre expression (319-321). In our breeding scheme, the LysM-Cre allele was transmitted by the father, but we cannot discard the possibility of different parental Cre inheritance contributing for the differences in recombination rates observed between these two studies. Besides, we detected heterogeneity between littermates regarding the proportion of cells expressing EYFP, which further supports the notion that Cre efficiency is variable within the same Cre-strain.

Although LysM is known to be expressed by different myeloid populations, it was previously shown that both erythrocytes and lymphocytes (T and B cells) can also exhibit evidence of previous LysM activity, given that a subset of HSCs was found to express the LysM gene, and that these cells could originate differentiated hematopoietic cells from other lineages besides myeloid (315). Thus, we additionally analyzed these cell lineages in parallel to evaluate the possibility of Cre recombinase activity. In particular, using the reporter strain we observed minor percentages of T and B cells expressing EYFP ($\approx 5-10\%$), which supports the notion that a fraction of lymphoid cells derive from multipotent progenitors that expressed the Lysozyme M gene (315). This suggests that a fraction of T and B cells in LysM-Cre/R26iDTR mice can express DTR and be ablated by DT treatment.

Moreover, to evaluate the efficiency of our model, we performed an immunofluorescence analysis to confirm DTR expression in peritoneal macrophages of LysM-Cre/R26iDTR mice. Double-staining with F4/80 and DTR revealed co-expression of these proteins exclusively in macrophages from LysM-Cre $^{+}$ /R26 $^{+iDTR}$ mice, validating that Cre-mediated loxP recombination occurred in myeloid cells. In the absence of Cre recombinase (LysM-Cre $^{-}$ /R26 $^{+iDTR}$ mice - controls) no DTR expression was found.

4. Optimization of Diphtheria Toxin-driven myeloid cell ablation *in vivo*

After demonstrating both specificity and efficiency of the LysM-Cre strain, we attempted to deplete myeloid cells by administering i.p. DT to LysM-Cre/R26iDTR mice. Variable doses of DT and different administration regimens were tested. Flow cytometry analysis of PB, lymphoid organs (BM, spleen, thymus) and peritoneal exudate was performed to monitor myeloid cell depletion. We started by injecting a single dose of DT at 50 ng/g and analyzed at different timepoints to evaluate the dynamics of myeloid cell depletion and recovery. Flow cytometry revealed a decrease ($\approx 35\%$) in peritoneal macrophages from LysM-Cre $^{+}$ /R26 $^{+iDTR}$ mice 24 h post-injection, which persisted for 3 more days. We also detected a significant decrease in thymic macrophages and BM DCs. The latter persisted for 6 days, suggesting that DC lifespan is longer than 6 days. Indeed, previous studies have shown that DC half-life in lymphoid organs is around 5-7 days (322, 323), which supports our findings.

Moreover, no significant differences in erythrocyte or lymphocyte proportions between the two groups were found 1 day after DT treatment, although B cells in the peritoneal cavity exhibited a decreasing tendency after DT injection in LysM-Cre⁺/R26^{+/iDTR} mice. This strong decrease was found for one of two mice, so more mice should be analyzed. At this stage we do not know if this reduction in B lymphocytes is due to LysM-Cre activity in a fraction of B cells, since we observed about 5% B cells expressing EYFP in LysM-Cre⁺/R26^{+/EYFP} mice, or if F4/80⁺ peritoneal macrophages were included in the B220-positive population (data not shown), similarly to what was reported by Turchyn *et al.* (316). If so, the decrease found in B220-positive cells after DT treatment, may actually result from a decrease in peritoneal macrophages. Besides, peritoneal B cells are not tissue-resident, being able to move between this compartment and omentum (intraperitoneal visceral fat), or other organs (secondary lymphoid organs and intestine) (324, 325), which may justify some variability found in B cells proportion. Nevertheless, it was reported that peritoneal macrophages from the same strain (LysM-Cre/R26iDTR) were more efficiently depleted with lower DT concentrations (< 50 ng/g) (326, 327). There are several factors that could influence cell ablation efficiency besides variability in Cre expression, such as the DT itself and the route of administration. It was previously reported that DT is very sensitive to freeze-thawing cycles, so we prepared single-use aliquots with the concentration of interest to avoid degradation and loss of activity. Besides, Lucas *et al.* have shown that intravenous administration of 25 ng/g of DT was more efficient at depleting resident macrophages in LysM-Cre/R26iDTR mice than several intraperitoneal injections at the same dose (326). Since DT has a short half-life (7.5 h) (328), the intravenous route might facilitate systemic distribution and potentiate its action.

Moreover, we tested a regimen including an initial higher concentration of DT (100 ng/g) followed by three daily lower doses (50 ng/g) of DT for maintenance, and thus try to attenuate recovery and maintain depletion of DTR-expressing cells. Flow cytometry analysis demonstrated that 100 ng/g of DT induced within 24 h moderate circulating monocyte depletion in LysM-Cre⁺/R26^{+/iDTR} mice, but in turn promoted an increase in circulating neutrophils. This may suggest a compensatory mechanism, that was previously observed in a study using also a DTR-system (329), characterized by an increased circulation of neutrophils in the first 2/3 days after DT treatment in response to monocyte reduction. Upon the administration of the daily doses of DT at 50 ng/g, the compensatory mechanism was no longer observed, with neutrophils decreasing and monocytes returning to near normal levels. Given that neutrophils and monocytes have comparable half-lives in circulation (10 h vs. 17 h, respectively) (330), the difference in response to DT daily treatments may result from the fact that neutrophils display higher activity of LysM promoter

(≈80%) compared to PB monocytes (≈40%) (304), being more efficiently ablated. These results suggest that to enhance myeloid cell depletion is important to consider both DT concentration and administration frequency. With the exception of tissue-resident macrophages (331), the majority of myeloid cells, namely the circulating ones, exhibit a short turnover (170, 179, 188, 330, 332), as so the administration of more frequent and concentrated DT doses is likely required to bypass compensatory mechanisms and/or total recovery of myeloid subsets.

In line with this idea, we tested an even higher concentration of DT to maximize myeloid cell depletion. FACS analysis revealed that a single i.p injection of DT at 200 ng/g was very efficient in depleting both macrophages and DCs from peritoneal cavity and spleen of LysM-Cre[+]/R26^{EYFP/iDTR} mice, without affecting CD11b⁺ cells, whereas the cumulative effect of two consecutive administrations did not improve myeloid cell depletion extent. However, parallel histologic analyses revealed lung and liver congestion in both LysM-Cre[+]/R26^{EYFP/iDTR} and LysM-Cre[+]/R26^{+/-EYFP} mice after treatment with 200 ng/g of DT. We excluded the possibility of ectopic Cre expression by performing anti-DTR or anti-GFP immunofluorescence analysis. The fact that non-DTR expressing mice still exhibited lesions induced by the drug suggests that murine DT natural resistance was compromised. Indeed, Cha *et al.* demonstrated that WT mice were resistant to DT up to a concentration of 100 ng/g, collapsing at 1000 ng/g (333). This suggests that DT over 100 ng/g is toxic to mice, causing heart failure and congestion of several organs, such as lungs and liver (333). This hypothesis supports our findings.

As a strategy to obtain long-term myeloid cell depletion in leukemic mice without causing toxic side-effects, we decided to inject first higher doses of DT at 100 ng/g (first phase), followed by lower doses at 50 ng/g for maintenance. For this experiment we tested on 6-week-old TJ2/LysM-Cre/R26^{+/-iDTR} mice, by crossing TJ2-Tg mice with LysM-Cre/R26^{+/-iDTR}, since we intend to assess how myeloid cell deficiency may impact TJ2-induced leukemogenesis. However, after the first phase of treatment, we observed signals of drug toxicity in some animals of both genotypes. Therefore, we decided to change the initial dose to 80 ng/g, to try to enhance mice survival while ensuring efficient myeloid cell depletion. We found that after the induction phase with 80 ng/g, TJ2/LysM-Cre[+]/R26^{+/-iDTR} mice displayed complete depletion of circulating neutrophils and Gr-1⁺ monocytes, which validated the efficiency of 80 ng/g of DT at inducing myeloid cells depletion. Thus, TJ2-Tg/LysM-Cre[-]/R26^{+/-iDTR} and TJ2-Tg/LysM-Cre[+]/R26^{+/-iDTR} mice underwent full DT treatment until leukemia development and euthanasia. Long-term DT treatment promoted the significant depletion of thymic CD8⁺ cDCs, splenic CD11b⁺ and CD8⁺ cDC subsets and macrophages in TJ2-Tg/LysM-Cre[+]/R26^{iDTR} mice. We also found a reduction in cDCs

and macrophages in peripheral LNs and a tendency for splenic Gr-1⁺ monocyte proportion to decrease in DT-treated mice. Therefore, we can conclude that prolonged DT treatment efficiently depleted DCs and macrophages but not CD11b⁺ cells in TJ2-Tg/LysM-Cre[+]/R26iDTR mice. Impaired depletion of CD11b⁺ cells, including PMNs/MDSCs and monocytes, may result from the fact that these cells exhibit the faster turnover among myeloid subsets or because they are more resistant to DT toxicity (170, 188, 330, 332). Besides, under cancer context occurs abnormal myelopoiesis that promotes the expansion of immunosuppressive myeloid cells, namely myeloid derived-suppressor cells (both PMN-MDSCs and M-MDSCs) (159, 172), which may compromise CD11b⁺ cells ablation.

Still, more experiments with increased number of LysM-Cre/R26iDTR animals would be recommended to further optimize DT-mediated myeloid cell ablation and minimize toxicity side-effects.

4.1 Alternative approaches to deplete myeloid cells *in vivo*

As a way of improving myeloid cell depletion, we could use LysM-Cre/R26iDTR mice homozygous for the DTR allele, which was previously shown to promote higher depletion of peritoneal and tissue-resident macrophages in the spleen, skin and liver, compared to heterozygous mice (326). Nevertheless, when using a Cre-loxP system we can still observe toxicity and off-target effects as we described before. In fact, LysM gene was also recently found to be expressed by neurons (334), besides alveolar epithelial cells (335). Likewise, the CD11c-DTR system, for instance, is strongly associated with promiscuous cellular depletion (304, 336) and mice lethally (337) driven by chronic application of DT. As so, to avoid non-specific deletion and alleviate cytotoxic effects driven by DT administration, we could generate bone marrow chimeras, in which LysM expression is restricted to the hematopoietic compartment. The only disadvantage of using these BM chimeras is that we can only deplete myeloid cells that are replaced by BM progenitors, which means that most of the tissue-resident macrophages, derived from fetal precursors (212), would not be eliminated. As an option for Cre-loxP system, we could use clodronate liposomes. These are detected as a foreign and quickly uptaken by phagocytic cells, including macrophages (338), monocytes (339) and DCs (340). Upon the digestion of liposome and release of clodronate into myeloid cells cytosol, the cellular metabolism is inhibited and cells die by apoptosis (341, 342). This method is very efficient at promoting systemic myeloid cell depletion and exhibits very low toxicity once only phagocytic cells are sensitive to liposomes. Besides, any free clodronate released from dead macrophages/DCs will promptly leave circulation (short half-life) without re-entering easily in any cells (338, 342).

5. Depletion of myeloid cells did not alter TEL-JAK2-induced leukemia time of onset

The myeloid ablative DT treatment had no impact on the age of leukemia onset of TJ2-Tg/LysM-Cre[-]/R26iDTR and TJ2-Tg/LysM-Cre[+]/R26iDTR mice. This suggests that cDC and macrophage reduction in lymphoid organs does not hamper leukemia development. Lyu *et al.* have shown that DC deficiency alone in T-ALL mice did not delay disease progression. However, when DCs were depleted in combination with other myeloid subsets, including macrophages and monocytes, T-ALL mouse survival was significantly prolonged (294). We may not have obtained any significant differences between genotypes regarding mouse survival because of the low number of animals used, and possibly due to a weaker myeloid cell depletion as that obtained by Lyu *et al.* Thus, experiments with larger mouse cohorts are required to ascertain the role of myeloid cell deficiency in TJ2 leukemia development.

6. Myeloid cell ablative treatment of leukemic mice was associated with increased number of circulating peripheral blood leukocytes

TJ2-Tg mice are characterized by an increase in circulating white blood cells (299). Although not statistically significant, TJ2-Tg/LysM-Cre[+]/R26^{+iDTR} leukemic mice treated with DT exhibited substantially increased white blood cell counts compared to TJ2-Tg/LysM-Cre[-]/R26^{+iDTR} control mice. Considering that DT treatment induced cDC and macrophage depletion, these results suggest that these cells could influence leukemic cell dissemination, by antagonizing their migration from the thymus and/or the spleen or LNs to the bloodstream. In contrast, Lyu *et al.* have shown that myeloid cell depletion was significantly associated with decreased number of leukemic cells in the PB of T-ALL transplanted mice (294). Nevertheless, it is important to note that the ETV6-JAK2 transgenic and LN3-transplanted mouse models of T-ALL display different leukemia times of onset (4 and 15 weeks, respectively) and clinical manifestation (T-ALL/LBL and T-ALL, respectively). In addition, Lyu *et al.* exhibited a more pronounced depletion of different myeloid populations, including cDC1s, cDC2s, macrophages and monocytes (294). These conflicting data suggest two non-mutually exclusive hypotheses: i) more efficient myeloid cell depletion may be associated with antagonistic effects in T-ALL migration; and ii) myeloid subsets other than cDCs and macrophages, such as monocytes, may play an important role in T-ALL distribution. Indeed, Yang and colleagues recently demonstrated that mice deficient for Ly6C⁺ monocytes exhibited a significant reduction in T-ALL cells in circulation and consequently in extramedullary organs (293).

7. Alterations in leukemia infiltration patterns in leukemic mice after myeloid cell ablative treatment

Diseased TJ2-Tg mice display increased lymphoid and non-lymphoid organs weight, due to leukemic cell accumulation and proliferation (299). We observed a substantial decrease in thymus weight and cellularity and increase in spleen and lymph node weight and cellularity in DT-treated TJ2-Tg/LysM-Cre⁺/R26^{+/iDTR} mice, as compared to TJ2-Tg/LysM-Cre⁻/R26^{+/iDTR} mice. Non-lymphoid organ weights were similar between the two groups. Variations in lymphoid organ weight and cellularity were linked to the levels of leukemic cell infiltration, as determined by flow cytometry detection of (CD8⁺CD25⁺) leukemic cells. These data and the higher circulating white blood cell counts in TJ2-Tg/LysM-Cre⁺/R26^{+/iDTR} mice suggest that cDC and macrophage deficiency in lymphoid organs may play a role in spleen and lymph node invasion by leukemic cells.

Overall, these results suggest that thymic CD8⁺ cDCs in TJ2-Tg mice hamper leukemic cells egress from the thymus towards secondary lymphoid organs, and perhaps display a central role in leukemic cell survival in the thymic TME. Supporting the survival hypothesis, Triplett *et al.* have shown that tumor-associated thymic DCs were necessary and sufficient to support T-ALL cells *ex vivo*, by promoting activation of IGF1R signaling (156). However, a following study from the same lab revealed that mice deficient for DCs succumbed faster to T-ALL, suggesting that DCs are not fundamental for disease development (294). Since these authors previously demonstrated that CD11b⁺ myeloid cells were also able to support T-ALL *in vitro*, they proposed that other myeloid subsets could be compensating for the reduction of DCs. Indeed, they found a significant increase in macrophages in DC-deficient tumor-bearing mice and further demonstrated that both murine tumor-associated cDC2s, as well as PB monocyte-derived DCs from T-ALL patients exhibited pro T-ALL activity *in vitro*. These results suggest that DCs display a leukemogenic role in T-ALL, which supports our hypothesis.

Still, it is important to note that leukemia may develop through alternative paths in different T-ALL models, and, more importantly, different tumor microenvironments may be associated with distinct selective pressures on T-ALL cells. The cDC2s appear to be more prominent in the splenic T-ALL microenvironment, where they were the most increased DCs and promoted LN3 T-ALL cells survival (294). The cDC1s may play the major supportive role in the TJ2-Tg thymic TME, where they were most expanded. So, in the absence of CD8⁺ cDCs, TJ2⁺ cells could face a more adverse microenvironment, and thus be “forced” to migrate to more supportive microenvironments. This led us to hypothesize that CD8⁺ cDCs antagonize leukemia dissemination. Under normal conditions, T-cell egress from the

thymus is dependent on sphingosine-1-phosphate gradients, which are found at higher levels in the plasma as compared to the thymic parenchyma, due to S1P lyase (SLP)-mediated metabolism. SLP is expressed by both TECs and DCs, but when the SLP gene is deleted in DCs, S1P gradients are disrupted, and S1P1-mediated T-cell egress, is impaired. These findings suggest that DCs behave as gatekeepers of T-cell emigration (343). In this line, S1P was shown to be involved in T-ALL and T-LBL blasts migration, as well (344). Assuming that DCs modulate thymic export, the reduction of CD8⁺ cDCs would promote T-ALL cell retention and not the opposite. Nevertheless, T-ALL cells could exploit alternative trafficking mechanisms, such as CXCR4/CXCL12 (132, 154, 345) and CCR7/CCL19/CCL21 (136, 346) signaling, which also can contribute for leukemic cell egress.

DT-mediated deficiency of cDCs and macrophages within the LNs and spleen of TJ2-Tg/LysM-Cre[+]/R26^{+/iDTR} mice does not seem to impair T-ALL cell homing/engraftment at these secondary sites. This suggests that these myeloid cells are not fundamental for leukemia progression *in vivo*. Contradicting this hypothesis, *in vitro* studies revealed that both tumor-associated splenic macrophages (285) and DCs (156) exhibit an efficient pro T-ALL activity *ex vivo*. Similarly, T-ALL cells from metastatic LNs were shown to be sensitive to myeloid support (156). In line with a fundamental role for myeloid cells in supporting T-ALL *in vivo*, Lyu *et al.* have recently shown that acute myeloid cell ablation resulted in significant lower tumor burden and prolonged T-ALL mice survival (294). Besides, co-cultures with tumor-associated myeloid cells further revealed that macrophages were the most efficient cell type (followed by monocytes and cDC2s) at supporting T-ALL cells *ex vivo*, partly through activation of IGF1R signaling. Based on these data and our observation that the reduction in myeloid subsets was not complete, we surmise that the remaining cDCs and macrophages in the splenic/LNs tumor microenvironments of DT-treated TJ2-Tg mice could still be sustaining T-ALL cells. There is also the possibility of other myeloid populations that were not as efficiently depleted (i.e., monocytes) to play a role in peripheral lymphoid organ expansion, as suggested by Lyu *et al.* Supporting the leukemogenic role of monocytes, Yang and colleagues demonstrated that T-ALL mice exhibit a significant increase in extramedullary Ly6C⁺ monocyte-derived LAMs that contribute for extramedullary T-ALL distribution via CCL8–CCR1/CCR2 and CCL9/10–CCR1 axes (293). Since we did not observed a reduction in splenic Ly6C⁺ monocytes in DT-treated TJ2-Tg/LysM-Cre[+]/R26^{+/iDTR} mice, these cell populations may be supporting T-ALL cells in the splenic microenvironment, thus compensating for the depletion of cDCs and macrophages.

8. Future Perspectives

With this work it became clear that different myeloid cells, namely conventional dendritic cells, are increased in diseased TJ2-Tg mice, which might suggest a possible role in T-ALL development. Previous studies demonstrated that mice presenting defects on thymic stromal cells exhibited delayed TEL-JAK2-induced leukemogenesis (150, 302). This indicates that modulation of the thymic tumor microenvironment may influence leukemia development. Here, the deletion of myeloid cells, namely cDCs and macrophages, had no effect on disease onset. However, upon flow cytometry analysis and WBC assessment, we hypothesized that CD8⁺ cDCs play a fundamental role in supporting TJ2⁺ cells in thymic lymphoma microenvironment, and consequently on leukemic cell dissemination. How these DCs and other myeloid cells could be supporting T-ALL cells remains elusive. Previous reports have shown that IGF1R signaling appears to be a common/major signaling employed by different myeloid cells to support T-ALL (156, 294), however, other myeloid-derived factors most likely contribute to T-ALL progression *in vivo*.

Based on our results and to confirm pro T-ALL activity of myeloid cells at different tumor microenvironments, we could co-culture primary TJ2⁺ cells with tumor-associated myeloid cells, including cDCs, macrophages and monocytes. Then, we could generate TJ2-Tg/LysM-Cre[+]/R26EYFP reporter mice and isolate lymphoma-infiltrating EYFP⁺ myeloid cells and perform transcriptomics (RNA-seq) to search for specific gene signatures and important modulators favoring the interaction with and growth of leukemic cells. Since we observed that CD8⁺ cDCs appear to modulate TJ2⁺ leukemic cell egress from the thymus towards secondary lymphoid organs, it would be also interesting to inject murine CD45.1-expressing TJ2⁺ cells into CD45.2 LysM-Cre/R26iDTR hosts to evaluate if myeloid cell deficiency affects T-ALL cell engraftment/homing to lymphoid and non-lymphoid organs, as well. Moreover, and to confirm if CD8⁺ cDCs may play a fundamental role in TJ2 leukemia development, we could generate *IRF8*^{-/-} mice, lacking CD8⁺ cDCs (347), to cross with TJ2-Tg mice and evaluate how CD8⁺ cDC deficiency impacts leukemia time of onset.

These experiments would help us understand better the role of myeloid cells in T-ALL development, and eventually identify new molecular mechanisms by which myeloid cells are modulating disease, opening the door for possible novel therapeutic approaches.

CONCLUSION

Here we demonstrated that different myeloid cells, namely cDCs, are increased in both thymic and splenic tumor microenvironments of diseased TEL-JAK2 transgenic mice.

Ablation experiments targeting LysM-expressing cells in TJ2-Tg mice, reveal that thymic CD8⁺ cDCs reduction is associated with lower thymic tumor burden and higher dissemination of leukemic cells. The depletion of cDCs and macrophages in secondary lymphoid organs did not affect T-ALL cells colonization capacity at these sites. This suggests that either myeloid cells have no role in secondary lymphoid organ colonization or other myeloid subsets may be compensating for DC and macrophage deficiency in those organs.

Hence, we propose a model where CD8⁺ cDCs play a central role in thymic lymphoma microenvironment, and consequently in leukemia dissemination and secondary lymphoid organs invasion (Figure 52).

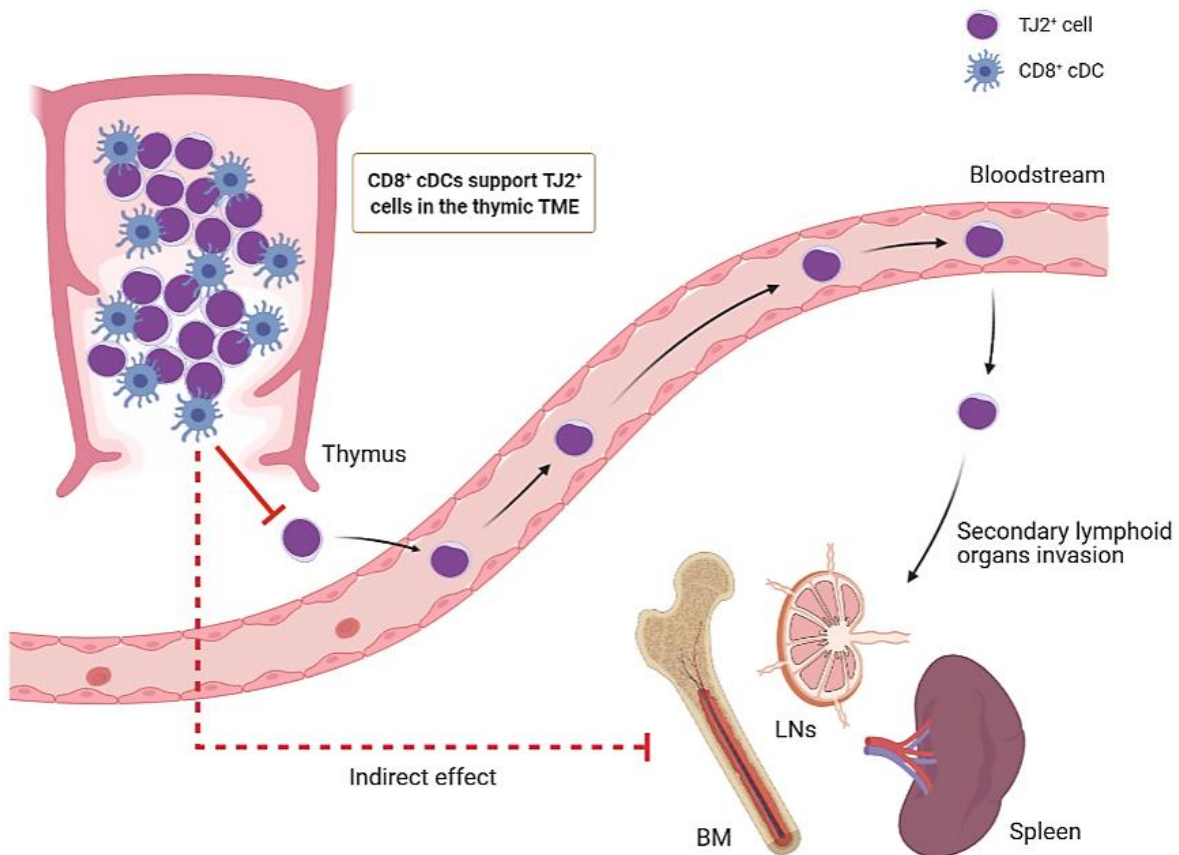


Figure 52. Potential leukemogenic role of CD8⁺ cDCs in the thymic TME of TEL-JAK2 transgenic mice. CD8⁺ cDCs appear to play a fundamental role at supporting TJ2⁺ cells in thymic lymphoma microenvironment, and consequently at impairing leukemia dissemination and secondary lymphoid organs infiltration.

REFERENCES

1. You MJ, Medeiros LJ, Hsi ED. T-lymphoblastic leukemia/lymphoma. *Am J Clin Pathol.* 2015;144(3):411-22.
2. Vadillo E, Dorantes-Acosta E, Pelayo R, Schnoor M. T cell acute lymphoblastic leukemia (T-ALL): New insights into the cellular origins and infiltration mechanisms common and unique among hematologic malignancies. *Blood Rev.* 2018;32(1):36-51.
3. Passaro D, Quang CT, Ghysdael J. Microenvironmental cues for T-cell acute lymphoblastic leukemia development. *Immunol Rev.* 2016;271(1):156-72.
4. Kaushansky K, Williams WJ. *Williams hematology.* 9th ed: McGraw-Hill Education; 2016.
5. Seita J, Weissman IL. Hematopoietic stem cell: self-renewal versus differentiation. *Wiley Interdiscip Rev Syst Biol Med.* 2010;2(6):640-53.
6. Sitnicka E. Early cellular pathways of mouse natural killer cell development. *J Innate Immun.* 2011;3(4):329-36.
7. Sultana DA, Zhang SL, Todd SP, Bhandoola A. Expression of functional P-selectin glycoprotein ligand 1 on hematopoietic progenitors is developmentally regulated. *J Immunol.* 2012;188(9):4385-93.
8. Zlotoff DA, Sambandam A, Logan TD, Bell JJ, Schwarz BA, Bhandoola A. CCR7 and CCR9 together recruit hematopoietic progenitors to the adult thymus. *Blood.* 2010;115(10):1897-905.
9. Sultana DA, Bell JJ, Zlotoff DA, De Obaldia ME, Bhandoola A. Eliciting the T cell fate with Notch. *Seminars in immunology.* 2010;22(5):254-60.
10. Bell JJ, Bhandoola A. The earliest thymic progenitors for T cells possess myeloid lineage potential. *Nature.* 2008;452(7188):764-7.
11. De Obaldia ME, Bell JJ, Bhandoola A. Early T-cell progenitors are the major granulocyte precursors in the adult mouse thymus. *Blood.* 2013;121(1):64-71.
12. Karrman K, Johansson B. Pediatric T-cell acute lymphoblastic leukemia. *Genes Chromosomes Cancer.* 2017;56(2):89-116.
13. Takahama Y. Journey through the thymus: stromal guides for T-cell development and selection. *Nat Rev Immunol.* 2006;6(2):127-35.
14. Blackburn CC, Manley NR. Developing a new paradigm for thymus organogenesis. *Nat Rev Immunol.* 2004;4(4):278-89.
15. Lee JY, Love PE. Assessment of T Cell Development by Flow Cytometry. *Methods Mol Biol.* 2016;1323:47-64.
16. Vantourout P, Hayday A. Six-of-the-best: unique contributions of gammadelta T cells to immunology. *Nat Rev Immunol.* 2013;13(2):88-100.
17. Roche PA, Cresswell P. Antigen Processing and Presentation Mechanisms in Myeloid Cells. *Microbiol Spectr.* 2016;4(3):10.1128/microbiolspec.MCHD-0008-2015.
18. Ueno T, Saito F, Gray DHD, Kuse S, Hieshima K, Nakano H, et al. CCR7 signals are essential for cortex-medulla migration of developing thymocytes. *The Journal of experimental medicine.* 2004;200(4):493-505.
19. Williams JA, Tai X, Hodes RJ. CD28-CD80/86 and CD40-CD40L Interactions Promote Thymic Tolerance by Regulating Medullary Epithelial Cell and Thymocyte Development. *Crit Rev Immunol.* 2015;35(1):59-76.
20. Xing Y, Hogquist KA. T-cell tolerance: central and peripheral. *Cold Spring Harb Perspect Biol.* 2012;4(6):a006957.
21. Derbinski J, Gäbler J, Brors B, Tierling S, Jonnakuty S, Hergenhausen M, et al. Promiscuous gene expression in thymic epithelial cells is regulated at multiple levels. *J Exp Med.* 2005;202(1):33-45.

22. Eldershaw SA, Sansom DM, Narendran P. Expression and function of the autoimmune regulator (Aire) gene in non-thymic tissue. *Clin Exp Immunol.* 2011;163(3):296-308.
23. Savage PA, Klawon DEJ, Miller CH. Regulatory T Cell Development. *Annu Rev Immunol.* 2020;38:421-53.
24. Matloubian M, Lo CG, Cinamon G, Lesneski MJ, Xu Y, Brinkmann V, et al. Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature.* 2004;427(6972):355-60.
25. Allende ML, Dreier JL, Mandala S, Proia RL. Expression of the sphingosine 1-phosphate receptor, S1P1, on T-cells controls thymic emigration. *J Biol Chem.* 2004;279(15):15396-401.
26. White AJ, Baik S, Parnell SM, Holland AM, Brombacher F, Jenkinson WE, et al. A type 2 cytokine axis for thymus emigration. *The Journal of experimental medicine.* 2017;214(8):2205-16.
27. Ueno T, Hara K, Willis MS, Malin MA, Höpken UE, Gray DH, et al. Role for CCR7 ligands in the emigration of newly generated T lymphocytes from the neonatal thymus. *Immunity.* 2002;16(2):205-18.
28. Jurlander J. Hematological malignancies, leukemias and lymphomas. *Encyclopedia of Cancer: Springer-Verlag Berlin Heidelberg;* 2011. p. 1640-4.
29. Campo E, Swerdlow SH, Harris NL, Pileri S, Stein H, Jaffe ES. The 2008 WHO classification of lymphoid neoplasms and beyond: evolving concepts and practical applications. *Blood.* 2011;117(19):5019-32.
30. Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Le Beau MM, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. *Blood.* 2016;127(20):2391-405.
31. Chiaretti S, Zini G, Bassan R. Diagnosis and subclassification of acute lymphoblastic leukemia. *Mediterr J Hematol Infect Dis.* 2014;6(1):e2014073.
32. Swerdlow SH, Campo E, Pileri SA, Harris NL, Stein H, Siebert R, et al. The 2016 revision of the World Health Organization classification of lymphoid neoplasms. *Blood.* 2016;127(20):2375-90.
33. Terwilliger T, Abdul-Hay M. Acute lymphoblastic leukemia: a comprehensive review and 2017 update. *Blood Cancer J.* 2017;7(6):e577.
34. James SL, Abate D, Abate KH, Abay SM, Abbafati C, Abbasi N, et al. Global, regional, and national incidence, prevalence, and years lived with disability for 354 diseases and injuries for 195 countries and territories, 1990–2017: a systematic analysis for the Global Burden of Disease Study 2017. *The Lancet.* 2018;392(10159):1789-858.
35. Baleyrier F, Decouvelaere AV, Bergeron J, Gaulard P, Canioni D, Bertrand Y, et al. T cell receptor genotyping and HOXA/TLX1 expression define three T lymphoblastic lymphoma subsets which might affect clinical outcome. *Clin Cancer Res.* 2008;14(3):692-700.
36. Karrman K, Forestier E, Heyman M, Andersen MK, Autio K, Blennow E, et al. Clinical and cytogenetic features of a population-based consecutive series of 285 pediatric T-cell acute lymphoblastic leukemias: rare T-cell receptor gene rearrangements are associated with poor outcome. *Genes Chromosomes Cancer.* 2009;48(9):795-805.
37. Wakeford R. Childhood leukaemia following medical diagnostic exposure to ionizing radiation in utero or after birth. *Radiat Prot Dosimetry.* 2008;132(2):166-74.
38. Eden T. Aetiology of childhood leukaemia. *Cancer Treat Rev.* 2010;36(4):286-97.
39. Del Principe MI, Maurillo L, Buccisano F, Sconocchia G, Cefalo M, De Santis G, et al. Central nervous system involvement in adult acute lymphoblastic leukemia: diagnostic tools, prophylaxis, and therapy. *Mediterr J Hematol Infect Dis.* 2014;6(1):e2014075.

40. Patel JL, Smith LM, Anderson J, Abromowitch M, Campana D, Jacobsen J, et al. The immunophenotype of T-lymphoblastic lymphoma in children and adolescents: a Children's Oncology Group report. *Br J Haematol*. 2012;159(4):454-61.
41. Hoelzer D, Gokbuget N. T-cell lymphoblastic lymphoma and T-cell acute lymphoblastic leukemia: a separate entity? *Clin Lymphoma Myeloma*. 2009;9 Suppl 3:S214-21.
42. Coustan-Smith E, Mullighan CG, Onciu M, Behm FG, Raimondi SC, Pei D, et al. Early T-cell precursor leukaemia: a subtype of very high-risk acute lymphoblastic leukaemia. *Lancet Oncol*. 2009;10(2):147-56.
43. Litzow MR, Ferrando AA. How I treat T-cell acute lymphoblastic leukemia in adults. *Blood*. 2015;126(7):833-41.
44. Raetz EA, Perkins SL, Bhojwani D, Smock K, Philip M, Carroll WL, et al. Gene expression profiling reveals intrinsic differences between T-cell acute lymphoblastic leukemia and T-cell lymphoblastic lymphoma. *Pediatr Blood Cancer*. 2006;47(2):130-40.
45. Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer [Internet]. 2020. Available from: <https://mitelmandatabase.isb-cgc.org>.
46. Karrman K, Castor A, Behrendtz M, Forestier E, Olsson L, Ehinger M, et al. Deep sequencing and SNP array analyses of pediatric T-cell acute lymphoblastic leukemia reveal NOTCH1 mutations in minor subclones and a high incidence of uniparental isodisomies affecting CDKN2A. *J Hematol Oncol*. 2015;8:42.
47. Yu L, Slovak ML, Mannoor K, Chen C, Hunger SP, Carroll AJ, et al. Microarray detection of multiple recurring submicroscopic chromosomal aberrations in pediatric T-cell acute lymphoblastic leukemia. *Leukemia*. 2011;25(6):1042-6.
48. Ferrando AA, Neuberg DS, Staunton J, Loh ML, Huard C, Raimondi SC, et al. Gene expression signatures define novel oncogenic pathways in T cell acute lymphoblastic leukemia. *Cancer Cell*. 2002;1(1):75-87.
49. Van Vlierberghe P, Pieters R, Beverloo HB, Meijerink JP. Molecular-genetic insights in paediatric T-cell acute lymphoblastic leukaemia. *Br J Haematol*. 2008;143(2):153-68.
50. Quintás-Cardama A, Tong W, Manshouri T, Vega F, Lennon PA, Cools J, et al. Activity of tyrosine kinase inhibitors against human NUP214-ABL1-positive T cell malignancies. *Leukemia*. 2008;22(6):1117-24.
51. Cheng Z, Yi Y, Xie S, Yu H, Peng H, Zhang G. The effect of the JAK2 inhibitor TG101209 against T cell acute lymphoblastic leukemia (T-ALL) is mediated by inhibition of JAK-STAT signaling and activation of the crosstalk between apoptosis and autophagy signaling. *Oncotarget*. 2017;8(63).
52. Roberts KG, Li Y, Payne-Turner D, Harvey RC, Yang YL, Pei D, et al. Targetable kinase-activating lesions in Ph-like acute lymphoblastic leukemia. *N Engl J Med*. 2014;371(11):1005-15.
53. Lacronique V, Boureux A, Valle VD, Poirel H, Quang CT, Mauchauffé M, et al. A TEL-JAK2 fusion protein with constitutive kinase activity in human leukemia. *Science*. 1997;278(5341):1309-12.
54. Mullighan CG, Phillips LA, Su X, Ma J, Miller CB, Shurtleff SA, et al. Genomic analysis of the clonal origins of relapsed acute lymphoblastic leukemia. *Science (New York, NY)*. 2008;322(5906):1377-80.
55. Gutierrez A, Sanda T, Grebliunaite R, Carracedo A, Salmena L, Ahn Y, et al. High frequency of PTEN, PI3K, and AKT abnormalities in T-cell acute lymphoblastic leukemia. *Blood*. 2009;114(3):647-50.
56. Tosello V, Mansour MR, Barnes K, Paganin M, Sulis ML, Jenkinson S, et al. WT1 mutations in T-ALL. *Blood*. 2009;114(5):1038-45.
57. Gutierrez A, Sanda T, Ma W, Zhang J, Grebliunaite R, Dahlberg S, et al. Inactivation of LEF1 in T-cell acute lymphoblastic leukemia. *Blood*. 2010;115(14):2845-51.

58. Kleppe M, Lahortiga I, El Chaar T, De Keersmaecker K, Mentens N, Graux C, et al. Deletion of the protein tyrosine phosphatase gene PTPN2 in T-cell acute lymphoblastic leukemia. *Nat Genet.* 2010;42(6):530-5.
59. Gutierrez A, Kentsis A, Sanda T, Holmfeldt L, Chen SC, Zhang J, et al. The BCL11B tumor suppressor is mutated across the major molecular subtypes of T-cell acute lymphoblastic leukemia. *Blood.* 2011;118(15):4169-73.
60. Ntziachristos P, Tsirigos A, Van Vlierberghe P, Nedjic J, Trimarchi T, Flaherty MS, et al. Genetic inactivation of the polycomb repressive complex 2 in T cell acute lymphoblastic leukemia. *Nat Med.* 2012;18(2):298-301.
61. Van Vlierberghe P, Palomero T, Khiabani H, Van der Meulen J, Castillo M, Van Roy N, et al. PHF6 mutations in T-cell acute lymphoblastic leukemia. *Nat Genet.* 2010;42(4):338-42.
62. Vicente C, Schwab C, Broux M, Geerdens E, Degryse S, Demeyer S, et al. Targeted sequencing identifies associations between IL7R-JAK mutations and epigenetic modulators in T-cell acute lymphoblastic leukemia. *Haematologica.* 2015;100(10):1301-10.
63. Clappier E, Cuccuini W, Kalota A, Crinquette A, Cayuela JM, Dik WA, et al. The C-MYB locus is involved in chromosomal translocation and genomic duplications in human T-cell acute leukemia (T-ALL), the translocation defining a new T-ALL subtype in very young children. *Blood.* 2007;110(4):1251-61.
64. Girardi T, Vicente C, Cools J, De Keersmaecker K. The genetics and molecular biology of T-ALL. *Blood.* 2017;129(9):1113-23.
65. Paganin M, Ferrando A. Molecular pathogenesis and targeted therapies for NOTCH1-induced T-cell acute lymphoblastic leukemia. *Blood Rev.* 2011;25(2):83-90.
66. Dos Santos NR, Ghezzi MN, da Silva RC, Fernandes MT. NF-kappaB in T-cell Acute Lymphoblastic Leukemia: Oncogenic Functions in Leukemic and in Microenvironmental Cells. *Cancers (Basel).* 2010;2(4):1838-60.
67. Osipo C, Golde TE, Osborne BA, Miele LA. Off the beaten pathway: the complex cross talk between Notch and NF-kappaB. *Lab Invest.* 2008;88(1):11-7.
68. Ribeiro D, Melao A, Barata JT. IL-7R-mediated signaling in T-cell acute lymphoblastic leukemia. *Adv Biol Regul.* 2013;53(2):211-22.
69. Mazzucchelli R, Durum SK. Interleukin-7 receptor expression: intelligent design. *Nat Rev Immunol.* 2007;7(2):144-54.
70. Ribeiro D, Melão A, van Bortel R, Santos CI, Silva A, Silva MC, et al. STAT5 is essential for IL-7-mediated viability, growth, and proliferation of T-cell acute lymphoblastic leukemia cells. *Blood Adv.* 2018;2(17):2199-213.
71. Bongiovanni D, Saccomani V, Piovan E. Aberrant Signaling Pathways in T-Cell Acute Lymphoblastic Leukemia. *International Journal of Molecular Sciences.* 2017;18(9).
72. Vilimas T, Mascarenhas J, Palomero T, Mandal M, Buonamici S, Meng F, et al. Targeting the NF-kappaB signaling pathway in Notch1-induced T-cell leukemia. *Nat Med.* 2007;13(1):70-7.
73. O'Neil J, Ventura J-J, Cusson N, Kelliher M. NF-κB activation in premalignant mouse tal-1/scl thymocytes and tumors. *Blood.* 2003;102(7):2593-6.
74. dos Santos NR, Williams M, Gachet S, Cormier F, Janin A, Weih D, et al. RelB-dependent stromal cells promote T-cell leukemogenesis. *PLoS One.* 2008;3(7):e2555.
75. Kordes U, Krappmann D, Heissmeyer V, Ludwig WD, Scheidereit C. Transcription factor NF-kappaB is constitutively activated in acute lymphoblastic leukemia cells. *Leukemia.* 2000;14(3):399-402.
76. Bellavia D, Campese AF, Alesse E, Vacca A, Felli MP, Balestri A, et al. Constitutive activation of NF-kappaB and T-cell leukemia/lymphoma in Notch3 transgenic mice. *EMBO J.* 2000;19(13):3337-48.

77. Espinosa L, Cathelin S, D'Altri T, Trimarchi T, Statnikov A, Guiu J, et al. The Notch/Hes1 pathway sustains NF- κ B activation through CYLD repression in T cell leukemia. *Cancer Cell*. 2010;18(3):268-81.
78. Soares-Silva M, Diniz FF, Gomes GN, Bahia D. The Mitogen-Activated Protein Kinase (MAPK) Pathway: Role in Immune Evasion by Trypanosomatids. *Frontiers in Microbiology*. 2016;7(183).
79. Guo YJ, Pan WW, Liu SB, Shen ZF, Xu Y, Hu LL. ERK/MAPK signalling pathway and tumorigenesis (Review). *Exp Ther Med*. 2020;19(3):1997-2007.
80. Ferrando AA. The role of NOTCH1 signaling in T-ALL. *Hematology*. 2009;2009(1):353-61.
81. Oswald F, Täuber B, Dobner T, Bourteele S, Kostezka U, Adler G, et al. p300 acts as a transcriptional coactivator for mammalian Notch-1. *Mol Cell Biol*. 2001;21(22):7761-74.
82. Erarslan-Uysal B, Kunz JB, Rausch T, Richter-Pechańska P, van Belzen IA, Fris mantas V, et al. Chromatin accessibility landscape of pediatric T-lymphoblastic leukemia and human T-cell precursors. *EMBO Mol Med*. 2020;12(9):e12104.
83. Huether R, Dong L, Chen X, Wu G, Parker M, Wei L, et al. The landscape of somatic mutations in epigenetic regulators across 1,000 paediatric cancer genomes. *Nat Commun*. 2014;5:3630-.
84. Zhang J, Ding L, Holmfeldt L, Wu G, Heatley SL, Payne-Turner D, et al. The genetic basis of early T-cell precursor acute lymphoblastic leukaemia. *Nature*. 2012;481(7380):157-63.
85. Soto-Feliciano YM, Bartlebaugh JME, Liu Y, Sanchez-Rivera FJ, Bhutkar A, Weintraub AS, et al. PHF6 regulates phenotypic plasticity through chromatin organization within lineage-specific genes. *Genes Dev*. 2017;31(10):973-89.
86. Mavrakis KJ, Van Der Meulen J, Wolfe AL, Liu X, Mets E, Taghon T, et al. A cooperative microRNA-tumor suppressor gene network in acute T-cell lymphoblastic leukemia (T-ALL). *Nat Genet*. 2011;43(7):673-8.
87. Ivor J. Benjamin RCG, Edward J. Wing, Greg Fitz. Andreoli and Carpenter's Cecil Essentials of Medicine. 9th ed: Elsevier Saunders; 2015.
88. Fattizzo B, Rosa J, Giannotta JA, Baldini L, Fracchiolla NS. The Physiopathology of T- Cell Acute Lymphoblastic Leukemia: Focus on Molecular Aspects. *Front Oncol*. 2020;10:273.
89. Pinnix CC, Yahalom J, Specht L, Dabaja BS. Radiation in Central Nervous System Leukemia: Guidelines From the International Lymphoma Radiation Oncology Group. *Int J Radiat Oncol Biol Phys*. 2018;102(1):53-8.
90. Su W, Thompson M, Sheu R-D, Steinberg A, Isola L, Stock R, et al. Low-dose cranial boost in high-risk adult acute lymphoblastic leukemia patients undergoing bone marrow transplant. *Practical Radiation Oncology*. 2017;7(2):103-8.
91. Cassileth PA, Harrington DP, Hines JD, Oken MM, Mazza JJ, McGlave P, et al. Maintenance chemotherapy prolongs remission duration in adult acute nonlymphocytic leukemia. *J Clin Oncol*. 1988;6(4):583-7.
92. Duration and intensity of maintenance chemotherapy in acute lymphoblastic leukaemia: overview of 42 trials involving 12 000 randomised children. *Lancet*. 1996;347(9018):1783-8.
93. Ribera J-M, Genescà E, Ribera J. Who Should Receive an Allogeneic Transplant in First Complete Remission? *Clinical Lymphoma Myeloma and Leukemia*. 2020;20:S48-S51.
94. Hoelzer D, Bassan R, Dombret H, Fielding A, Ribera JM, Buske C, et al. Acute lymphoblastic leukaemia in adult patients: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 2016;27(suppl 5):v69-v82.

95. Goldstone AH, Richards SM, Lazarus HM, Tallman MS, Buck G, Fielding AK, et al. In adults with standard-risk acute lymphoblastic leukemia, the greatest benefit is achieved from a matched sibling allogeneic transplantation in first complete remission, and an autologous transplantation is less effective than conventional consolidation/maintenance chemotherapy in all patients: final results of the International ALL Trial (MRC UKALL XII/ECOG E2993). *Blood*. 2008;111(4):1827-33.
96. Bond J, Graux C, Lhermitte L, Lara D, Cluzeau T, Leguay T, et al. Early Response-Based Therapy Stratification Improves Survival in Adult Early Thymic Precursor Acute Lymphoblastic Leukemia: A Group for Research on Adult Acute Lymphoblastic Leukemia Study. *J Clin Oncol*. 2017;35(23):2683-91.
97. Schrauder A, Reiter A, Gadner H, Niethammer D, Klingebiel T, Kremens B, et al. Superiority of allogeneic hematopoietic stem-cell transplantation compared with chemotherapy alone in high-risk childhood T-cell acute lymphoblastic leukemia: results from ALL-BFM 90 and 95. *J Clin Oncol*. 2006;24(36):5742-9.
98. Balduzzi A, Valsecchi MG, Uderzo C, De Lorenzo P, Klingebiel T, Peters C, et al. Chemotherapy versus allogeneic transplantation for very-high-risk childhood acute lymphoblastic leukaemia in first complete remission: comparison by genetic randomisation in an international prospective study. *The Lancet*. 2005;366(9486):635-42.
99. Dahlke J, Kröger N, Zabelina T, Ayuk F, Fehse N, Wolschke C, et al. Comparable results in patients with acute lymphoblastic leukemia after related and unrelated stem cell transplantation. *Bone Marrow Transplant*. 2006;37(2):155-63.
100. Kiehl MG, Kraut L, Schwerdtfeger R, Hertenstein B, Remberger M, Kroeger N, et al. Outcome of allogeneic hematopoietic stem-cell transplantation in adult patients with acute lymphoblastic leukemia: no difference in related compared with unrelated transplant in first complete remission. *J Clin Oncol*. 2004;22(14):2816-25.
101. Bassan R, Maino E, Cortelazzo S. Lymphoblastic lymphoma: an updated review on biology, diagnosis, and treatment. *Eur J Haematol*. 2016;96(5):447-60.
102. Weng AP, Ferrando AA, Lee W, Morris JPt, Silverman LB, Sanchez-Irizarry C, et al. Activating mutations of NOTCH1 in human T cell acute lymphoblastic leukemia. *Science*. 2004;306(5694):269-71.
103. Zweidler-McKay PA, DeAngelo DJ, Douer D, Dombret H, Ottmann OG, Vey N, et al. The Safety and Activity of BMS-906024, a Gamma Secretase Inhibitor (GSI) with Anti-Notch Activity, in Patients with Relapsed T-Cell Acute Lymphoblastic Leukemia (T-ALL): Initial Results of a Phase 1 Trial. *Blood*. 2014;124(21):968-.
104. Real PJ, Tosello V, Palomero T, Castillo M, Hernando E, de Stanchina E, et al. Gamma-secretase inhibitors reverse glucocorticoid resistance in T cell acute lymphoblastic leukemia. *Nat Med*. 2009;15(1):50-8.
105. Wei P, Walls M, Qiu M, Ding R, Denlinger RH, Wong A, et al. Evaluation of selective gamma-secretase inhibitor PF-03084014 for its antitumor efficacy and gastrointestinal safety to guide optimal clinical trial design. *Mol Cancer Ther*. 2010;9(6):1618-28.
106. Hagenbeek TJ, Wu X, Choy L, Sanchez-Irizarry C, Seshagiri S, Stinson J, et al. Murine Pten(-/-) T-ALL requires non-redundant PI3K/mTOR and DLL4/Notch1 signals for maintenance and gammac/TCR signals for thymic exit. *Cancer Lett*. 2014;346(2):237-48.
107. Minuzzo S, Agnusdei V, Pusceddu I, Pinazza M, Moserle L, Masiero M, et al. DLL4 regulates NOTCH signaling and growth of T acute lymphoblastic leukemia cells in NOD/SCID mice. *Carcinogenesis*. 2015;36(1):115-21.
108. Degryse S, de Bock CE, Cox L, Demeyer S, Gielen O, Mentens N, et al. JAK3 mutants transform hematopoietic cells through JAK1 activation, causing T-cell acute lymphoblastic leukemia in a mouse model. *Blood*. 2014;124(20):3092-100.
109. Subramaniam Prem S, Whye Dosh W, Efimenko E, Chen J, Tosello V, De Keersmaecker K, et al. Targeting Nonclassical Oncogenes for Therapy in T-ALL. *Cancer Cell*. 2012;21(4):459-72.

110. Falà F, Blalock WL, Tazzari PL, Cappellini A, Chiarini F, Martinelli G, et al. Proapoptotic activity and chemosensitizing effect of the novel Akt inhibitor (2S)-1-(1H-Indol-3-yl)-3-[5-(3-methyl-2H-indazol-5-yl)pyridin-3-yl]oxypropan-2-amine (A443654) in T-cell acute lymphoblastic leukemia. *Mol Pharmacol*. 2008;74(3):884-95.
111. Evangelisti C, Evangelisti C, Chiarini F, Lonetti A, Buontempo F, Bressanin D, et al. Therapeutic potential of targeting mTOR in T-cell acute lymphoblastic leukemia (Review). *Int J Oncol*. 2014;45(3):909-18.
112. Martelli AM, Chiarini F, Evangelisti C, Cappellini A, Buontempo F, Bressanin D, et al. Two hits are better than one: targeting both phosphatidylinositol 3-kinase and mammalian target of rapamycin as a therapeutic strategy for acute leukemia treatment. *Oncotarget*. 2012;3(4):371-94.
113. Mendez-Ferrer S, Bonnet D, Steensma DP, Hasserjian RP, Ghobrial IM, Gribben JG, et al. Bone marrow niches in haematological malignancies. *Nat Rev Cancer*. 2020;20(5):285-98.
114. Rupec RA, Jundt F, Rebholz B, Eckelt B, Weindl G, Herzinger T, et al. Stroma-mediated dysregulation of myelopoiesis in mice lacking I kappa B alpha. *Immunity*. 2005;22(4):479-91.
115. Walkley CR, Olsen GH, Dworkin S, Fabb SA, Swann J, McArthur GA, et al. A microenvironment-induced myeloproliferative syndrome caused by retinoic acid receptor gamma deficiency. *Cell*. 2007;129(6):1097-110.
116. Raaijmakers MH, Mukherjee S, Guo S, Zhang S, Kobayashi T, Schoonmaker JA, et al. Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. *Nature*. 2010;464(7290):852-7.
117. Chiarini F, Lonetti A, Evangelisti C, Buontempo F, Orsini E, Evangelisti C, et al. Advances in understanding the acute lymphoblastic leukemia bone marrow microenvironment: From biology to therapeutic targeting. *Biochim Biophys Acta*. 2016;1863(3):449-63.
118. Tabe Y, Konopleva M. Advances in understanding the leukaemia microenvironment. *Br J Haematol*. 2014;164(6):767-78.
119. Wang W, Zimmerman G, Huang X, Yu S, Myers J, Wang Y, et al. Aberrant Notch Signaling in the Bone Marrow Microenvironment of Acute Lymphoid Leukemia Suppresses Osteoblast-Mediated Support of Hematopoietic Niche Function. *Cancer Res*. 2016;76(6):1641-52.
120. Hawkins ED, Duarte D, Akinduro O, Khorshed RA, Passaro D, Nowicka M, et al. T-cell acute leukaemia exhibits dynamic interactions with bone marrow microenvironments. *Nature*. 2016;538(7626):518-22.
121. Liu J, Masurekar A, Johnson S, Chakraborty S, Griffiths J, Smith D, et al. Stromal cell-mediated mitochondrial redox adaptation regulates drug resistance in childhood acute lymphoblastic leukemia. *Oncotarget*. 2015;6(40):43048-64.
122. Battula VL, Chen Y, Cabreira Mda G, Ruvolo V, Wang Z, Ma W, et al. Connective tissue growth factor regulates adipocyte differentiation of mesenchymal stromal cells and facilitates leukemia bone marrow engraftment. *Blood*. 2013;122(3):357-66.
123. Cahu X, Calvo J, Poglio S, Prade N, Colsch B, Arcangeli ML, et al. Bone marrow sites differently imprint dormancy and chemoresistance to T-cell acute lymphoblastic leukemia. *Blood Adv*. 2017;1(20):1760-72.
124. Uzan B, Poglio S, Gerby B, Wu CL, Gross J, Armstrong F, et al. Interleukin-18 produced by bone marrow-derived stromal cells supports T-cell acute leukaemia progression. *EMBO Mol Med*. 2014;6(6):821-34.
125. Ehsanipour EA, Sheng X, Behan JW, Wang X, Butturini A, Avramis VI, et al. Adipocytes cause leukemia cell resistance to L-asparaginase via release of glutamine. *Cancer Res*. 2013;73(10):2998-3006.

126. Kwong-Lam F, Chi-Fung CG. Vincristine could partly suppress stromal support to T-ALL blasts during pegylated arginase I treatment. *Exp Hematol Oncol*. 2013;2(1):11-.
127. Winter SS, Sweatman JJ, Lawrence MB, Rhoades TH, Hart AL, Larson RS. Enhanced T-lineage acute lymphoblastic leukaemia cell survival on bone marrow stroma requires involvement of LFA-1 and ICAM-1. *Br J Haematol*. 2001;115(4):862-71.
128. Benito J, Shi Y, Szymanska B, Carol H, Boehm I, Lu H, et al. Pronounced hypoxia in models of murine and human leukemia: high efficacy of hypoxia-activated prodrug PR-104. *PLoS One*. 2011;6(8):e23108.
129. Maynard MA, Ohh M. The role of hypoxia-inducible factors in cancer. *Cellular and Molecular Life Sciences*. 2007;64(16):2170-80.
130. Giambra V, Jenkins CE, Lam SH, Hoofd C, Belmonte M, Wang X, et al. Leukemia stem cells in T-ALL require active Hif1 α and Wnt signaling. *Blood*. 2015;125(25):3917-27.
131. Pitt LA, Tikhonova AN, Hu H, Trimarchi T, King B, Gong Y, et al. CXCL12-Producing Vascular Endothelial Niches Control Acute T Cell Leukemia Maintenance. *Cancer Cell*. 2015;27(6):755-68.
132. Passaro D, Irigoyen M, Catherinet C, Gachet S, Da Costa De Jesus C, Lasgi C, et al. CXCR4 Is Required for Leukemia-Initiating Cell Activity in T Cell Acute Lymphoblastic Leukemia. *Cancer Cell*. 2015;27(6):769-79.
133. Melo RdCC, Longhini AL, Bigarella CL, Baratti MO, Traina F, Favaro P, et al. CXCR7 Is Highly Expressed in Acute Lymphoblastic Leukemia and Potentiates CXCR4 Response to CXCL12. *PLOS ONE*. 2014;9(1):e85926.
134. O'Neill HC. Niches for Extramedullary Hematopoiesis in the Spleen. *Niche* 2012;1(1):12-6.
135. Shuster JJ, Falletta JM, Pullen DJ, Crist WM, Humphrey GB, Dowell BL, et al. Prognostic factors in childhood T-cell acute lymphoblastic leukemia: a Pediatric Oncology Group study. *Blood*. 1990;75(1):166-73.
136. Ma S, Shi Y, Pang Y, Dong F, Cheng H, Hao S, et al. Notch1-induced T cell leukemia can be potentiated by microenvironmental cues in the spleen. *J Hematol Oncol*. 2014;7:71-.
137. Di Grande A, Piers S, Van Vlierberghe P, Ni Chonghaile T. The Splenic Microenvironment Is a Novel Site of Relapse Following ABT-199 Treatment. *Blood*. 2019;134(Supplement_1):3800-.
138. Di Grande A, Peirs S, Donovan PD, Van Timpont M, Morscio J, Lintermans B, et al. The spleen as a sanctuary site for residual leukemic cells following ABT-199 monotherapy in ETP-ALL. *Blood Adv*. 2021;5(7):1963-76.
139. Kaplan HS. Influence of thymectomy, splenectomy, and gonadectomy on incidence of radiation-induced lymphoid tumors in strain C57 black mice. *J Natl Cancer Inst*. 1950;11(1):83-90.
140. Hiai H, Nishi Y, Miyazawa T, Matsudaira Y, Nishizuka Y. Mouse lymphoid leukemias: symbiotic complexes of neoplastic lymphocytes and their microenvironments. *J Natl Cancer Inst*. 1981;66(4):713-22.
141. Attarbaschi A, Mann G, Dworzak M, Wiesbauer P, Schrappe M, Gadner H. Mediastinal mass in childhood T-cell acute lymphoblastic leukemia: significance and therapy response. *Med Pediatr Oncol*. 2002;39(6):558-65.
142. Mazzucchelli RI, Warming S, Lawrence SM, Ishii M, Abshari M, Washington AV, et al. Visualization and identification of IL-7 producing cells in reporter mice. *PLoS One*. 2009;4(11):e7637.
143. Peschon JJ, Morrissey PJ, Grabstein KH, Ramsdell FJ, Maraskovsky E, Gliniak BC, et al. Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice. *Journal of Experimental Medicine*. 1994;180(5):1955-60.

144. Oliveira ML, Akkapeddi P, Ribeiro D, Melao A, Barata JT. IL-7R-mediated signaling in T-cell acute lymphoblastic leukemia: An update. *Adv Biol Regul.* 2019;71:88-96.
145. Scupoli MT, Vinante F, Krampera M, Vincenzi C, Nadali G, Zampieri F, et al. Thymic epithelial cells promote survival of human T-cell acute lymphoblastic leukemia blasts: the role of interleukin-7. *Haematologica.* 2003;88(11):1229-37.
146. Scupoli MT, Perbellini O, Krampera M, Vinante F, Cioffi F, Pizzolo G. Interleukin 7 requirement for survival of T-cell acute lymphoblastic leukemia and human thymocytes on bone marrow stroma. *Haematologica.* 2007;92(2):264-6.
147. Barata J, Cardoso AA, Nadler L, Boussiotis VA. Interleukin-7 promotes survival and cell cycle progression of T-cell acute lymphoblastic leukemia cells by down-regulating the cyclin-dependent kinase inhibitor p27(kip1). *Blood.* 2001;98:1524-31.
148. Silva A, Laranjeira AB, Martins LR, Cardoso BA, Demengeot J, Yunes JA, et al. IL-7 contributes to the progression of human T-cell acute lymphoblastic leukemias. *Cancer Res.* 2011;71(14):4780-9.
149. Laouar Y, Crispe IN, Flavell RA. Overexpression of IL-7R alpha provides a competitive advantage during early T-cell development. *Blood.* 2004;103(6):1985-94.
150. Ghezzo MN, Fernandes MT, Pacheco-Leyva I, Rodrigues PM, Machado RS, Araujo MAS, et al. FoxN1-dependent thymic epithelial cells promote T-cell leukemia development. *Carcinogenesis.* 2018;39(12):1463-76.
151. Calderon L, Boehm T. Synergistic, context-dependent, and hierarchical functions of epithelial components in thymic microenvironments. *Cell.* 2012;149(1):159-72.
152. Chen L, Xiao S, Manley NR. Foxn1 is required to maintain the postnatal thymic microenvironment in a dosage-sensitive manner. *Blood.* 2009;113(3):567-74.
153. Tomczkowski J, Frick D, Schwinzer B, Wittner N, Ludwig WD, Reiter A, et al. Expression and regulation of c-kit receptor and response to stem cell factor in childhood malignant T-lymphoblastic cells. *Leukemia.* 1998;12(8):1221-9.
154. Ferrandino F, Bernardini G, Tsaouli G, Grazioli P, Campese AF, Noce C, et al. Intrathymic Notch3 and CXCR4 combinatorial interplay facilitates T-cell leukemia propagation. *Oncogene.* 2018;37(49):6285-98.
155. Mirandola L, Chiriva-Internati M, Montagna D, Locatelli F, Zecca M, Ranzani M, et al. Notch1 regulates chemotaxis and proliferation by controlling the CC-chemokine receptors 5 and 9 in T cell acute lymphoblastic leukaemia. *J Pathol.* 2012;226(5):713-22.
156. Triplett TA, Cardenas KT, Lancaster JN, Hu Z, Selden HJ, Jasso GJ, et al. Endogenous dendritic cells from the tumor microenvironment support T-ALL growth via IGF1R activation. *Proc Natl Acad Sci U S A.* 2016;113(8):E1016-25.
157. Talmadge JE, Gabrilovich DI. History of myeloid-derived suppressor cells. *Nature Reviews Cancer.* 2013;13(10):739-52.
158. Gabrilovich DI, Ostrand-Rosenberg S, Bronte V. Coordinated regulation of myeloid cells by tumours. *Nat Rev Immunol.* 2012;12(4):253-68.
159. Kumar V, Patel S, Tcyganov E, Gabrilovich DI. The Nature of Myeloid-Derived Suppressor Cells in the Tumor Microenvironment. *Trends in Immunology.* 2016;37(3):208-20.
160. Noy R, Pollard Jeffrey W. Tumor-Associated Macrophages: From Mechanisms to Therapy. *Immunity.* 2014;41(1):49-61.
161. Coffelt SB, Wellenstein MD, de Visser KE. Neutrophils in cancer: neutral no more. *Nature Reviews Cancer.* 2016;16(7):431-46.
162. Kitamura T, Qian B-Z, Pollard JW. Immune cell promotion of metastasis. *Nature Reviews Immunology.* 2015;15(2):73-86.
163. Engblom C, Pfirschke C, Pittet MJ. The role of myeloid cells in cancer therapies. *Nature Reviews Cancer.* 2016;16(7):447-62.

164. Fleming V, Hu X, Weber R, Nagibin V, Groth C, Altevogt P, et al. Targeting Myeloid-Derived Suppressor Cells to Bypass Tumor-Induced Immunosuppression. *Front Immunol.* 2018;9:398.
165. DeNardo DG, Ruffell B. Macrophages as regulators of tumour immunity and immunotherapy. *Nat Rev Immunol.* 2019;19(6):369-82.
166. Geissmann F, Manz MG, Jung S, Sieweke MH, Merad M, Ley K. Development of monocytes, macrophages, and dendritic cells. *Science.* 2010;327(5966):656-61.
167. Serbina NV, Jia T, Hohl TM, Pamer EG. Monocyte-mediated defense against microbial pathogens. *Annu Rev Immunol.* 2008;26:421-52.
168. Zhu YP, Thomas GD, Hedrick CC. 2014 Jeffrey M. Hoeg Award Lecture: Transcriptional Control of Monocyte Development. *Arterioscler Thromb Vasc Biol.* 2016;36(9):1722-33.
169. Yanez A, Coetzee SG, Olsson A, Muench DE, Berman BP, Hazelett DJ, et al. Granulocyte-Monocyte Progenitors and Monocyte-Dendritic Cell Progenitors Independently Produce Functionally Distinct Monocytes. *Immunity.* 2017;47(5):890-902 e4.
170. Ginhoux F, Jung S. Monocytes and macrophages: developmental pathways and tissue homeostasis. *Nat Rev Immunol.* 2014;14(6):392-404.
171. Cortez-Retamozo V, Etzrodt M, Newton A, Rauch PJ, Chudnovskiy A, Berger C, et al. Origins of tumor-associated macrophages and neutrophils. *Proc Natl Acad Sci U S A.* 2012;109(7):2491-6.
172. Wu C, Ning H, Liu M, Lin J, Luo S, Zhu W, et al. Spleen mediates a distinct hematopoietic progenitor response supporting tumor-promoting myelopoiesis. *J Clin Invest.* 2018;128(8):3425-38.
173. McKim DB, Yin W, Wang Y, Cole SW, Godbout JP, Sheridan JF. Social Stress Mobilizes Hematopoietic Stem Cells to Establish Persistent Splenic Myelopoiesis. *Cell Rep.* 2018;25(9):2552-62 e3.
174. Swirski FK, Nahrendorf M, Etzrodt M, Wildgruber M, Cortez-Retamozo V, Panizzi P, et al. Identification of splenic reservoir monocytes and their deployment to inflammatory sites. *Science.* 2009;325(5940):612-6.
175. Auffray C, Fogg D, Garfa M, Elain G, Join-Lambert O, Kayal S, et al. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. *Science.* 2007;317(5838):666-70.
176. Wolf AA, Yanez A, Barman PK, Goodridge HS. The Ontogeny of Monocyte Subsets. *Front Immunol.* 2019;10:1642.
177. Kratofil RM, Kubes P, Deniset JF. Monocyte Conversion During Inflammation and Injury. *Arterioscler Thromb Vasc Biol.* 2017;37(1):35-42.
178. Carlin LM, Stamatiades EG, Auffray C, Hanna RN, Glover L, Vizcay-Barrena G, et al. Nr4a1-dependent Ly6C(low) monocytes monitor endothelial cells and orchestrate their disposal. *Cell.* 2013;153(2):362-75.
179. Yona S, Kim KW, Wolf Y, Mildner A, Varol D, Breker M, et al. Fate mapping reveals origins and dynamics of monocytes and tissue macrophages under homeostasis. *Immunity.* 2013;38(1):79-91.
180. Bain CC, Bravo-Blas A, Scott CL, Perdiguero EG, Geissmann F, Henri S, et al. Constant replenishment from circulating monocytes maintains the macrophage pool in the intestine of adult mice. *Nat Immunol.* 2014;15(10):929-37.
181. Calderon B, Carrero JA, Ferris ST, Sojka DK, Moore L, Epelman S, et al. The pancreas anatomy conditions the origin and properties of resident macrophages. *J Exp Med.* 2015;212(10):1497-512.
182. Tamoutounour S, Guilliams M, Montanana Sanchis F, Liu H, Terhorst D, Malosse C, et al. Origins and functional specialization of macrophages and of conventional and monocyte-derived dendritic cells in mouse skin. *Immunity.* 2013;39(5):925-38.

183. Epelman S, Lavine KJ, Beaudin AE, Sojka DK, Carrero JA, Calderon B, et al. Embryonic and adult-derived resident cardiac macrophages are maintained through distinct mechanisms at steady state and during inflammation. *Immunity*. 2014;40(1):91-104.
184. Puhr S, Lee J, Zvezdova E, Zhou YJ, Liu K. Dendritic cell development-History, advances, and open questions. *Semin Immunol*. 2015;27(6):388-96.
185. Schlitzer A, Sivakamasundari V, Chen J, Sumatoh HR, Schreuder J, Lum J, et al. Identification of cDC1- and cDC2-committed DC progenitors reveals early lineage priming at the common DC progenitor stage in the bone marrow. *Nat Immunol*. 2015;16(7):718-28.
186. Merad M, Sathe P, Helft J, Miller J, Mortha A. The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol*. 2013;31:563-604.
187. Varol C, Vallon-Eberhard A, Elinav E, Aychek T, Shapira Y, Luche H, et al. Intestinal lamina propria dendritic cell subsets have different origin and functions. *Immunity*. 2009;31(3):502-12.
188. Liu K, Nussenzweig MC. Origin and development of dendritic cells. *Immunol Rev*. 2010;234(1):45-54.
189. Bogunovic M, Ginhoux F, Helft J, Shang L, Hashimoto D, Greter M, et al. Origin of the lamina propria dendritic cell network. *Immunity*. 2009;31(3):513-25.
190. Shigematsu H, Reizis B, Iwasaki H, Mizuno S, Hu D, Traver D, et al. Plasmacytoid dendritic cells activate lymphoid-specific genetic programs irrespective of their cellular origin. *Immunity*. 2004;21(1):43-53.
191. Karsunky H, Merad M, Cozzio A, Weissman IL, Manz MG. Flt3 Ligand Regulates Dendritic Cell Development from Flt3+ Lymphoid and Myeloid-committed Progenitors to Flt3+ Dendritic Cells In Vivo. *Journal of Experimental Medicine*. 2003;198(2):305-13.
192. Ishikawa F, Niino H, Iino T, Yoshida S, Saito N, Onohara S, et al. The developmental program of human dendritic cells is operated independently of conventional myeloid and lymphoid pathways. *Blood*. 2007;110(10):3591-660.
193. Musumeci A, Lutz K, Winheim E, Krug AB. What Makes a pDC: Recent Advances in Understanding Plasmacytoid DC Development and Heterogeneity. *Front Immunol*. 2019;10:1222.
194. Guermonprez P, Gerber-Ferder Y, Vaivode K, Bourdely P, Helft J. Origin and development of classical dendritic cells. *Int Rev Cell Mol Biol*. 2019;349:1-54.
195. Williams JW, Tjota MY, Clay BS, Vander Lugt B, Bandukwala HS, Hrusch CL, et al. Transcription factor IRF4 drives dendritic cells to promote Th2 differentiation. *Nat Commun*. 2013.
196. Schlitzer A, McGovern N, Teo P, Zelante T, Atarashi K, Low D, et al. IRF4 transcription factor-dependent CD11b+ dendritic cells in human and mouse control mucosal IL-17 cytokine responses. *Immunity*. 2013;38(5):970-83.
197. Kaiko GE, Horvat JC, Beagley KW, Hansbro PM. Immunological decision-making: how does the immune system decide to mount a helper T-cell response? *Immunology*. 2008;123(3):326-38.
198. Chistiakov DA, Orekhov AN, Sobenin IA, Bobryshev YV. Plasmacytoid dendritic cells: development, functions, and role in atherosclerotic inflammation. *Front Physiol*. 2014;5:279.
199. Gabrilovich DI. Myeloid-Derived Suppressor Cells. *Cancer Immunol Res*. 2017;5(1):3-8.
200. Lee YS, Radford KJ. The role of dendritic cells in cancer. *Int Rev Cell Mol Biol*. 2019;348:123-78.
201. Gabrilovich DI, Nagaraj S. Myeloid-derived suppressor cells as regulators of the immune system. *Nat Rev Immunol*. 2009;9(3):162-74.

202. Schmielau J, Finn OJ. Activated granulocytes and granulocyte-derived hydrogen peroxide are the underlying mechanism of suppression of t-cell function in advanced cancer patients. *Cancer Res.* 2001;61(12):4756-60.
203. Mazzoni A, Bronte V, Visintin A, Spitzer JH, Apolloni E, Serafini P, et al. Myeloid suppressor lines inhibit T cell responses by an NO-dependent mechanism. *J Immunol.* 2002;168(2):689-95.
204. Rodriguez PC, Quiceno DG, Zabaleta J, Ortiz B, Zea AH, Piazuelo MB, et al. Arginase I production in the tumor microenvironment by mature myeloid cells inhibits T-cell receptor expression and antigen-specific T-cell responses. *Cancer Res.* 2004;64(16):5839-49.
205. Srivastava MK, Sinha P, Clements VK, Rodriguez P, Ostrand-Rosenberg S. Myeloid-derived suppressor cells inhibit T-cell activation by depleting cystine and cysteine. *Cancer Res.* 2010;70(1):68-77.
206. Yu J, Du W, Yan F, Wang Y, Li H, Cao S, et al. Myeloid-derived suppressor cells suppress antitumor immune responses through IDO expression and correlate with lymph node metastasis in patients with breast cancer. *J Immunol.* 2013;190(7):3783-97.
207. Geiger R, Rieckmann JC, Wolf T, Basso C, Feng Y, Fuhrer T, et al. L-Arginine Modulates T Cell Metabolism and Enhances Survival and Anti-tumor Activity. *Cell.* 2016;167(3):829-42.e13.
208. Rodriguez PC, Quiceno DG, Ochoa AC. L-arginine availability regulates T-lymphocyte cell-cycle progression. *Blood.* 2007;109(4):1568-73.
209. Kumar V, Cheng P, Condamine T, Mony S, Languino LR, McCaffrey JC, et al. CD45 Phosphatase Inhibits STAT3 Transcription Factor Activity in Myeloid Cells and Promotes Tumor-Associated Macrophage Differentiation. *Immunity.* 2016;44(2):303-15.
210. Bizymi N, Bjelica S, Kittang AO, Mojsilovic S, Velegraki M, Pontikoglou C, et al. Myeloid-Derived Suppressor Cells in Hematologic Diseases: Promising Biomarkers and Treatment Targets. *Hemasphere.* 2019;3(1):e168.
211. Haas L, Obenauf AC. Allies or Enemies-The Multifaceted Role of Myeloid Cells in the Tumor Microenvironment. *Front Immunol.* 2019;10:2746.
212. Ginhoux F, Guilliams M. Tissue-Resident Macrophage Ontogeny and Homeostasis. *Immunity.* 2016;44(3):439-49.
213. Hirayama D, Iida T, Nakase H. The Phagocytic Function of Macrophage-Enforcing Innate Immunity and Tissue Homeostasis. *International journal of molecular sciences.* 2017;19(1):92.
214. Li Y, You MJ, Yang Y, Hu D, Tian C. The Role of Tumor-Associated Macrophages in Leukemia. *Acta Haematol.* 2020;143(2):112-7.
215. Osaki T, Hashimoto W, Gambotto A, Okamura H, Robbins PD, Kurimoto M, et al. Potent antitumor effects mediated by local expression of the mature form of the interferon- γ inducing factor, interleukin-18 (IL-18). *Gene Therapy.* 1999;6(5):808-15.
216. Qian BZ, Pollard JW. Macrophage diversity enhances tumor progression and metastasis. *Cell.* 2010;141(1):39-51.
217. Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. *Nat Immunol.* 2010;11(10):889-96.
218. Mantovani A, Sica A, Allavena P, Garlanda C, Locati M. Tumor-associated macrophages and the related myeloid-derived suppressor cells as a paradigm of the diversity of macrophage activation. *Hum Immunol.* 2009;70(5):325-30.
219. Mantovani A, Sica A. Macrophages, innate immunity and cancer: balance, tolerance, and diversity. *Current Opinion in Immunology.* 2010;22(2):231-7.
220. Ruffell B, Coussens LM. Macrophages and therapeutic resistance in cancer. *Cancer Cell.* 2015;27(4):462-72.

221. Zhang Q-w, Liu L, Gong C-y, Shi H-s, Zeng Y-h, Wang X-z, et al. Prognostic Significance of Tumor-Associated Macrophages in Solid Tumor: A Meta-Analysis of the Literature. *PLOS ONE*. 2012;7(12):e50946.
222. Steidl C, Lee T, Shah SP, Farinha P, Han G, Nayar T, et al. Tumor-associated macrophages and survival in classic Hodgkin's lymphoma. *N Engl J Med*. 2010;362(10):875-85.
223. Komohara Y, Jinushi M, Takeya M. Clinical significance of macrophage heterogeneity in human malignant tumors. *Cancer Sci*. 2014;105(1):1-8.
224. Dave SS, Wright G, Tan B, Rosenwald A, Gascoyne RD, Chan WC, et al. Prediction of Survival in Follicular Lymphoma Based on Molecular Features of Tumor-Infiltrating Immune Cells. *New England Journal of Medicine*. 2004;351(21):2159-69.
225. Lin EY, Li JF, Gnatovskiy L, Deng Y, Zhu L, Grzesik DA, et al. Macrophages regulate the angiogenic switch in a mouse model of breast cancer. *Cancer Res*. 2006;66(23):11238-46.
226. Riabov V, Gudima A, Wang N, Mickley A, Orekhov A, Kzhyshkowska J. Role of tumor associated macrophages in tumor angiogenesis and lymphangiogenesis. *Front Physiol*. 2014;5:75.
227. Sangaletti S, Di Carlo E, Gariboldi S, Miotti S, Cappetti B, Parenza M, et al. Macrophage-derived SPARC bridges tumor cell-extracellular matrix interactions toward metastasis. *Cancer Res*. 2008;68(21):9050-9.
228. Chen J, Yao Y, Gong C, Yu F, Su S, Chen J, et al. CCL18 from tumor-associated macrophages promotes breast cancer metastasis via PITPNM3. *Cancer cell*. 2011;19(4):541-55.
229. Qian B, Deng Y, Im JH, Muschel RJ, Zou Y, Li J, et al. A distinct macrophage population mediates metastatic breast cancer cell extravasation, establishment and growth. *PLoS One*. 2009;4(8):e6562.
230. Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. *Nature medicine*. 2013;19(11):1423-37.
231. Zheng Y, Cai Z, Wang S, Zhang X, Qian J, Hong S, et al. Macrophages are an abundant component of myeloma microenvironment and protect myeloma cells from chemotherapy drug-induced apoptosis. *Blood*. 2009;114(17):3625-8.
232. Shiao SL, Ruffell B, DeNardo DG, Faddegon BA, Park CC, Coussens LM. TH2-Polarized CD4(+) T Cells and Macrophages Limit Efficacy of Radiotherapy. *Cancer Immunol Res*. 2015;3(5):518-25.
233. Mitchem JB, Brennan DJ, Knolhoff BL, Belt BA, Zhu Y, Sanford DE, et al. Targeting tumor-infiltrating macrophages decreases tumor-initiating cells, relieves immunosuppression, and improves chemotherapeutic responses. *Cancer Res*. 2013;73(3):1128-41.
234. Strachan DC, Ruffell B, Oei Y, Bissell MJ, Coussens LM, Pryer N, et al. CSF1R inhibition delays cervical and mammary tumor growth in murine models by attenuating the turnover of tumor-associated macrophages and enhancing infiltration by CD8(+) T cells. *Oncoimmunology*. 2013;2(12):e26968.
235. Pyonteck SM, Akkari L, Schuhmacher AJ, Bowman RL, Sevenich L, Quail DF, et al. CSF-1R inhibition alters macrophage polarization and blocks glioma progression. *Nat Med*. 2013;19(10):1264-72.
236. DeNardo DG, Brennan DJ, Rexhepaj E, Ruffell B, Shiao SL, Madden SF, et al. Leukocyte complexity predicts breast cancer survival and functionally regulates response to chemotherapy. *Cancer Discov*. 2011;1(1):54-67.
237. Paulus P, Stanley ER, Schäfer R, Abraham D, Aharinejad S. Colony-stimulating factor-1 antibody reverses chemoresistance in human MCF-7 breast cancer xenografts. *Cancer Res*. 2006;66(8):4349-56.

238. Ruffell B, Chang-Strachan D, Chan V, Rosenbusch A, Ho CM, Pryer N, et al. Macrophage IL-10 blocks CD8⁺ T cell-dependent responses to chemotherapy by suppressing IL-12 expression in intratumoral dendritic cells. *Cancer Cell*. 2014;26(5):623-37.
239. Priceman SJ, Sung JL, Shaposhnik Z, Burton JB, Torres-Collado AX, Moughon DL, et al. Targeting distinct tumor-infiltrating myeloid cells by inhibiting CSF-1 receptor: combating tumor evasion of antiangiogenic therapy. *Blood*. 2010;115(7):1461-71.
240. Zhu Y, Knolhoff BL, Meyer MA, Nywening TM, West BL, Luo J, et al. CSF1/CSF1R blockade reprograms tumor-infiltrating macrophages and improves response to T-cell checkpoint immunotherapy in pancreatic cancer models. *Cancer research*. 2014;74(18):5057-69.
241. Dalod M, Chelbi R, Malissen B, Lawrence T. Dendritic cell maturation: functional specialization through signaling specificity and transcriptional programming. *EMBO J*. 2014;33(10):1104-16.
242. Böttcher JP, Reis e Sousa C. The Role of Type 1 Conventional Dendritic Cells in Cancer Immunity. *Trends in Cancer*. 2018;4(11):784-92.
243. Cancel J-C, Crozat K, Dalod M, Mattiuz R. Are Conventional Type 1 Dendritic Cells Critical for Protective Antitumor Immunity and How? *Frontiers in Immunology*. 2019;10(9).
244. Edelson BT, Kc W, Juang R, Kohyama M, Benoit LA, Klekotka PA, et al. Peripheral CD103⁺ dendritic cells form a unified subset developmentally related to CD8alpha⁺ conventional dendritic cells. *J Exp Med*. 2010;207(4):823-36.
245. Hildner K, Edelson BT, Purtha WE, Diamond M, Matsushita H, Kohyama M, et al. Batf3 deficiency reveals a critical role for CD8alpha⁺ dendritic cells in cytotoxic T cell immunity. *Science*. 2008;322(5904):1097-100.
246. Roberts EW, Broz ML, Binnewies M, Headley MB, Nelson AE, Wolf DM, et al. Critical Role for CD103(+)/CD141(+) Dendritic Cells Bearing CCR7 for Tumor Antigen Trafficking and Priming of T Cell Immunity in Melanoma. *Cancer Cell*. 2016;30(2):324-36.
247. Spranger S, Dai D, Horton B, Gajewski TF. Tumor-Residing Batf3 Dendritic Cells Are Required for Effector T Cell Trafficking and Adoptive T Cell Therapy. *Cancer Cell*. 2017;31(5):711-23.e4.
248. Chow MT, Ozga AJ, Servis RL, Frederick DT, Lo JA, Fisher DE, et al. Intratumoral Activity of the CXCR3 Chemokine System Is Required for the Efficacy of Anti-PD-1 Therapy. *Immunity*. 2019;50(6):1498-512.e5.
249. Binnewies M, Muijal AM, Pollack JL, Combes AJ, Hardison EA, Barry KC, et al. Unleashing Type-2 Dendritic Cells to Drive Protective Antitumor CD4(+) T Cell Immunity. *Cell*. 2019;177(3):556-71.e16.
250. Laoui D, Keirsse J, Morias Y, Van Overmeire E, Geeraerts X, Elkrim Y, et al. The tumour microenvironment harbours ontogenically distinct dendritic cell populations with opposing effects on tumour immunity. *Nat Commun*. 2016;7:13720-.
251. Bailey SR, Nelson MH, Himes RA, Li Z, Mehrotra S, Paulos CM. Th17 cells in cancer: the ultimate identity crisis. *Front Immunol*. 2014;5:276.
252. Swiecki M, Colonna M. The multifaceted biology of plasmacytoid dendritic cells. *Nature reviews Immunology*. 2015;15(8):471-85.
253. Sisirak V, Faget J, Vey N, Blay JY, Menetrier-Caux C, Caux C, et al. Plasmacytoid dendritic cells deficient in IFNalpha production promote the amplification of FOXP3(+) regulatory T cells and are associated with poor prognosis in breast cancer patients. *Oncoimmunology*. 2013;2(1):e22338.
254. Treilleux I, Blay JY, Bendriss-Vermare N, Ray-Coquard I, Bachelot T, Guastalla JP, et al. Dendritic cell infiltration and prognosis of early stage breast cancer. *Clin Cancer Res*. 2004;10(22):7466-74.

255. Labidi-Galy SI, Sisirak V, Meeus P, Gobert M, Treilleux I, Bajard A, et al. Quantitative and functional alterations of plasmacytoid dendritic cells contribute to immune tolerance in ovarian cancer. *Cancer research*. 2011;71(16):5423-34.
256. Jensen TO, Schmidt H, Møller HJ, Donskov F, Høyer M, Sjoegren P, et al. Intratumoral neutrophils and plasmacytoid dendritic cells indicate poor prognosis and are associated with pSTAT3 expression in AJCC stage I/II melanoma. *Cancer*. 2012;118(9):2476-85.
257. Hartmann E, Wollenberg B, Rothenfusser S, Wagner M, Wellisch D, Mack B, et al. Identification and functional analysis of tumor-infiltrating plasmacytoid dendritic cells in head and neck cancer. *Cancer Res*. 2003;63(19):6478-87.
258. Aspord C, Leccia M-T, Charles J, Plumas J. Melanoma hijacks plasmacytoid dendritic cells to promote its own progression. *Oncoimmunology*. 2014;3(1):e27402-e.
259. Veglia F, Gabrilovich DI. Dendritic cells in cancer: the role revisited. *Current opinion in immunology*. 2017;45:43-51.
260. Kuhn S, Yang J, Ronchese F. Monocyte-Derived Dendritic Cells Are Essential for CD8(+) T Cell Activation and Antitumor Responses After Local Immunotherapy. *Front Immunol*. 2015;6:584.
261. Kuhn S, Hyde EJ, Yang J, Rich FJ, Harper JL, Kirman JR, et al. Increased numbers of monocyte-derived dendritic cells during successful tumor immunotherapy with immune-activating agents. *J Immunol*. 2013;191(4):1984-92.
262. Ma Y, Adjemian S, Mattarollo SR, Yamazaki T, Aymeric L, Yang H, et al. Anticancer chemotherapy-induced intratumoral recruitment and differentiation of antigen-presenting cells. *Immunity*. 2013;38(4):729-41.
263. Anguille S, Smits EL, Lion E, van Tendeloo VF, Berneman ZN. Clinical use of dendritic cells for cancer therapy. *The Lancet Oncology*. 2014;15(7):e257-e67.
264. Oyama T, Ran S, Ishida T, Nadaf S, Kerr L, Carbone DP, et al. Vascular endothelial growth factor affects dendritic cell maturation through the inhibition of nuclear factor-kappa B activation in hemopoietic progenitor cells. *J Immunol*. 1998;160(3):1224-32.
265. Menetrier-Caux C, Montmain G, Dieu MC, Bain C, Favrot MC, Caux C, et al. Inhibition of the differentiation of dendritic cells from CD34(+) progenitors by tumor cells: role of interleukin-6 and macrophage colony-stimulating factor. *Blood*. 1998;92(12):4778-91.
266. Kobie JJ, Wu RS, Kurt RA, Lou S, Adelman MK, Whitesell LJ, et al. Transforming Growth Factor β Inhibits the Antigen-Presenting Functions and Antitumor Activity of Dendritic Cell Vaccines. *Cancer Research*. 2003;63(8):1860-4.
267. Mariathasan S, Turley SJ, Nickles D, Castiglioni A, Yuen K, Wang Y, et al. TGF β attenuates tumour response to PD-L1 blockade by contributing to exclusion of T cells. *Nature*. 2018;554(7693):544-8.
268. Tauriello DVF, Palomo-Ponce S, Stork D, Berenguer-Llargo A, Badia-Ramentol J, Iglesias M, et al. TGF β drives immune evasion in genetically reconstituted colon cancer metastasis. *Nature*. 2018;554(7693):538-43.
269. Cassim S, Pouyssegur J. Tumor Microenvironment: A Metabolic Player that Shapes the Immune Response. *Int J Mol Sci*. 2019;21(1).
270. Gottfried E, Kunz-Schughart LA, Ebner S, Mueller-Klieser W, Hoves S, Andreesen R, et al. Tumor-derived lactic acid modulates dendritic cell activation and antigen expression. *Blood*. 2006;107(5):2013-21.
271. Herber DL, Cao W, Nefedova Y, Novitskiy SV, Nagaraj S, Tyurin VA, et al. Lipid accumulation and dendritic cell dysfunction in cancer. *Nature medicine*. 2010;16(8):880-6.
272. Ramakrishnan R, Tyurin VA, Veglia F, Condamine T, Amoscato A, Mohammadyani D, et al. Oxidized lipids block antigen cross-presentation by dendritic cells in cancer. *J Immunol*. 2014;192(6):2920-31.

273. Gao F, Liu C, Guo J, Sun W, Xian L, Bai D, et al. Radiation-driven lipid accumulation and dendritic cell dysfunction in cancer. *Sci Rep.* 2015;5:9613-.
274. Spranger S, Gajewski TF. Impact of oncogenic pathways on evasion of antitumour immune responses. *Nature reviews Cancer.* 2018;18(3):139-47.
275. Spranger S, Bao R, Gajewski TF. Melanoma-intrinsic β -catenin signalling prevents anti-tumour immunity. *Nature.* 2015;523(7559):231-5.
276. Böttcher JP, Bonavita E, Chakravarty P, Blees H, Cabeza-Cabrerizo M, Sammicheli S, et al. NK Cells Stimulate Recruitment of cDC1 into the Tumor Microenvironment Promoting Cancer Immune Control. *Cell.* 2018;172(5):1022-37.e14.
277. Comi M, Amodio G, Gregori S. Interleukin-10-Producing DC-10 Is a Unique Tool to Promote Tolerance Via Antigen-Specific T Regulatory Type 1 Cells. *Frontiers in immunology.* 2018;9:682-.
278. Lv M, Wang K, Huang X-J. Myeloid-derived suppressor cells in hematological malignancies: friends or foes. *J Hematol Oncol.* 2019;12(1):105-.
279. Salem ML, El-Shanshory MR, Abdou SH, Attia MS, Sobhy SM, Zidan MF, et al. Chemotherapy alters the increased numbers of myeloid-derived suppressor and regulatory T cells in children with acute lymphoblastic leukemia. *Immunopharmacol Immunotoxicol.* 2018;40(2):158-67.
280. Liu YF, Chen YY, He YY, Wang JY, Yang JP, Zhong SL, et al. Expansion and activation of granulocytic, myeloid-derived suppressor cells in childhood precursor B cell acute lymphoblastic leukemia. *J Leukoc Biol.* 2017;102(2):449-58.
281. Komohara Y, Niino D, Ohnishi K, Ohshima K, Takeya M. Role of tumor-associated macrophages in hematological malignancies. *Pathol Int.* 2015;65(4):170-6.
282. Petty AJ, Yang Y. Tumor-Associated Macrophages in Hematologic Malignancies: New Insights and Targeted Therapies. *Cells.* 2019;8(12).
283. Hanna BS, McClanahan F, Yazdanparast H, Zaborsky N, Kalter V, Rossner PM, et al. Depletion of CLL-associated patrolling monocytes and macrophages controls disease development and repairs immune dysfunction in vivo. *Leukemia.* 2016;30(3):570-9.
284. Yang X, Feng W, Wang R, Yang F, Wang L, Chen S, et al. Repolarizing heterogeneous leukemia-associated macrophages with more M1 characteristics eliminates their pro-leukemic effects. *Oncoimmunology.* 2018;7(4):e1412910.
285. Chen SY, Yang X, Feng WL, Liao JF, Wang LN, Feng L, et al. Organ-specific microenvironment modifies diverse functional and phenotypic characteristics of leukemia-associated macrophages in mouse T cell acute lymphoblastic leukemia. *J Immunol.* 2015;194(6):2919-29.
286. Yang X, Feng W, Wang R, Yang F, Wang L, Chen S, et al. Hepatic leukemia-associated macrophages exhibit a pro-inflammatory phenotype in Notch1-induced acute T cell leukemia. *Immunobiology.* 2018;223(1):73-80.
287. Chen S, Yang X, Feng W, Yang F, Wang R, Chen C, et al. Characterization of peritoneal leukemia-associated macrophages in Notch1-induced mouse T cell acute lymphoblastic leukemia. *Mol Immunol.* 2017;81:35-41.
288. Hilden JM, Dinndorf PA, Meerbaum SO, Sather H, Villaluna D, Heerema NA, et al. Analysis of prognostic factors of acute lymphoblastic leukemia in infants: report on CCG 1953 from the Children's Oncology Group. *Blood.* 2006;108(2):441-51.
289. Thomas X, Le QH. Prognostic factors in adult acute lymphoblastic leukemia. *Hematology.* 2003;8(4):233-42.
290. Guastafierro S, Falcone U, Celentano M, Carbone A, Ferrara MG, Cappabianca S, et al. Chronic lymphocytic leukemia presenting with symptomatic peritoneal infiltration. *Leuk Res.* 2010;34(4):e115-6.

291. Ko SY, Ladanyi A, Lengyel E, Naora H. Expression of the homeobox gene HOXA9 in ovarian cancer induces peritoneal macrophages to acquire an M2 tumor-promoting phenotype. *The American journal of pathology*. 2014;184(1):271-81.
292. Yamaguchi T, Fushida S, Yamamoto Y, Tsukada T, Kinoshita J, Oyama K, et al. Tumor-associated macrophages of the M2 phenotype contribute to progression in gastric cancer with peritoneal dissemination. *Gastric Cancer*. 2016;19(4):1052-65.
293. Yang F, Feng W, Wang H, Wang L, Liu X, Wang R, et al. Monocyte-Derived Leukemia-Associated Macrophages Facilitate Extramedullary Distribution of T-cell Acute Lymphoblastic Leukemia Cells. *Cancer Res*. 2020;80(17):3677-91.
294. Lyu A, Triplett TA, Nam SH, Hu Z, Arasappan D, Godfrey WH, et al. Tumor-associated myeloid cells provide critical support for T-ALL. *Blood*. 2020;136(16):1837-50.
295. Galati D, Corazzelli G, De Filippi R, Pinto A. Dendritic cells in hematological malignancies. *Crit Rev Oncol Hematol*. 2016;108:86-96.
296. Mami NB, Mohty M, Chambost H, Gaugler B, Olive D. Blood dendritic cells in patients with acute lymphoblastic leukaemia. *Br J Haematol*. 2004;126(1):77-80.
297. Maecker B, Mougiakakos D, Zimmermann M, Behrens M, Hollander S, Schrauder A, et al. Dendritic cell deficiencies in pediatric acute lymphoblastic leukemia patients. *Leukemia*. 2006;20(4):645-9.
298. Valencia J, L MF-S, Fraile-Ramos A, Sacedon R, Jimenez E, Vicente A, et al. Acute Lymphoblastic Leukaemia Cells Impair Dendritic Cell and Macrophage Differentiation: Role of BMP4. *Cells*. 2019;8(7).
299. Carron C, Cormier F, Janin A, Lacronique V, Giovannini M, Daniel MT, et al. TEL-JAK2 transgenic mice develop T-cell leukemia. *Blood*. 2000;95(12):3891-9.
300. Preibisch S, Saalfeld S, Tomancak P. Globally optimal stitching of tiled 3D microscopic image acquisitions. *Bioinformatics*. 2009;25(11):1463-5.
301. dos Santos NR, Rickman DS, de Reynies AI, Cormier Fo, Williams M, Blanchard C, et al. Pre-TCR expression cooperates with TEL-JAK2 to transform immature thymocytes and induce T-cell leukemia. *Blood*. 2006;109(9):3972-81.
302. Fernandes MT, Ghezzi MN, Silveira AB, Kalathur RK, Póvoa V, Ribeiro AR, et al. Lymphotoxin- β receptor in microenvironmental cells promotes the development of T-cell acute lymphoblastic leukaemia with cortical/mature immunophenotype. *Br J Haematol*. 2015;171(5):736-51.
303. Kim HJ, Alonzo ES, Dorothee G, Pollard JW, Sant'Angelo DB. Selective depletion of eosinophils or neutrophils in mice impacts the efficiency of apoptotic cell clearance in the thymus. *PLoS One*. 2010;5(7):e11439.
304. Abram CL, Roberge GL, Hu Y, Lowell CA. Comparative analysis of the efficiency and specificity of myeloid-Cre deleting strains using ROSA-EYFP reporter mice. *J Immunol Methods*. 2014;408:89-100.
305. Rose S, Misharin A, Perlman H. A novel Ly6C/Ly6G-based strategy to analyze the mouse splenic myeloid compartment. *Cytometry A*. 2012;81(4):343-50.
306. Mebius RE, Kraal G. Structure and function of the spleen. *Nat Rev Immunol*. 2005;5(8):606-16.
307. Lokmic Z, Lammermann T, Sixt M, Cardell S, Hallmann R, Sorokin L. The extracellular matrix of the spleen as a potential organizer of immune cell compartments. *Semin Immunol*. 2008;20(1):4-13.
308. Shi J, Hua L, Harmer D, Li P, Ren G. Cre Driver Mice Targeting Macrophages. *Methods Mol Biol*. 2018;1784:263-75.
309. Buch T, Heppner FL, Tertilt C, Heinen TJ, Kremer M, Wunderlich FT, et al. A Cre-inducible diphtheria toxin receptor mediates cell lineage ablation after toxin administration. *Nat Methods*. 2005;2(6):419-26.

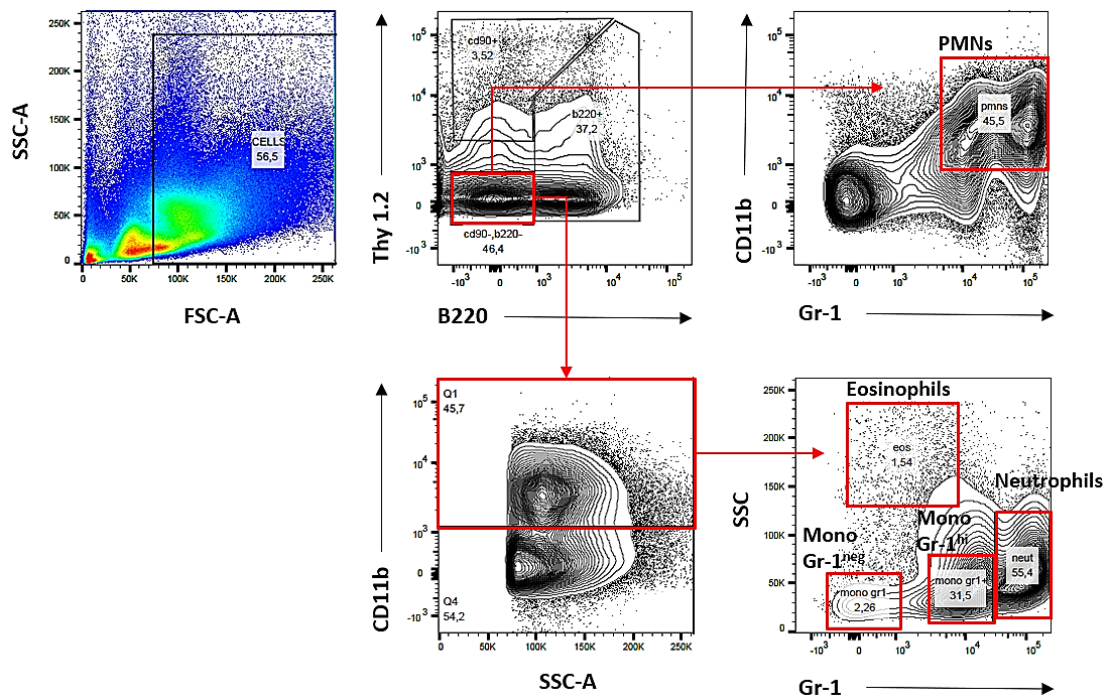
310. Clausen BE, Burkhardt C, Reith W, Renkawitz R, Förster I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. *Transgenic Res.* 1999;8(4):265-77.
311. Faust N, Varas F, Kelly LM, Heck S, Graf T. Insertion of enhanced green fluorescent protein into the lysozyme gene creates mice with green fluorescent granulocytes and macrophages. *Blood.* 2000;96(2):719-26.
312. Fierer J, Looney D, Pechère J-C. Nature and Pathogenicity of Micro-organisms. *Infectious Diseases.* 2017;4-25.e1.
313. Ghosn EE, Cassado AA, Govoni GR, Fukuhara T, Yang Y, Monack DM, et al. Two physically, functionally, and developmentally distinct peritoneal macrophage subsets. *Proc Natl Acad Sci U S A.* 2010;107(6):2568-73.
314. Cross M, Mangelsdorf I, Wedel A, Renkawitz R. Mouse lysozyme M gene: isolation, characterization, and expression studies. *Proc Natl Acad Sci U S A.* 1988;85(17):6232-6.
315. Ye M, Iwasaki H, Laiosa CV, Stadtfeld M, Xie H, Heck S, et al. Hematopoietic Stem Cells Expressing the Myeloid Lysozyme Gene Retain Long-Term, Multilineage Repopulation Potential. *Immunity.* 2003;19(5):689-99.
316. Turchyn LR, Baginski TJ, Renkiewicz RR, Lesch CA, Mobley JL. Phenotypic and functional analysis of murine resident and induced peritoneal macrophages. *Comp Med.* 2007;57(6):574-80.
317. Zhang X, Goncalves R, Mosser DM. The isolation and characterization of murine macrophages. *Curr Protoc Immunol.* 2008;Chapter 14:Unit 14 1.
318. Sisirak V, Faget J, Vey N, Blay J-Y, Ménétrier-Caux C, Caux C, et al. Plasmacytoid dendritic cells deficient in IFN α production promote the amplification of FOXP3(+) regulatory T cells and are associated with poor prognosis in breast cancer patients. *Oncoimmunology.* 2013;2(1):e22338-e.
319. Schmidt-Supprian M, Rajewsky K. Vagaries of conditional gene targeting. *Nat Immunol.* 2007;8(7):665-8.
320. Hébert JM, McConnell SK. Targeting of cre to the Foxg1 (BF-1) locus mediates loxP recombination in the telencephalon and other developing head structures. *Dev Biol.* 2000;222(2):296-306.
321. Heffner CS, Herbert Pratt C, Babiuk RP, Sharma Y, Rockwood SF, Donahue LR, et al. Supporting conditional mouse mutagenesis with a comprehensive cre characterization resource. *Nat Commun.* 2012;3:1218-.
322. Ginhoux F, Liu K, Helft J, Bogunovic M, Greter M, Hashimoto D, et al. The origin and development of nonlymphoid tissue CD103+ DCs. *J Exp Med.* 2009;206(13):3115-30.
323. Liu K, Waskow C, Liu X, Yao K, Hoh J, Nussenzweig M. Origin of dendritic cells in peripheral lymphoid organs of mice. *Nat Immunol.* 2007;8(6):578-83.
324. Berberich S, Förster R, Pabst O. The peritoneal microenvironment commits B cells to home to body cavities and the small intestine. *Blood.* 2007;109(11):4627-34.
325. Ansel KM, Harris RB, Cyster JG. CXCL13 is required for B1 cell homing, natural antibody production, and body cavity immunity. *Immunity.* 2002;16(1):67-76.
326. Lucas T, Waisman A, Ranjan R, Roes J, Krieg T, Muller W, et al. Differential roles of macrophages in diverse phases of skin repair. *J Immunol.* 2010;184(7):3964-77.
327. Lee B, Qiao L, Kinney B, Feng GS, Shao J. Macrophage depletion disrupts immune balance and energy homeostasis. *PLoS One.* 2014;9(6):e99575.
328. Yamaizumi M, Uchida T, Takamatsu K, Okada Y. Intracellular stability of diphtheria toxin fragment A in the presence and absence of anti-fragment A antibody. *Proc Natl Acad Sci U S A.* 1982;79(2):461-5.
329. Tittel AP, Heuser C, Ohliger C, Llanto C, Yona S, Hämmerling GJ, et al. Functionally relevant neutrophilia in CD11c diphtheria toxin receptor transgenic mice. *Nat Methods.* 2012;9(4):385-90.

330. O'Connell KE, Mikkola AM, Stepanek AM, Vernet A, Hall CD, Sun CC, et al. Practical murine hematopathology: a comparative review and implications for research. *Comp Med*. 2015;65(2):96-113.
331. Hashimoto D, Chow A, Noizat C, Teo P, Beasley MB, Leboeuf M, et al. Tissue-resident macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. *Immunity*. 2013;38(4):792-804.
332. Simon SI, Kim MH. A day (or 5) in a neutrophil's life. *Blood*. 2010;116(4):511-2.
333. Cha JH, Chang MY, Richardson JA, Eidels L. Transgenic mice expressing the diphtheria toxin receptor are sensitive to the toxin. *Mol Microbiol*. 2003;49(1):235-40.
334. Orthgiess J, Gericke M, Immig K, Schulz A, Hirrlinger J, Bechmann I, et al. Neurons exhibit Lyz2 promoter activity in vivo: Implications for using LysM-Cre mice in myeloid cell research. *Eur J Immunol*. 2016;46(6):1529-32.
335. McCubbrey AL, Allison KC, Lee-Sherick AB, Jakubzick CV, Janssen WJ. Promoter Specificity and Efficacy in Conditional and Inducible Transgenic Targeting of Lung Macrophages. *Frontiers in immunology*. 2017;8:1618-.
336. Probst HC, Tschannen K, Odermatt B, Schwendener R, Zinkernagel RM, Van Den Broek M. Histological analysis of CD11c-DTR/GFP mice after in vivo depletion of dendritic cells. *Clin Exp Immunol*. 2005;141(3):398-404.
337. Jung S, Unutmaz D, Wong P, Sano G, De los Santos K, Sparwasser T, et al. In vivo depletion of CD11c⁺ dendritic cells abrogates priming of CD8⁺ T cells by exogenous cell-associated antigens. *Immunity*. 2002;17(2):211-20.
338. van Rooijen N, Hendrikx E. Liposomes for specific depletion of macrophages from organs and tissues. *Methods Mol Biol*. 2010;605:189-203.
339. Sunderkötter C, Nikolic T, Dillon MJ, Van Rooijen N, Stehling M, Drevets DA, et al. Subpopulations of mouse blood monocytes differ in maturation stage and inflammatory response. *J Immunol*. 2004;172(7):4410-7.
340. Zhang Y, Shlomchik WD, Joe G, Louboutin JP, Zhu J, Rivera A, et al. APCs in the liver and spleen recruit activated allogeneic CD8⁺ T cells to elicit hepatic graft-versus-host disease. *J Immunol*. 2002;169(12):7111-8.
341. Moreno SG. Depleting Macrophages In Vivo with Clodronate-Liposomes. *Methods Mol Biol*. 2018;1784:259-62.
342. van Rooijen N, van Kesteren-Hendrikx E. Clodronate liposomes: perspectives in research and therapeutics. *J Liposome Res*. 2002;12(1-2):81-94.
343. Zamora-Pineda J, Kumar A, Suh JH, Zhang M, Saba JD. Dendritic cell sphingosine-1-phosphate lyase regulates thymic egress. *J Exp Med*. 2016;213(12):2773-91.
344. Messias CV, Santana-Van-Vliet E, Lemos JP, Moreira OC, Cotta-de-Almeida V, Savino W, et al. Sphingosine-1-Phosphate Induces Dose-Dependent Chemotaxis or Fugetaxis of T-ALL Blasts through S1P1 Activation. *PLoS One*. 2016;11(1):e0148137.
345. Jost TR, Borga C, Radaelli E, Romagnani A, Perruzza L, Omodho L, et al. Role of CXCR4-mediated bone marrow colonization in CNS infiltration by T cell acute lymphoblastic leukemia. *J Leukoc Biol*. 2016;99(6):1077-87.
346. Buonamici S, Trimarchi T, Ruocco MG, Reavie L, Cathelin S, Mar BG, et al. CCR7 signalling as an essential regulator of CNS infiltration in T-cell leukaemia. *Nature*. 2009;459(7249):1000-4.
347. Seillet C, Jackson JT, Markey KA, Brady HJ, Hill GR, Macdonald KP, et al. CD8 α ⁺ DCs can be induced in the absence of transcription factors Id2, Nfil3, and Batf3. *Blood*. 2013;121(9):1574-83.

ANNEXES

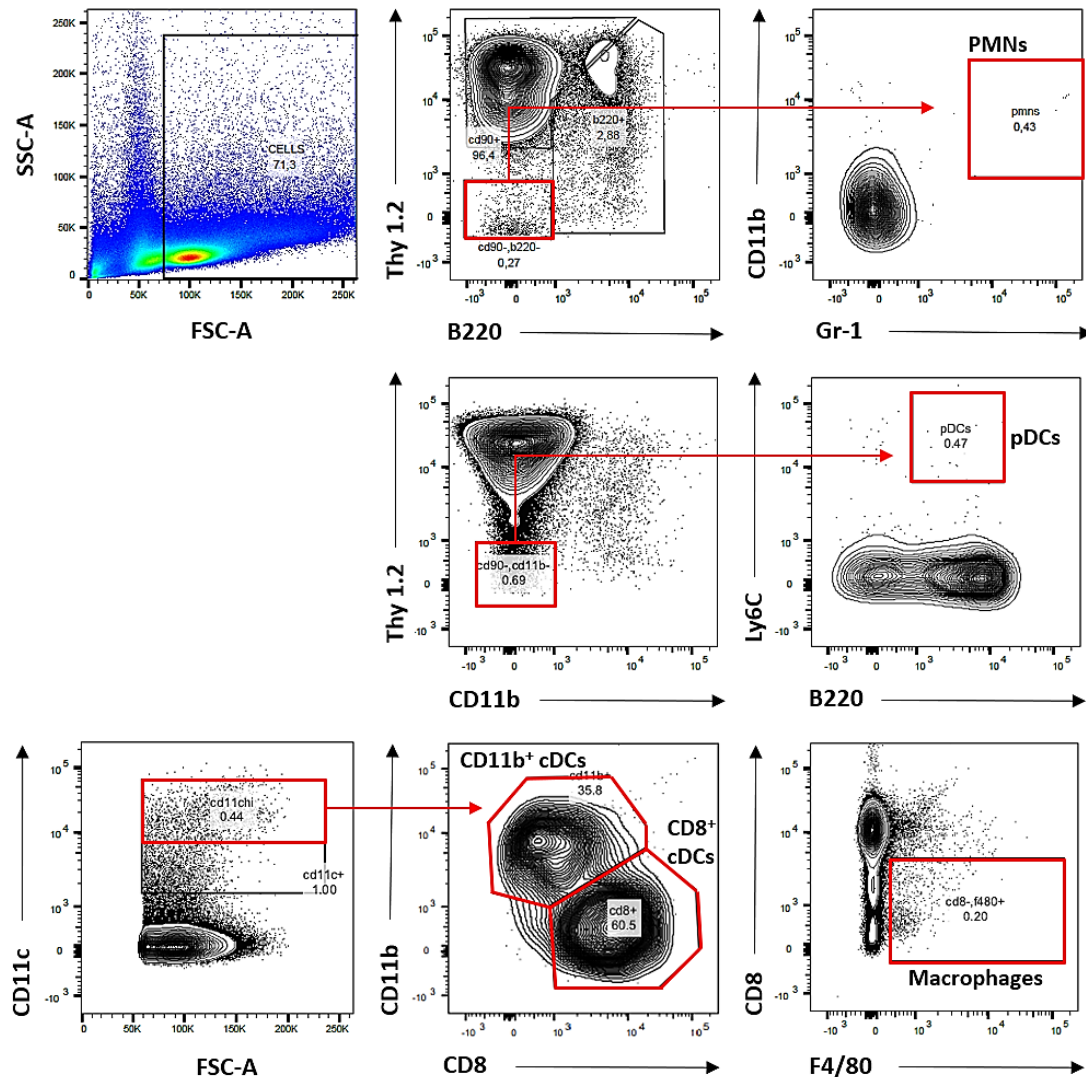
Annex 1.

Bone Marrow



Representative gating strategy to identify myeloid subsets in the bone marrow of TJ2-Tg mice. Medullary cell suspensions from TJ2-Tg and WT mice were analyzed by flow cytometry. Viable cells were selected based on Forward (FSC) versus Side scatter (SSC) gating. Lineage negative cells ($Thy1.2^{neg}B220^{neg}$) were analyzed to identify polymorphonuclear cells ($CD11b^{+}Gr-1^{+}$) and other myeloid $CD11b$ -expressing cells, including neutrophils ($CD11b^{+}SSC^{int}Gr-1^{hi}$), eosinophils ($CD11b^{+}SSC^{hi}Gr-1^{int}$), and both monocyte subsets, $Gr-1^{lo/neg}$ ($CD11b^{+}SSC^{low}Gr-1^{low/neg}$) and $Gr-1^{hi}$ ($CD11b^{+}SSC^{low}Gr-1^{+}$). PMNs, polymorphonuclear cells; Mono $Gr-1^{hi}$, $Gr-1^{hi}$ monocytes; Mono $Gr-1^{neg}$, $Gr-1^{neg}$ monocytes

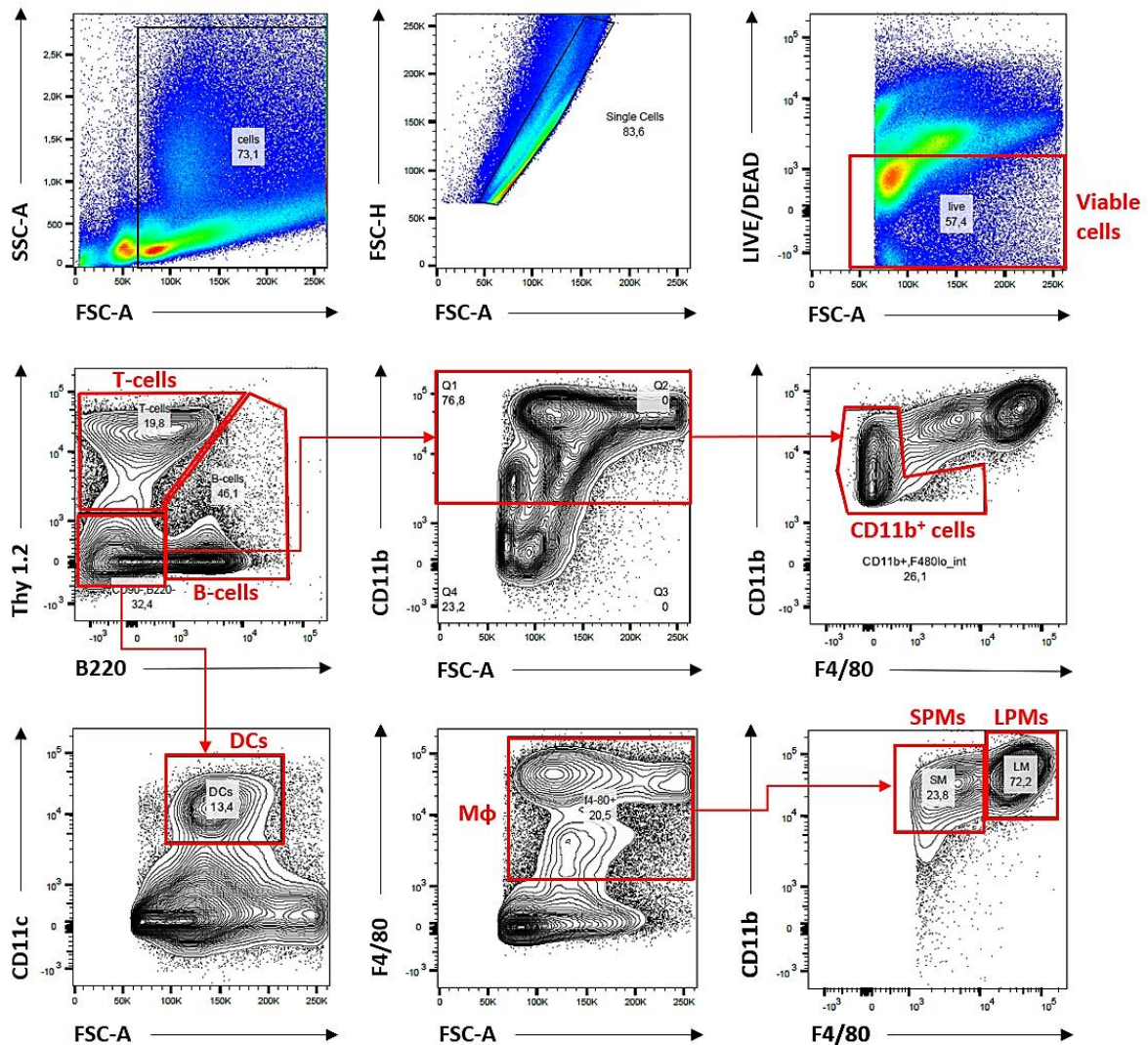
Thymus



Representative gating strategy to identify myeloid subsets in the thymus of TJ2-Tg mice. Thymic cell-suspensions from TJ2-Tg and WT mice were analyzed by flow cytometry. Viable cells were selected based on Forward (FSC) versus Side scatter (SSC) gating. Lineage negative cells (Thy1.2^{neg}B220^{neg}) were analyzed to identify polymorphonuclear cells (CD11b⁺Gr-1⁺). Additionally, macrophages (F4/80⁺CD8^{neg}), pDCs (CD11b^{neg}B220⁺Ly6C^{hi}) and cDCs (CD11c^{hi}) were assessed. The latter were divided into cDC1/CD8⁺ cDCs (CD11c^{hi}CD8⁺) and cDC2/CD11b⁺ cDCs (CD11c^{hi}CD11b⁺). PMNs, polymorphonuclear cells; pDCs, plasmacytoid DCs; cDCs, conventional DCs.

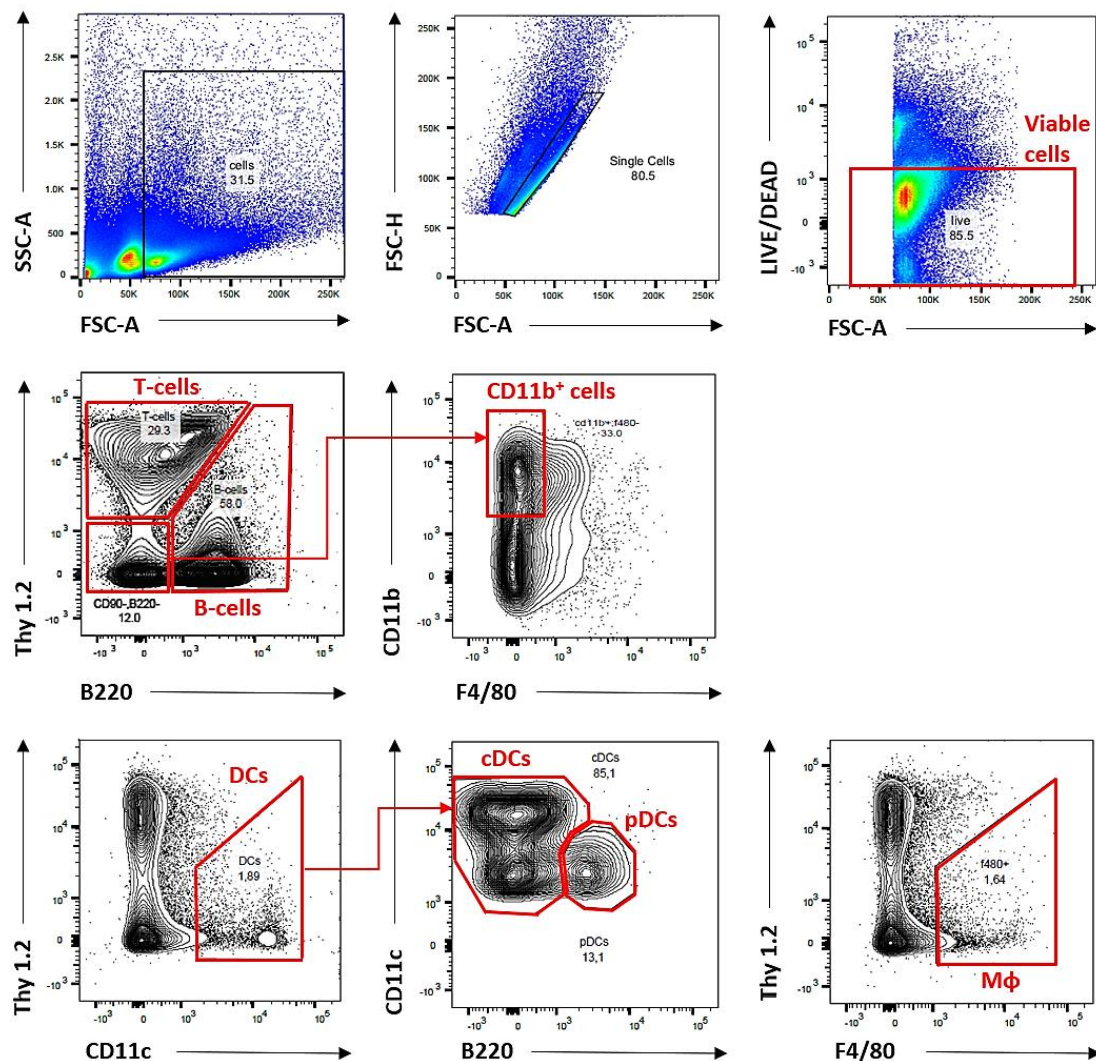
Annex 2.

Peritoneal Cavity



Representative gating strategy to identify myeloid subsets in the peritoneal cavity of LysM-Cre/R26EYFP mice. Cell suspension from the peritoneal cavity of LysM-Cre[+]/R26^{+/EYFP} mice were analyzed by flow cytometry. Viable cells were selected based on Live/Dead staining. Lineage negative cells (Thy1.2^{neg}B220^{neg}) were analyzed to identify CD11b⁺ cells (excepting for Mφ) (CD11b⁺F4/80^{low/int}) and DCs (CD11c^{hi}). Moreover, peritoneal macrophages (F4/80⁺) were assessed and further characterized into SPMs (CD11b^{hi}F4/80⁺) and LPMs (CD11b^{hi}F4/80^{hi}). CD11b⁺, CD11b-positive myeloid cells; DCs, dendritic cells; Mφ, macrophages; SPMs, small peritoneal macrophages; LPMs, large peritoneal macrophages

Lymphoid organs



Representative gating strategy to identify myeloid subsets in the lymphoid organs (spleen and thymus) of LysM-Cre/R26EYFP mice. Splenic (sample represented) and thymic cell-suspensions from LysM-Cre[+]/R26^{+/EYFP} mice were analyzed by flow cytometry. Viable cells were selected based on Life/Death staining. Lineage negative cells (Thy1.2^{neg}B220^{neg}) were analyzed to identify CD11b⁺ cells (excepting for Mφ) (CD11b⁺F4/80^{lo/neg}). Additionally, macrophages (F4/80⁺Thy1.2^{neg}) and DCs (CD11c⁺) were assessed. The latter were divided into cDCs (CD11c⁺B220⁻) and pDCs (CD11c⁺B220⁺). CD11b⁺, CD11b-positive myeloid cells; Mφ, macrophages; DCs, dendritic cells; cDCs, conventional DCs; pDCs, plasmacytoid DCs