Unraveling the role of lymphotoxin signaling in T-cell acute leukemia molecular crosstalk with microenvironmental cells

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Trabalho efetuado sob a orientação do
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(Principal Researcher at i3S)

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My sincere thanks!!!

Titulo: O papel da sinalização da linfotoxina na comunicação com as células do microambiente em leucemia linfoblástica aguda de linfócitos

RESUMO

O microambiente tumoral influencia a iniciação e a progressão de doenças malignas hematológicas. Contudo, sabe-se relativamente pouco sobre a identidade e a função de fatores microambientais que participam no desenvolvimento da leucemia linfoblástica aguda de linfócitos T (LLA-T). A LLA-T é uma doença maligna agressiva, fatal na ausência de diagnóstico precoce e terapia apropriada, que afeta principalmente crianças e adolescentes e tem mau prognóstico após recidiva. Nos pacientes LLA-T, as células malignas podem se encontrar em nichos microambientais distintos, como o sangue, a medula óssea e os órgãos linfáticos. Estes nichos e fatores moleculares produzidos por eles serão importantes para a manutenção e progressão da doença, mas até agora apenas alguns fatores microambientais demonstraram favorecer a progressão de LLA-T em murganhos, como por exemplo a interleucina 7 e certas quimiocinas. Murganhos transgénicos que expressam a proteína de fusão TEL-JAK2 desenvolvem LLA-T com latência rápida e penetrância completa. Verificou-se que as células leucémicas TEL-JAK2 expressam proteínas linfotoxina (LT) desde os estadios iniciais da doença 1. Indicando que a linfotoxina promove a leucemogénese, observou-se que a inativação do gene codificante do recetor da LT-β (LTβR) em murganhos TEL-JAK2 aumentou a latência de desenvolvimento de LLA-T 1. Estes resultados sugerem que a linfotoxina medeia a comunicação molecular entre células malignas e células não malignas do estroma, favorecendo assim a leucemogénese. No seguimento destes resultados, o nosso objetivo foi compreender como a ativação de LTβR nas células do estroma pode facilitar a leucemogénese dos linfócitos T. Para este fim, testamos uma hipótese: as células leucémicas que expressam linfotoxina ativam a sinalização LTβR nas células estromais. Afim de testar esta hipótese, co-cultivamos as células leucémicas que expressam LT com linhas celulares derivadas de estroma da medula óssea (MS-5) ou fibroblásticas (NIH3T3), previamente transduzidas de forma estável com uma construção lentiviral que expressa um gene repórter de luciferase sob o controle de um promotor transcricional responsivo a NF-κB. Assim, verificámos que as células leucémicas ativaram o repórter NF-κB em células MS-5 e fibroblastos NIH3T3, e confirmámos que estas linhas celulares que expressam LTβR. Em seguida, observámos que a atividade de luciferase em co-culturas foi bloqueada por uma proteína inibidora solúvel (LTβR-Fc), mostrando assim que a ativação de NF-κB foi de fato mediada pela estimulação de LTβR. Além disso, a indução do repórter de NF-κB por células leucémicas co-cultivadas foi diminuída em fibroblastos embrionários de murganho deficientes do gene codificante do LTβR. Em conclusão, as células leucémicas que expressam LT podem de facto ativar a sinalização LTβR em células estromais vizinhas. Considerando estes resultados, de futuro iremos identificar programas de transcrição dependentes de LTβR induzidos em células de estroma por células leucémicas.

Palavras-chave: leucemia linfoblástica aguda de linfócitos T, microambiente, via NF-κB, via de sinalização LTα1β2/LTβR, modelo murganho transgénico TEL-JAK2

Title: Unraveling the role of lymphotoxin signaling in T-cell acute leukemia molecular crosstalk with microenvironmental cells

ABSTRACT

The tumor microenvironment influences the initiation and progression of hematological malignancies. However, relatively little is known about the identity and function of microenvironmental factors participating in T-cell acute lymphoblastic leukemia (T-ALL) development. T-ALL is an aggressive malignancy, fatal without early diagnosis and therapy, affecting mainly children and adolescents, and with poor prognosis upon relapse. In T-ALL patients, malignant cells may occur in distinct microenvironmental niches such as blood, bone marrow (BM), and lymphoid organs. Such niches and molecular factors there produced are likely important for maintenance and disease progression, but so far only a few microenvironmental factors have been shown to favor T-ALL progression in mice, such as interleukin 7 and certain chemokines. Transgenic mice expressing the TEL-JAK2 fusion protein develop T-ALL with short latency and full penetrance. It was found that TEL-JAK2 leukemic cells express lymphotoxin (LT) proteins from the early stages of the disease 1. Supporting the notion that lymphotoxin promotes leukemogenesis, it was found that genetic inactivation of the LTβ receptor (LTβR) in TEL-JAK2 mice impaired T-ALL development 1. These results suggested that LT mediates molecular crosstalk between malignant and stromal non-malignant cells, thus favoring leukemogenesis. Therefore, we aimed to understand how LTβR activation in stromal cells can facilitate T-cell leukemogenesis. To this end, we tested a hypothesis: lymphotoxin-expressing leukemic cells activate LTβR signaling in stromal cells. To test this hypothesis, we co-cultured LT-expressing leukemic cells with fibroblast (NIH3T3) or BM-derived stromal (MS-5) cell lines stably transduced with a lentiviral construct expressing a luciferase reporter gene under control of an NF-κB-responsive transcriptional promoter. We found that leukemic cells activated the NF-κB reporter in transduced MS-5 cells and NIH3T3 fibroblasts, and confirmed that these cell line express LTβR. Next, we observed that luciferase activity in co-cultures was blocked by LTβR-Fc, a soluble inhibitory protein, thus showing that NF-κB activation was indeed mediated through LTβR stimulation. In addition, NF-κB reporter induction by co-cultured leukemic cells was impaired in LTβR gene-deficient mouse embryonic fibroblasts. In conclusion, LT-expressing leukemic cells can indeed activate LTβR signaling in neighboring stromal cells. Considering these results, we will in the future identify LTβR-dependent transcriptional programs induced in stromal cells by leukemic cells.

Key words: T-cell acute lymphoblastic leukemia, microenvironment, NF-κB pathway, LTα1β2/LTβR signaling pathway, TEL-JAK2 mouse model.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

A

ADP: Adenosine diphosphate

Aire: Autoimmune regulator

ALL: Acute lymphoblastic leukemia

ARF: Alternative reading frame

ATP: Adenosine triphosphate

B

BAFF-R: B-cell activating factor receptor

bHLH: Basic helix-loop-helix

BM: Bone marrow

BrdU: 5-bromo-2'-deoxyuridine

C

CAC: Colitis associated colon cancer

CCR: Chemokine (C-C motif) receptor

CD#: Cluster of differentiation

CD: Cytoplasmic domain

CDKN2A: Cyclin-dependent kinase Inhibitor 2A

Chr: Chromosome

CIAP: Calf intestinal alkaline phosphatase

CMJ: Corticomedullary junction

CMP: Common myeloid progenitor

CNS: Central nervous system

CRD: Cysteine-rich domain

cTEC: Cortical TEC

CXCL: Chemokine (C-X-C motif) ligand

D

DC: Dendritic cell

DMEM: Dulbecco's modified eagle medium

DMSO: Dimethyl sulfoxide

DN: Double-negative

DNA: Deoxyribonucleic acid

DP: Double positive

dTom: dTomate a dimeric variant of DsRed fluorescent protein

E

ECD: Extracellular domain

EDTA: Ethylenediamine tetraacetic acid

ETPs: Early T cell progenitors

F

FACS: Fluorescence-activated cell sorting

FBS: Fetal bovine serum

FITC: Fluorescein isothiocyanate

Fn14: Fibroblast growth factor-inducible 14

G

GFP: green fluorescent protein

H

h: hours

HBV: Hepatitis B virus

HEBS: Hepes buffered saline

HEVs: High endothelial venules

HIV-1: Human immunodeficiency virus 1

***HOX*:** Homeobox gene

HSC: Hematopoietic stem cells

I

ICAM: Intercellular adhesion molecule

ICD: Intracellular domain

IEC: Intestinal epithelial cells

IFN: Interferon

IgG: Immunoglobulin G

IGF: Insulin-like growth factor

IkB: Inhibitor of kappa B

IKKα/ IKK1: Inhibitor of nuclear factor kappa-B kinase subunit alpha (one)

IKKβ/IKK2: Inhibitor of nuclear factor kappa-B kinase subunit beta (two)

IKKγ/ NEMO: Inhibitor of nuclear factor kappa-B kinase subunit gamma / NF-kappaB essential modulator

IL#: Interleukin

Iono: Ionomycin

IRAK: Interleukin-1 receptor-associated kinase

J

JAK: Janus kinase

JNK: c-Jun N-terminal Kinase

K

***KO*:** Gene knockout

L

LBL: Lymphoblastic lymphoma

LBP: Lipopolysaccharide binding protein

Leuk: Leukemic

LIGHT/TNFSF14: Tumor necrosis factor superfamily member 14

LMO: LIM domain only

LMPP: Lymphoid-primed multipotent progenitor

LN: Lymph node

LPS: Lipopolysaccharide

LT: Lymphotoxin

LT-HSC: "Long-term" HSC

LTα1β2: Heterotrimer of LTα and two LTβ

LTβR: Lymphotoxin beta receptor

Luc: Luciferase

LYL1: Lymphoblastic leukemia-derived

M

MAP Kinase: Mitogen Activated Protein Kinases

MEF: Mouse embryonic fibroblast

MEMα: Minimum essential medium alpha

MEP: Megakaryocyte erythroid progenitor

MHC: Major histocompatibility complex

MKP: Megakaryocyte progenitor

MOI: Multiplicity of infection

MPP: Multipotent progenitor

mTEC: Medullary TEC

MYD88: Myeloid differentiation primary response

N

NF-κappaB/NF-κB: Nuclear factor κappa-light-chain-enhancer of activated B cells

NFKB1: Nuclear factor NF-κappa-B p105 subunit

NFKB2: Nuclear factor NF-κappa-B p100 subunit

NIK: NF-κappaB inducing kinase

NK: Natural killer cells

NMS: Normal mouse serum

NOTCH1: Notch homolog1, translocation-associated (Drosophila)

P

P14/ARF: ARF tumor suppressor

P16 or p16/INK4A: Cyclin-dependent kinase inhibitor 2A, multiple tumor suppressor 1

PB: Peripheral blood

PBS: Phosphate-buffered saline

PCM1: Pericentriolar material 1

PCR: Polymerase chain reaction

PE: Phycoerythrin

PE-Cy5: Phycoerythrin-cyanine 5

PGE2

pH: Potential of hydrogen

PI: Propidium iodide

PI3K: Phosphoinositide 3-kinase

PMA: Phorbol myristate acetate

pTECs: Common progenitors TEC

R

RANK: Receptor activator of NF-κB

RANKL: RANK ligand

RBC: Red blood cell

RelA: V-rel avian reticuloendotheliosis viral oncogene homolog A - Transcription factor RelA

RelB: V-rel avian reticuloendotheliosis viral oncogene homolog B - Transcription factor RelB

RHD: Rh blood group, D antigen

RIP1: Receptor-interacting protein 1

RNA: Ribonucleic acid

RPMI: Roswell Park Memorial Institute medium

RRE: Rev response elements

RT: Room temperature

RT-qPCR: quantitative real-time polymerase chain reaction

S

S1P: Via sphingosine-1 phosphate

SIP-1R: Via sphingosine-1 phosphate receptor

SHH: Sonic Hedgehog

SP: Single-positive

STAT: Signal transducer and activator of transcription

ST-HSC: "Short-term" HSC

T

TAK: TGF-beta activated kinase

TAL: T-cell acute lymphocytic leukemia protein

T-ALL: T-cell acute lymphoblastic leukemia

TCR: T-cell receptor

TE: Tris-EDTA buffer

TEC: Thymic epithelial cell

TEL: Translocated ets leukemia

TEL-JAK2: Fusion protein, human

TJ2-Tg: TEL-JAK2 transgenic

TLR: Toll-like receptors

TLX: T-cell leukemia homeobox

TM: Temperature of melting

TMD: Transmembrane domain

TNFRSF: Tumor necrosis factor receptor superfamily

TNFα: Tumor necrosis factor alpha

TRA: Tissue-restricted antigen

TRAF: TNF Receptor-Associated Factor

TRIF: TIR-domain-containing adapter-inducing interferon-β

TSAs: Tissue-specific self-antigens

U

Ubc 13: Ubiquitin-conjugating enzyme 13

UBC: Human Ubiquitin C

Uev1A: Ubiquitin-conjugating enzyme E2 variant 1

UV: Ultraviolet

V

VSV-G: Vesicular stomatitis Indiana virus protein

CHAPTER 1 - INTRODUCTION

I. 1. T CELL DEVELOPMENT IN THE THYMUS

I.1.1. Hematopoiesis

All the cellular elements of the blood are originated from a common precursor in the BM - the hematopoietic stem cells (HSC) – in a process designated by hematopoiesis. Hematopoiesis is a highly dynamic and finely regulated developmental process that depends on both self-renewal and well-regulated differentiation of hematopoietic stem cells to maintain the capacity to generate all blood cells throughout the lifespan of mammals and other vertebrates [1–3]. It is a process that leads to the formation of all types of mature blood cells through the generation of intermediate progenitors that committing to a certain lineage lose progressively the differentiation potential for other cell lineages [4].

Single HSCs cell emergence during embryonic development (fetal pre-HSCs). Each major branch point in adult hematopoiesis, develop sequentially 3 populations: "long-term" HSC (LT-HSC) of the BM, that can self-renew practically for a lifetime, "short-term" HSC (ST-HSC) with capacity of limited self-renewal and contributing to the multi-lineage differentiation, and multipotent progenitors (MPP), which lose capacity of self-renewal [4–6].

The so-called composite model of hematopoiesis states that MPPs originate asymmetrically either a common myeloid progenitor (CMP) or a lymphoid-primed multipotent progenitor (LMPP) [6,7]. Each of these common progenitors have differentiation potentials restricted to all cell types within their respective lineage: CMPs originate megakaryocytes, erythrocytes, granulocytes, and macrophages, whereas LMPPs can originate myeloid descendants such as macrophages or neutrophilic (GMP), as well as lymphoid descendants such as T, B and natural killer (NK) lymphocytes (CLP), but not erythroid or megakaryocytic cells (**Figure I.1**). Nevertheless, the existence of LMPPs in the BM is controversial. In fact, some studies demonstrated that the B lineage is segregated of the T cell pathway before a branch point for T lineage versus myeloid lineage [6–10]. However, the notion that in normal conditions BM-derived progenitors migrate to the thymus in order to originate self-tolerant, functional T lymphocytes (also known as T cells) is widely accepted [11].

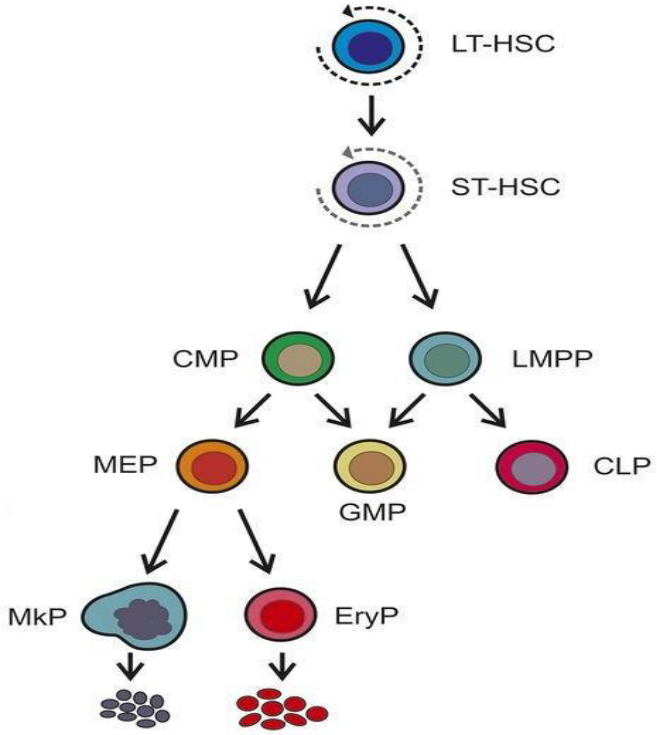


Figure I.1: Hematopoietic hierarchy. Adult HSCs first start to lose self-renewal abilities, differentiating from long-term to short-term (LT-HSCs), then differentiate in short-term or multipotent progenitors (ST-HSC/MPP). As time passes, multipotency potential is lost by differentiation into common lymphoid or myeloid progenitors (LMPP or CMP, respectively), which will further differentiate into the different cell types of each lineage. CLP common lymphoid progenitors. MEP indicates megakaryocyte erythroid progenitor. MKP indicates megakaryocyte progenitor. GMPs granulocyte-macrophage progenitors. EryP erythroid progenitor. [12].

I.1.2. T- cell lineage specification

Common lymphoid progenitors, which are BM cells with T and B lineage potential, and LMPPs retain the potential to generate diverse lymphocyte types, as well as NK, dendritic, and myeloid cells under somewhat different ranges of permissive conditions. After bloodborne migration, contact of these cells with the thymic environment efficiently blocks non-T cell lineage potentials through a variety of mechanisms. Signals provided by the thymic microenvironment are critical for T-cell lineage specification and thymocyte survival and proliferation [10]. Essential ligands expressed by stromal cells and driving early T-cell development include Notch ligands [10,13,14], interleukin 7 (IL7) [13,15,16], growth factors such as SCF [13], and morphogens such as Sonic Hedgehog (SHH) [13,17].

At the earliest thymic stage, as early T cells progenitors (ETPs) turn off Flt3 expression, they unconditionally lose their B-cell lineage potential. The mechanism depends on Notch signals and GATA expression [10,14]. Notch1 molecules on lymphoid precursors interact with Notch ligands (Delta-like 1 or 4) produced, in the thymic microenvironment, leading to activation of the T-cell-specific developmental program [10]. Notch regulates the decision of a lymphoid precursor to become a T versus a B lymphocyte. When a constitutively active version of Notch1, one of four versions of Notch, is overexpressed in hematopoietic cells, T cells rather than B cells develop in the BM. Reciprocally, when the Notch1 gene is knocked-out among hematopoietic precursors, B cells rather than T cells develop in the thymus [2,10,13]. Exclusion of myeloid and dendritic cell (DC) potentials is caused by removal of a positive regulator, PU.1,. When PU.1 is present, Notch signaling keeps myeloid potential under check. During the DN2b phage of thymocyte development (see later), PU.1 is silenced, under the influence of Runx and possibly TCF-1 or GATA-3, thus shutting down the myeloid potentials[10,18]. The loss of differentiation potential to NK and innate-like cell 2, in turn, is due to activation of a repressor, Bcl11b [19].

IL7/IL7R signaling is required in different steps of thymocyte development. Besides being critical to the survival and proliferation of DN2 thymocytes is also important in the regulation of T-cell commitment timing at this stage. Indeed, IL-7 signaling promotes the expression of stem/progenitor cell-specific genes of early progenitors, while inhibiting expression of T-cell transcription factors, notably Bcl11b [13,15,16].

Also, the BM microenvironment is highly heterogeneous, being composed of different cell types with specialized functions, as well as extracellular matrix, growth factors, and chemokines [13]. There are studies verifying that the BM HSC microenvironment play a role in regulating HSC function via multiple mechanisms including Scf and CXCL12 secretion from stromal cells [20–22], TGFβ [22,23], and the Notch pathway [22,24,25].

I.1.3. Thymic microenvironment

The thymus is an organ that provides a unique microenvironment composed of a variety of stromal cells, including thymic epithelial cells (TECs), endothelial cells, fibroblasts, and hematopoietic-derived stromal cells such as DCs and macrophages. These thymic stromal cells

provide essential signals for thymocyte development. In the absence of a thymus, T cells do not develop.

The thymus is subdivided into two histologically discrete regions: the cortex and medulla (**Figure I.2**). Each of these regions is filled by distinct subsets of thymic resident cells, creating microenvironment unique to each site and specialized to coordinate distinct selection events. For example, expression of a distinct proteasome subunit and unique lysosomal proteases within cortical epithelial cells (cTECs) generate a specialized peptidic repertoire to support positive selection. In contrast, a program of promiscuous gene expression in a subset of medullary thymic epithelial cells (mTECs) and broad expression of costimulatory ligands in the BM makes this locus particularly suitable for promoting negative selection [26,27]. The cTECs and mTECs are derived from common progenitor TEC (pTECs) progressing to a transitional progenitor stage and both exhibit distinct functions in the regulation of T cells development. FoxN1 is an important mediator of the development (mainly in the embryonic stage) and functions of TEC, since FoxN1 deficiency completely disrupts the development of thymic cell in animals and humans [26].

The ETPs migrate from the BM through the bloodstream and accumulate in the thymus through the expression and stimulation of specific chemokine receptors (such as CCR7, CCR9)[28]. ETPs enter the thymus through the cortico-medullar region, migrating then to the outer cortex [29]. However, at this phase, these progenitor cells are not yet committed to the T lineage (as mentioned above), and may differentiate to NK or DCs. Upon exposure a series of soluble factors from the thymic microenvironment, these cells survive, proliferate, and differentiate .to the T lineage.

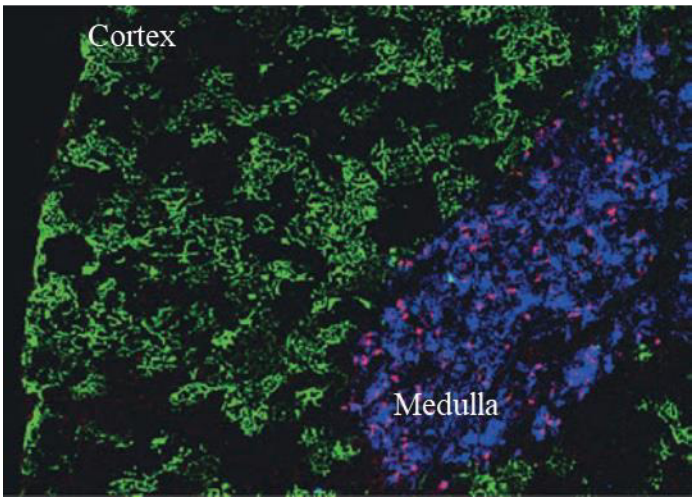


Figure I.2: The cortex–medulla architecture of the adult mouse thymus. Cortical thymic epithelial cells (cTECs) marked by expression of Venus fluorescent protein (green) under the and medullary TECs (mTECs) are marked by expression of autoimmune regulator (AIRE; red) and keratin 5 (blue) [28].

I.1.4. Stages of thymocyte differentiation

The combination of Notch ligand and IL 7 signals induces ETP, which high levels of CD5,to express surface CD1a molecules, thereby marking the transition to the next differentiation stage, usually referred to as pre-T cells [1].

Pre-T cells do not express CD4 or CD8 on their surface, so being defined as double negative (DN) cells [1,2]. Mouse DN thymocytes are further classified from DN1 to DN4 (**Figure I.3**) based on different profiles of CD117 (also known as c-kit), CD25 and CD44 cell surface expression [30]. DN1 thymocytes (corresponding to ETPs) are the first to enter the thymus, populating the cortico-medullary junction (CMJ). These cells are characterized by high levels of CD117 and CD44 expression, and low levels of CD25 ($CD117^{++}$ $CD44^{+}$ $CD25^{-}$). Then, they migrate to the cortex, where they proliferate and acquire CD25 expression, being designated DN2 thymocytes ($CD117^{++}$ $CD44^{+}$ $CD25^{+}$). At this critical stage of development, the gene segments coding for the T-cell receptor (TCR) γ, δ, and β chains begin each to undergo genetic recombination [2,31].

The DN2 stage is further subdivided into two subsets: DN2a and DN2b. At the late DN2 stage, T-cell precursors fully commit to the T-cell lineage and reduce expression of both CD117 and CD44 [2,31]. The transition from DN2b to DN3 ($CD24^{+}$ $CD25^{+}$ $CD44^{-}$ $CD117^{-}$) stage is marked by the upregulation of the pre-TCR α chain (pTα, encoded by the *Ptcra* gene).

The selection for the αβ T cell lineage begins in the subcapsular thymic zone. β selection consists of selective thymocyte survival, in which only thymocytes undergoing successful TCR β locus rearrangements and capable of expressing a functional β chain in a functional pre-TCR complex (together with pTα and CD3 proteins) receive specific survival and proliferation signals. Thymocytes that do not have a nonfunctional β chain, do not receive survival and/or proliferation signals and eventually die [1,2].

Then, thymocytes migrate to the inner cortex where they lose CD25 expression, stop proliferating, and enter the final DN developmental phase, DN4 ($CD24^+$ $CD25^-$ $CD44^-$ $CD117^-$). DN4 thymocytes proliferate and express CD4 and CD8 co-receptors, giving rise to CD4/CD8 double positive (DP) thymocytes. In the cortex, the Rag genes are reexpressed and DP thymocytes undergo TCRα-VJ rearrangement, thereby expressing αβTCR on the cell surface [10,26,31].

Once thymocytes reach the DP stage of development, they undergo TCR-mediated positive and negative selection through interaction with major histocompatibility (MHC)/peptide complexes expressed by cortical TECs (cTECs) and DCs [31,32]. DP thymocytes that do not perform a correct assembly of the αβTCR complex and therefore fail to recognize die by apoptosis (death by neglect). In contrast, DP that interact with low affinity with self-peptide–MHC complexes presented by cTECs are induced to survive and differentiate into $CD4^+$ $CD8^-$ or $CD4^-$ $CD8^+$ single-positive (SP) thymocytes. This process is called positive selection.

The TCR specificity for MHC during positive selection is associated with selective differentiation for CD4 or CD8 lineage, so that recognition of MHC class I molecules promotes the commits to the CD8 lineage, and in turn the recognition of MHC II molecules compromises the CD4 lineage. Positively selected thymocytes are induced to express chemokine receptor CCR7 and to migrate to the thymic medulla, where CCR7 ligands are abundant [26,28].

In the medulla, SP thymocytes that are reactive to tissue-specific antigens promiscuously presented by mTECs are deleted, a process termed negative selection. $CD4^+$ or $CD8^+$ autoreactive thymocytes with high affinity receptors for self-peptide-MHC complexes are extremely dangerous to the body, and can lead to auto-immunity if they are not eliminated by negative selection in the thymus. Thereby, the negative selection is essential for the establishment of central tolerance [26,33].

Finally, SP thymocytes that have completed the developmental program and repertoire selection are exported from the thymus into circulation. Thymocyte emigration through vessels near the cortico-medullary junctions is controlled by chemotactic signaling via sphingosine-1 phosphate (S1P) present in the blood, and expression of its receptor S1P1 by SP thymocytes.

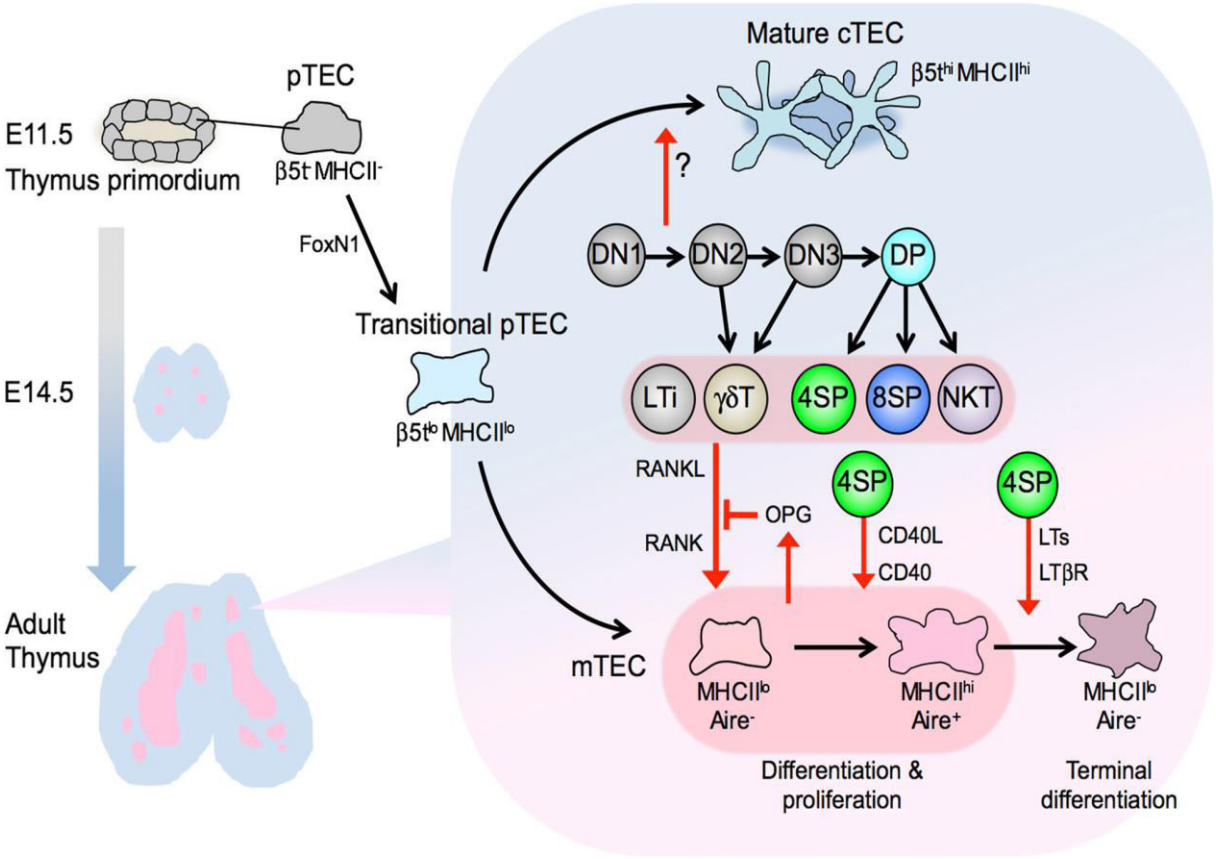


Figure I.3: Roles of thymic stromal cells in T cell development. cTECs and mTECs arise from common progenitor TECs (pTECs) differentiate into 'transitional' pTECs that express cTEC-associated genes such as b5t and IL-7, regulated by the transcription factor FoxN1 but independent of lymphocytes. cTEC and mTEC subpopulations coordinate the development and repertoire selection of T cells. SP thymocytes and NKT cells express RANKL to promote the differentiation and proliferation of Aire-expressing mTECs. RANKL-stimulated mTECs produce OPG to self-tune their development. CD40L expressed in CD4SP thymocytes cooperates with RANKL to promote mTEC development. CD4SP thymocytes also express LTs, which induce terminal differentiation of mTEC [26].

I.1.4. LTβR in mTEC development

Medullary TECs establish T cell self-tolerance through the expression of autoimmune regulator (Aire), which primary function is to upregulate the expression of many tissue-specific antigens

(TSAs). These antigens are processed and presented by mTECs directly to both $CD4^+$ and $CD8^+$ T cells, or acquired by DCs for processing and presentation to both $CD4^+$ and $CD8^+$ T cells. Recognition of these antigens with high affinity results in deletion of self-reactive T cells to avoid release of these cells into the periphery and thus provoke autoimmunity [34–36].

Several studies have shown the involvement of the NF-κB transcription factor in the development of mTECs [34]. The NF-κB signaling pathway in mTECs is activated by tumor necrosis factor receptor superfamily (TNFRSF) receptors, namely receptor activator of NF-κB (RANK), CD40, and lymphotoxin β receptor (LTβR). These receptors are expressed on mTECs, while the respective TNF superfamily ligands, RANKL, CD40L, and lymphotoxin (LT) αβ trimers, are expressed by thymocytes, mainly SP thymocytes [26]. This bidirectional exchange of signals between thymocytes and stromal cells is called thymic crosstalk and is crucial not only for thymocyte development but also for thymic stromal cell differentiation and maintenance [26,33].

Until now, it is not entirely clear what is the role of LTβR and its ligands in mTEC differentiation. However, it is known that LTβR is involved in supporting the three-dimensional structure of the thymus and mTECs differentiation [37–40], but *Ltbr* deficiency does not affect either Aire expression or Aire-dependent TSA gene expression in mTECs [41]. In a recent study by Cosway *et al.,* 2017 [42], reported that LTβR does not control the expression of Fezf2, an intrathymic TSAs regulator, as opposed to a recent study [38]in which LTβR was considered an essential regulator of Fezf2 expression in mTECs. Thus, this recent study [42] showed that the effect of LTβR on mTECs is separable from its importance in thymic tolerance, suggesting further that LTβR mediates tolerance by another mechanism. Indeed, in conditional $Ltbr^{-/-}$ mice it was found that despite a profound perturbation of mTECs caused by the specific elimination of LTβR in TECs, T cell tolerance remained intact. However, the authors identified a new thymic role for this TNFRSF member, reporting that LTβR expression in mesenchymal cells controls thymic DC differentiation and frequency [42]. How mesenchymal cells control thymic DCs is not currently known, although it is known that in both mesenchyme and endothelium, LTβR regulates the expression of chemokines and adhesion molecules [43,44] that can help thymus entry of DCs or DC progenitors [42].

I.2. T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA (T-ALL)

Acute lymphoblastic leukemia (ALL) is a heterogeneous disease at the level of cellular morphology, cytogenetic abnormalities, and immunological markers. As other malignancies, this pathology is a consequence of uncontrolled accumulation of immature lymphoid cells, either B or T cells, which develop in the BM or thymus, respectively [45–47]. ALL represents approximately 25% of all pediatric cancers, and has a poor prognosis after relapse. Currently the survival rate is about 80% in the case of children and 40-60% in adults [48].

The T-cell subtype of acute lymphoblastic leukemia (T-ALL) is a highly aggressive malignancy. About 15% of pediatric ALL patients present with a T-cell phenotype, while in adults the percentage increases up to 25% [49,50].

This disease begins with a malignant clone that likely arises in the thymus [51] and then proliferates and propagates throughout the body. Malignant cells may occur in various organs such as BM, peripheral blood, spleen, liver, lymph nodes, and central nervous system (CNS). Leukemia dissemination results in the signs and symptoms associated with T-ALL, such as suppressed BM function, leukocytosis, neurological abnormalities, and in some cases, a mediastinal mass present with or without pleural effusions, which may lead to respiratory distress [51,52].

I.2.1. T-ALL subtypes

T-ALL can be classified in different subtypes based on the presence or absence of immunophenotypic markers. The European Group for the Immunological Characterization of Leukemias (EGIL) classified T-ALL as pro-T or T-I ($CD7^+$ $CD2^-$ $CD5^-$), pre-T or T-II ($CD7^+$ $CD2^+$ $CD5^{+/-}$), cortical T or T-III ($CD1a^+$), and mature T or T-IV ($mCD3^+$ $CD1a^-$) [53], from the most immature to mature stage. An additional subgroup, early T-cell precursor (ETP)-ALL has more recently been identified. In parallel with normal ETPs, ETP-ALL is characterized by absence of CD1a and CD8, weak expression of CD5 and expression of one or more myeloid or stem cell-associated markers (CD117 CD34, human leukocyte antigen [HLA]-DR, CD13 CD33 CD11b or CD65) [54]. T-ALL can also be classified in genetic subgroups (i.e., immature, HOXA, proliferative, TLX1/3, and TAL/LMO) according to different gene expression profiles, which are associated with the underlying expression of particular oncogenes [55–57].

Moreover, a more recently proposed (but less used) TCR-based classification system, divides T-ALL in four stages: immature stage (cytoplasmic (c) TCRβ$^-$, surface (s) CD3$^-$), pre-αβ stage (cTCRβ$^+$ sCD3$^-$ pTα$^+$), TCRαβ (sCD3$^+$ TCRαβ$^+$), and TCRγδ (sCD3$^+$ TCRγδ$^+$) [58].

I.2.2. Genetic and molecular alterations in T-ALL

T-ALL is characterized by diverse genetic alterations that perturb normal hematopoietic differentiation and lead to malignant cellular transformation. Several oncogenic mutational steps are required for transformation, which likely occur at any stage of thymocyte development [59]. These alterations occur in genes that control diverse cellular processes, and include point mutations, gene fusions, translocations, inversions, and deletions [45,60] and have been classified into two categories: Type A and Type B [59].

Type A mutations are generally considered driving chromosomal abnormalities or initiating events that facilitate the arrest of thymocytes development at specific stages of differentiation. These mutations involve oncogenes that define the molecular subtypes mentioned above. Type B mutations include gene-specific small deletions, translocations, duplications and point mutations in proto-oncogenes or tumor suppressor genes that affect different cellular processes, such as cell cycle regulation, TCR signaling or T-cell differentiation [45,59]. These mutations can occur across the different molecular subtypes.

T-ALLs characteristically show chromosomal translocations (30-35% of T-ALL cases) or other genetic alterations leading to aberrant expression of transcription factor oncogenes, which constitute type A mutations. During the RAG-mediated TCR loci V(D)J recombination events in thymocyte development, other genes become vulnerable to the action of those recombinases. Strong promoter and enhancer elements of the TCR genes may then become juxtaposed to transcription factor proto-oncogenes. These oncogenic transcription factors include: basic helix-loop-helix (bHLH) family members such as TAL1, TAL2, LYL1, and BHLHB1; LIM-only domain (*LMO*) genes such as *LMO1* and *LMO2*; TLX proteins like TLX1/HOX11 and TLX3/HOX11L2, among others [61]. Among the different genetic changes that constitute type B mutations, the constitutive activation of *NOTCH1* signaling is the most outstanding oncogenic pathway in T cell transformation [61,62]. Furthermore, deletions of the *CDKN2A* locus in chromosome band 9p21, which involve the p16/INK4A and p14/ARF suppressor proteins, are present in more than 70% of all T-ALL cases [61].

On the other hand, in a study by Trinquand *et al.,* 2016, the authors observed that co-expression of the TEL-JAK2 oncogene with a transgene encoding TCR-HY, which induces negative selection only in male mice, specifically compromised leukemia onset in males. When leukemias obtained from females were transplanted in either male or female secondary recipients, only females succumbed to T-ALL [63–65]. This indicated that the strong/sustained TCR activation associated with the negatively selecting TCR-HY severely impaired leukemia maintenance [64,65].

The diversity of genetic lesions involved in the pathogenesis of T-ALL is further complicated by a number of recurrent cytogenetic and molecular alterations that are common between all molecular subtypes a (**Table I.1**) [61].

Table I.1: Most common genetic lesions in adult versus pediatric T-ALL [66,67].

GENE	GENETIC LESION(S)	FREQUENCY (%) Pediatric	Adult
TRANSCRIPTION FACTOR ONCOGENES			
TAL1	Chromosomal rearrangements/mutations/deletions/expression	30	34
TLX1	Chromosomal rearrangements/mutations/deletions/expression	8	20
TLX3	Chromosomal rearrangements/expression	19	9
MYB	Chromosomal rearrangements/duplications	7	17
NKX2.1/NKX2.2	Chromosomal rearrangements/expression	8 (only pediatric cases)	
LMO2	Chromosomal rearrangements/deletions/expression	13	21
HOXA (CALMAF10, MLL-ENL AND SET-NUP214)	Chromosomal rearrangements/inversions/expression	5	8
NOTCH1 PATHWAY			
NOTCH1	Activating mutation	50	57
FBXW7	Inactivating mutation	14	14
CELL CYCLE			
CDKN2A OR CDKN2B	9p21 deletion- Methylation	61 - 58	55 - 46
RB1	Deletion	12 (only pediatric cases)	
CDKN2B	12p13.2 deletion	12	12
TRANSCRIPTION FACTOR TUMOR SUPPRESSORS			
WT1	Inactivating mutation / deletion	19	11
LEF1	Inactivating mutation / deletion	10	2
ETV6	Inactivating mutation / deletion	8	14
BCL11B	Inactivating mutation / deletion	10	9
RUNX1	Inactivating mutation / deletion	8	10
SIGNAL TRANSDUCTION			
PTEN	Inactivating mutation / deletion	19	11
JAK1	Activating mutation	5	7
FLT3	Activating mutation	6	4
IL7R	Activating mutation	10	12
DNM2	Inactivating mutation	13	13
EZH2	Inactivating mutations/deletions	12	12
TRANSLATION AND RNA STABILITY			
MTOR	Activating mutations	5 (only adult cases)	
RPL5	Inactivating mutations	2	2
RPL10	Missense mutations	8	1
CNOT3	Missense mutations	3	8

I.2.3. Microenvironmental factors

It is known that the tumor microenvironment influences the initiation and progression of hematological malignancies [13]. Nevertheless, relatively little is known about the identity and roles of microenvironmental factors participating in T-cell acute lymphoblastic leukemia (T-

ALL) development. The lymphoid tumors cells and the microenvironment evolve together by continuous communication through paracrine and/or juxtacrine signaling, leading to the creation of a selective and permissive microenvironment that supports malignant cells to survive, grow and resist immune recognition and elimination [68].

The thymus provides a dynamic and essential microenvironment for the development of T cells, which depend on the interaction with thymic stromal cells. In addition, the thymus may provide a permissive location for T-cell leukemogenesis and tumor progression [51]. Indeed, several studies reports that the thymic microenvironment is crucial for tumorigenesis and that there are various factors that can contribute for T-ALL development [13].

It has long been known that human and mouse T-ALL cells often require stromal cell support to be maintained ex vivo [69,70] and several stromal cell-derived factors have been found to mediate that supportive action. In the study of Martins *et al.* 2014 about the role of the thymic microenvironment in T-cell progenitor competition, the authors have shown that blocking cellular competition between bone-marrow-derived progenitors and T-cell precursors developing in thymus leads to the development of T-cell leukemia. Thus, cellular competition in thymus acts as a tumor suppressor mechanism and protects the T cell progenitors from *in situ* ageing and malignant transformation [71].

The role of cytokines, chemokines or growth factors that are involved in the normal development of T cells as enhancers of leukemogenesis arose the interest of many authors. Thus, several cellular factors that may be involved in the development of T-ALL have been studied, including growth factors [e.g., interleukin 7 (IL7), IL18], insulin-like growth factor 1 (IGF1), Notch ligands (DLL4), chemokines such as CCR7 and CCR9 [72], and adhesion molecules (for example, ICAM1) [69,70,73–76]. Silva *et al.,* 2011 showed that IL7 produced by thymic and BM epithelial stromal cells is involved in the *in vivo* progression and dissemination of leukemia [70]. Another cytokine, IL18, which is produced by BM-derived stromal cells in inflammatory microenvironments, increases and acts as a proliferative factor of T-ALL cells [75].

In addition, several studies have shown that elements of the microenvironment can induce NOTCH1 signaling in leukemic cells thus leading to an oncogenic transcription program, promoting proliferation and survival of T-ALL lymphoblasts [77]. NOTCH1 signaling is involved in increased receptivity of leukemic cells to signals provided by the microenvironment by inducing expression of receptors for extracellular ligands, such as IL7 receptor [78].

As dos Santos *et al.,* 2008 [79] found, the expression of the NF-κB transcription factor RelB in non-hematopoietic stromal cells contributes to the development of T-ALL in a mouse model. Since RelB is activated after the engagement of TNFRSF members, such as the LTβR, the previous data suggested that signaling from a TNFRSF member was involved in T-ALL,

Finally, some of the signaling pathways aberrantly activated in T-ALL are induced by many microenvironmental factors rather than cell-intrinsic mechanisms, which are summarized in **Table 1.2.**

Table I.2: Microenvironmental factors involved in T-ALL development.

Factors involved

Stromal cells	T-ALL	Effect on T-ALL cells	References
ICAM-1	LFA-1	Survival	73
CXCL12	CXCR4	Chemotaxis, extramedullary organ infiltration	80
CCL25	CCR9	Chemotaxis, infiltration, resistance to apoptosis, proliferation	81 72
IL-7	IL-7R	Survival, proliferation, T-ALL progression	82,83 70
IL-18	IL-18R	Proliferation, T-ALL progression	75
DLL4	NOTCH3	Angiogenesis, tumor growth in vivo and escape from dormancy; T-ALL progression	84 76
IGF1	IGF1R	Survival, proliferation, LIC activity	74
UNKNOWN	Calcineurin	Survival, proliferation, motility, LIC activity	85 86
IL-2, IL-7, IL-9	Cytokine receptors	Proliferation, T-ALL leukemogenesis	87

I.3. NF-κB SIGNALING PATHWAY

NF-κB proteins are transcriptional regulators that bind cognate DNA elements as homo or heterodimers [88] and have been identified for being involved in many physiological processes, such as cellular responses to stress, immune responses, and inflammation [88]. Also, signals activating NF-κB pathways are critical for thymic medulla and thymocyte development [26,33].

The five members of this transcription factor family are p65 (RelA), RelB, c-Rel, NF-κB1 and NF-κB2. NF-κB1 and NF-κB2 are synthesized are longer isoforms p105 and p100, and then proteolytically processed to p50 and p52, respectively [89]. All family members contain the characteristic Rel homology domain (RHD), responsible for DNA binding, dimerization, and nuclear localization [88]. Normally, NF-κB factors resides in the cytoplasm of unstimulated cells as an inactive complex associated with a member of the IκB inhibitory protein family [89]. These inhibitors proteins are characterized by the presence of ankyrin repeats domains, which interact with and inhibit the RHD domain of NF-κB proteins [88,89].

The NF-κB pathway is often subdivided in two pathways designated as canonical or noncanonical (also called alternative) (**Figure I.4**). These two pathways differ in the type of stimulation, distinctive transcriptional programs regulated, and diverse biological functions where they are involved [88]. The canonical pathway is usually triggered either by exposure to proinflammatory cytokines [such as tumor-necrosis factor-a (TNFα)], by Toll-like receptor ligands, T-cell antigen receptor (TCR) or B-cell receptor antigenic stimulation, or in response to microbial and viral infections. Upon stimulation, the IkB kinase complex, which is composed by IKKα, IKKβ and NEMO (also known as IKKγ), phosphorylates IκBα which causes its proteasomal degradation by the 26S ubiquitin-dependent proteasome complex. RelA containing NF-κB are then released and translocated to the nucleus, thus activating target gene transcription [88–90].

In contrast, the alternative pathway of NF-κB activation, also designated as noncanonical pathway, is originated from different classes of receptors including BAFFR, LTβR, CD40, RANK, TNFR2 and Fn14 [89]. In non-stimulated cells, NIK is constitutively degraded by the proteasome through TRAF3-mediated ubiquitination by cIAP proteins [88]. Upon receptor activation, TRAF3 and the associated TRAF2, cIAP1, and cIAP2 proteins are degraded, and NIK is stabilized and activated. The activated NIK phosphorylates and activates predominantly IKKα, which in turn phosphorylates p100. When, phosphorylated on serine residues and

subsequently ubiquitinated, p100 is proteolytically processed to the p52 subunit. Thus, the RelB/p52 complex is released and can be translocated from the cytoplasm to the nucleus in order to induce expression of target genes [88–90].

In parallel, it has been demonstrated that p100, when undergoing proteolysis, activates not only RelB- but also RelA-containing dimers, though with slower kinetics than RelA heterodimers activated via the canonical pathway. Since the expression of the RelB and p100/NF-κB2 genes is induced by canonical NF-κB signaling, inactivation of this pathway also hampers indirectly noncanonical signaling [88].

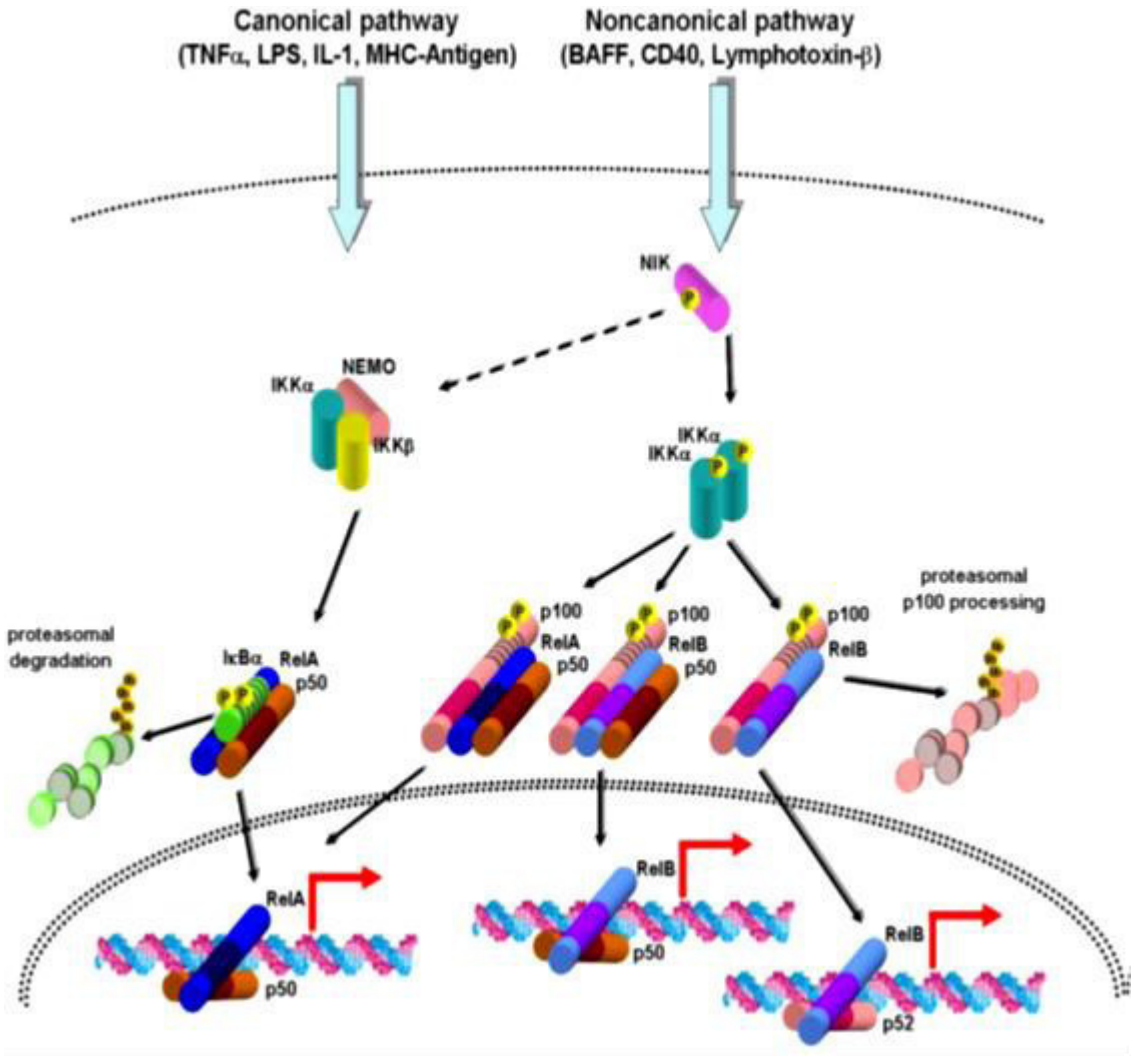


Figure I.4: Schematic representation of the main actors intervening in the canonical and non-canonical NF-κB activation pathways. The canonical NF-κB activation pathway, which is triggered by an array of stimuli such as TNFα, lipopolysaccharide (LPS), interleukin-1 (IL1), and MHC–coupled antigen activate, relies on IKKβ (IKK2)/IKKγ (NEMO)-dependent IκB phosphorylation and degradation and results in RelA and/or c-Rel activation. The non-canonical NF-κB activation pathway, which can be activated by specific members of the TNF receptor family (e.g., LTβR and BAFF receptor [BAFF-R]) depends on IKKα (IKK1) and NIK kinase activity but not on IKKβ or IKKγ [1]. Upon stimulation, IKKα phosphorylates p100 on C-terminal serine residues and induces

its ubiquitin-dependent processing to generate p52. When released from p100 sequestration, p52:RelB, p50:Rel1B, and, as recently shown, p50:RelA dimers shuttle to the nucleus to activate transcription of specific target genes [88].

Termination of NF-κB transcriptional activity is mainly achieved by the up-regulation of its inhibitors of the IκB family, in which newly synthesized IκBα enter the nucleus, remove NF-κB from the DNA and relocate it to the cytosol [89].

The NF-κB pathway promotes multiple cancer behaviors such as proliferation, survival, angiogenesis, and metastasis [89]. For research and therapeutic purposes, several NF-κB inhibitors have been developed. Burke *et al,*. 2003 [91] reported a potent inhibitor of the NF-κB pathway compound BMS-345541, which was shown to be highly selective for the IKK catalytic subunits versus numerous other kinases. In cells, only stimulus-induced phosphorylation of IκBα was inhibited by BMS-345541, whereas other signal transduction cascades were unaffected. BMS-345541 displayed an action against both IKKα (IKK1) and IKKβ (IKK2), but displaying 10-fold selectivity towards IKKβ [91,92].

I.3.1. TLR4/LPS signals activate NF-κB signaling

Lipopolysaccharide (LPS) is an important structural component of the outer membrane of Gram-negative bacteria that can trigger a variety of inflammatory reactions, including the release of pro-inflammatory cytokines and other soluble factors. LPS stimulation of mammalian cells occurs through a series of interactions with several proteins including: the LPS binding protein (LBP) which directly binds to LPS, the CD14, which facilitates the transfer of LPS to the TLR4/MD-2 receptor complex and modulates LPS recognition and the MD-2/TLR4 complex (**Figure I.5**) [93].

Toll-like receptors (TLRs) are pattern recognition receptors that play a central role in host cell recognition, responses to microbial pathogens and initiation of innate immune responses. These receptors are expressed at the membranes of leukocytes including DC, macrophages, NK cells, adaptive immunity cells (T and B lymphocytes) and non-immune cells (epithelial and endothelial cells, and fibroblasts) [93,94].

Evidence suggest that several pathogen-associated molecular patterns, such as LPS, can stimulate TLR 4 [93]. TLR signaling is largely divided into two pathways: either MyD88-dependent or TRIF-dependent (**Figure I.5**), although both pathways commonly activate the

canonical NF-κB pathway for the induction of inflammatory cytokines. Additionally, each pathway activates other proteins that defines their unique effector function [95].

In the MyD88-dependent pathway, the activation of NF-κB induces expression of proinflammatory cytokine genes [93] such as IL 1α/β, IL18, IL6, and TNFα. On the other hand, the TRIF-depend pathway involves the TRIF-related adaptor molecule (TRAM). NF-κB activation by this pathway activates the transcription of target genes, such as type I interferons (IFNs) [93,95]. TRIF can recruit TRAF3 and activate IRF3 through TBK1, inducing transcription of type I IFNs and IFN-inducible genes [96].

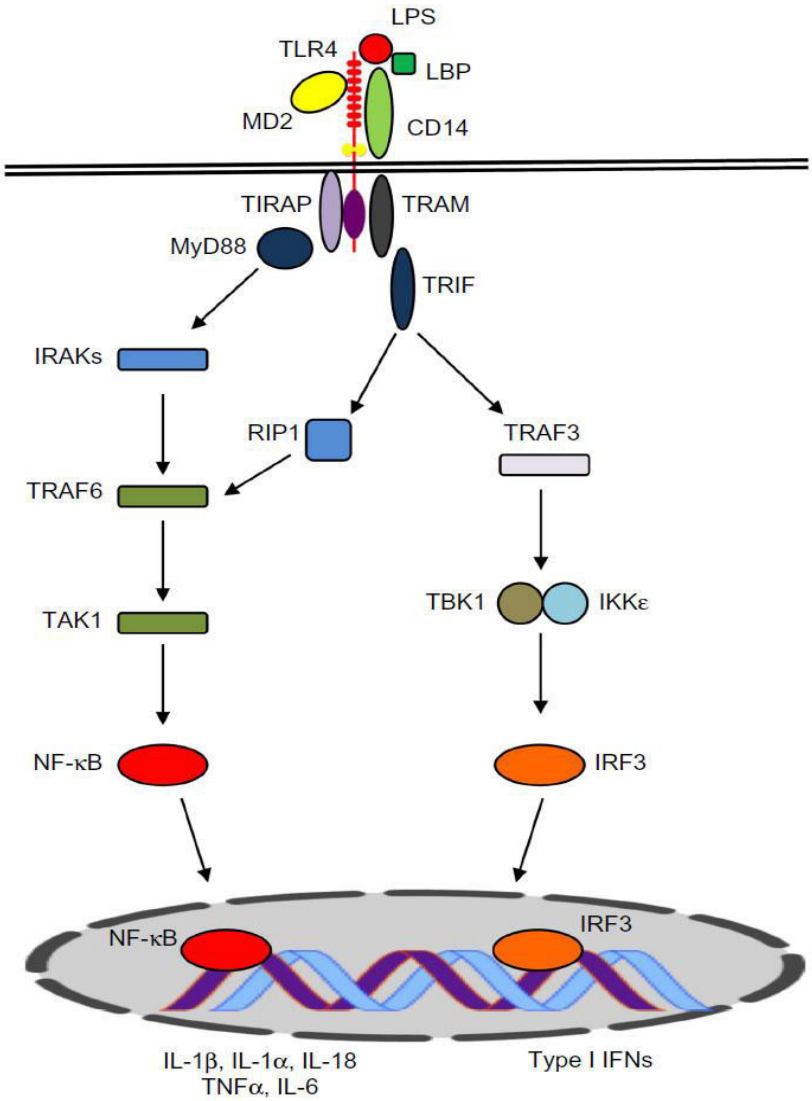


Figure I.5: LPS/TLR4 signaling. Upon LPS stimulation, TLR4 signaling can be separated into MyD88-dependent and TRIF-dependent independent pathways, which mediate the activation of proinflammatory cytokines and type I IFNs genes, respectively [96].

I.3.2. NF-κB signaling in inflammation and cancer

The NF-κB system is tightly controlled at various levels, and deregulations of NF-κB homeostasis has been implicated in a wide range of diseases, ranging from inflammatory and immune disorders to cancer. In particular, NF-κB is a key link between chronic inflammation and cancer transformation [97].

Inflammation in general and NF-κB in particular have a double-edged role in cancer. On one hand, activation of NF-κB is part of the immune defense, which targets and eliminates transformed cells [89]. The negative regulators of NF-κB have tumor suppressor functions, and are frequently inactivated either by genomic deletions or point mutations [97], such as stimulating neoangiogenesis and inducing pro-invasive/pro-metastatic inflammatory microenvironments [46,47,98]. This seems to be particularly true for acute inflammatory processes, where full activation of NF-κB is accompanied by a high activity of cytotoxic immune cells against cancer cells [89]. On the other hand, NF-κB is constitutively activated in many types of cancer and can exert a variety of pro-tumorigenic functions [89]. Key positive regulators of NF-κB signaling can act as oncogenes and are often prone to chromosomal translocation, amplifications, or activating mutations [97], making cancer cells resistant to apoptosis and/or making them highly proliferative [46,47,98].

The anti-tumorigenic function of the immune systems with NF-κB being an important effector of it, has been designated as tumor-immunosurveillance [89,99]. This immune defense against cancer cells, however, is normally not tight enough to eliminate all the aberrant cells, resulting in a shift to an equilibrium phase, which is often followed by an "escape" phase of the cancer cells, in which they outperform the immune system [89,100].

NF-κB activation usually results in the up-regulation of anti-apoptotic genes thereby providing cell survival mechanism to withstand the physiological stress that trigger the inflammatory response [89].

An evidence of the link between NF-κB and inflammation was found in colitis associated colon cancer (CAC). Classical inflammatory-driven cancer accounts for about 5% of sporadic colorectal cancers. The conditional ablation of IKKβ showed that activation of NF-κB in intestinal epithelial cells (IECs), in which β-catenin signaling was activated via mutation, provides premalignant progenitors with a survival advantage through the induction of antiapoptotic genes [101–103]. Despite this role in IECs, NF-κB in myeloid cells, most probably

lamina propria macrophages, also contributes to tumor growth and progression through the transcriptional activation of genes encoding growth factors that enhance the proliferation of premalignant IECs and their transformed derivatives [101,102]. Indeed, many inflammatory cytokines produced by lamina propria macrophages and DCs, such as TNF [104], IL6 and IL23 [105], have been identified as the main drivers of CAC growth [101,103].

In sum, the contribution of inflammation in general and NF-κB in particular to cancer initiation and progression is manifold and complex. For example, enhanced NF-κB activity can be directly induced by mutations of NF-κB genes and/or oncogenes that activate the NF-κB signaling pathway. Alternatively, a tumor can achieve elevated NF-κB activity through increased cytokine release from the tumor microenvironment [89,101].

I.3.2.1. Role of the NF-κB signaling pathway in T-ALL

Direct mutations of NF-κB signaling genes have so far been detected mainly in lymphoid malignancies [89,101]. Although mutations in genes of the NF-κB signaling pathway have not been described in T-ALL, the constitutive activation of both the canonical and noncanonical NF-κB pathways through different mechanisms, has been observed in murine and human T-cell acute lymphoblastic leukemia cells [88,106].

Adding to the widely known involvement of NF-κB signaling pathways in malignant cells, one study uncovered a role for RelB transcription factor in the crosstalk between thymic stromal and T-ALL cells [79]. This NF-κB family member activated by the alternative signaling pathway was shown to play a pro-oncogenic role, not in tumor cells, but rather in cells that compose the tumor microenvironment, thus leading to accelerated leukemia onset and increased severity of TEL-JAK2-induced mouse leukemia [79]. Therefore, NF-κB signaling appears to modulate T-cell leukemogenesis through its action on microenvironmental cells [88].

One of the most frequent changes in T-ALL are NOTCH1 mutations, occurring in more than 50% of the cases [88]. Several studies have described that NOTCH1 can activate the NF-κB pathway in T-cell ALL at various levels, for examples by interaction with and leading to increased IKK activity or by inducing the expression of *NF-κB2* and *RelB*, resulting in sustained NF-κB activity [88,107].

I.4. ROLE OF THE LYMPHOTOXIN- β RECEPTOR (LTβR) IN CANCER

I.4.1. The lymphotoxin-β receptor and its ligands: $LT\alpha_1\beta_2$ and LIGHT

The lymphotoxin-β receptor (LTβR) is a TNFRSF member involved in diverse biological processes such as differentiation of mTECs, liver regeneration, regulation of innate and adaptive immune response, and has a key mediator role in the control, development, organization, and homeostasis of lymphatic tissues and organs [47].

The human LTβR genetic locus (*TNFRSF3* or *LTBR*) contains 10 exons and it is located on the chromosome (Chr) 12 (mouse Chr 6), adjacently to the *TNFR1* gene (*TNFRSF1A*) and *CD27* genes [47,108,109]. The LTβR full-length transcript encodes a 435-amino acid type I glycosylated protein consisting of an extracellular domain (ECD), a transmembrane domain (TMD), and an intracellular domain (ICD), also known as cytoplasmic domain (CD). Also, LTβR contains four cysteine-rich domains (CRD), which confer the specificity and affinity for its cognate ligands [47].

The TNFRSF receptors can be subdivided as death and non-death receptors, according to their ICD domain. The LTβR does not contain a death domain but has rather two TRAF binding sites [47,110]. LTβR is activated by either of two ligands, $LT\alpha_1\beta_2$ heterotrimer or LIGHT/TNFSF14 homotrimer (**Figure I.6**) [47,110].

In the human genome, *lymphotoxin α* (*LTA* or *TNFSF1*) and *lymphotoxin beta* (*LTβ* or *TNFSF3*) genes are localized each flanking the gene encoding TNFα, being all tightly linked within the MHC class III region on chromosome 6 (mouse Chr17). The *LTβ*-encoding gene is oriented in the opposite direction of *TNFα* and *LTα*-encoding genes [47,109]. The *LTA* locus contains 4 exons and the full-length mRNA encodes a 205-amino acid type II glycosylated protein, whereas the *LTB* locus also contains 4 exons and is translated into a 244-amino acid type II glycosylated protein. *LTβ* contains a transmembrane domain as but not its homolog *LTα* [47,111]. Therefore, when expressed in the absence of LTβ, Ltα forms LTα3 homotrimers through interactions between hydrophobic and aromatic side-chains [47,110]. When LTα is expressed together with LTβ, these proteins oligomerize to form $LT\alpha_2\beta_1$ and $LT\alpha_1\beta_2$ heterotrimers. However, $LT\alpha_2\beta_1$ is a minor form that only represents less than 2% of total LTβ in activated T cells [47,110].

The *LIGHT* gene (or *TNFSF14*) is part of the human TNF superfamily and encodes also a LTβR ligand. It is localized within an MHC-like region on Chr 19 (Chr 17 in the mouse), recognized

as an MHC paralog region, in close proximity to other TNF superfamily member genes such as the CD27 ligand (*TNFSF7*) and the CD137 ligand (*TNFSF9*) [47]. The *LIGHT* gene is composed by 4 exons and the full-length transcript encodes a 29 kDa N-glycosylated type II transmembrane protein. LIGHT is expressed at the cell surface and is not secreted [47,110].

LTβR is constitutively expressed in a wide variety of cells in lymphoid and visceral tissues (for example: fibroblasts, epithelial cells, endothelial cells, and follicular DCs) and cells of myeloid lineage, but is not expressed on T or B cells. The LTα1β2 ligand is physiologically expressed on the surface of activated T, B and NK cells, while LIGHT is expressed primarily in activated T cells. This expression pattern hints that the signals mediating LTβR activation are paracrine or juxtracrine in nature [46,47].

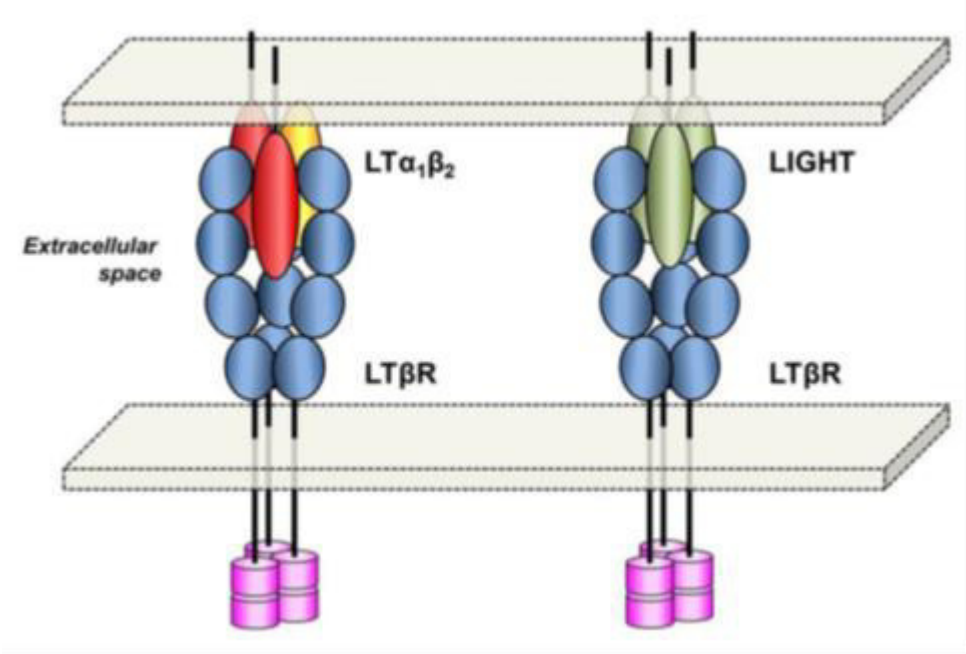


Figure I.6: Schematic representation of interaction between LTβR and its two main ligands. $LT\alpha_1\beta_2$ heterotrimers (left) and LIGHT homotrimers (right). Blue shapes represent LTβR cysteine-rich domains (CRDs) and pink shapes represent TRAF-binding domains [47].

The binding of $LT\alpha_1\beta_2$ or LIGHT to LTβR brings two molecules in close proximity and the LTβR self-interaction region in the cytoplasmic domain promotes receptor aggregation resulting in conformational changes. Afterwards, there is a sequential recruitment of cytosolic adaptor proteins of the TRAF family to the cytoplasmic region of LTβR. The respective proteins activate or repress the signaling initiation leading to gene transcription through different signaling pathways such as the classical and alternative NF-κB pathways, the c-Jun N-terminal

kinase (JNK) MAP kinase pathway, and other signaling pathways [47]. However, other biological processes like apoptosis/cell death, cell cycle progression, angiogenesis, taxis and adipogenic differentiation, were shown to be also potentially regulated by LTβR signaling (**Figure I.7**) [112].

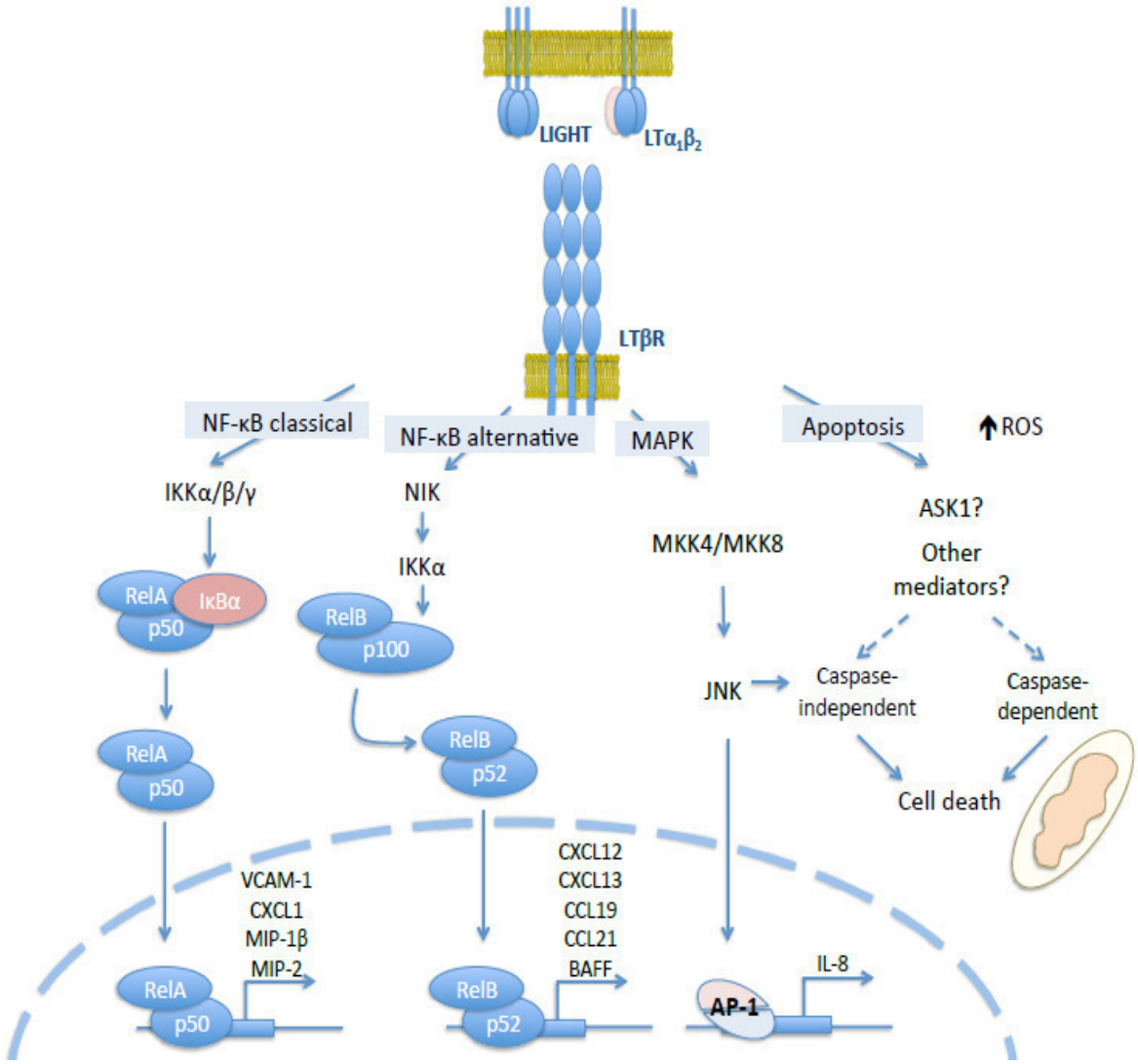


Figure I.7: Summary of LTβR-mediated signal transduction for target-gene expression. Activation of LTβR signaling axis by LIGHT or LTα1β2 induces gene expression and cell death. NF-κB classical pathway induction involves activation of IKK complex, IKK-mediated IκBα phosphorylation, and subsequent degradation, resulting in nuclear translocation of RelA/p50 heterodimers, which induce the expression of proinflammatory cytokines, chemokines, and adhesion molecules (see above). On the other hand, the alternative NF-κB pathway relies on NIK and IKKα-dependent processing of p100 into p52, leading to the translocation of RelB/p52 dimers to the nucleus where they activate the expression of genes mainly involved in lymphoid organogenesis and homeostasis. LTβR was also shown to activate JNK and AP-1-induced gene expression. Furthermore, LTβR activation induces cell death by poorly characterized mechanisms involving reactive oxygene species (ROS) production, ASK-1, and either caspase-independent or caspase-dependent apoptosis [113]

I.4.2. LTβR signaling in carcinogenesis

Several studies have shown a role for LTβR signaling in carcinogenesis. Earlier studies showed that LTβR can mediate anti-tumor effects by direct cytotoxicity of LTβR-expressing cancer cells, likely induced by immune cells expressing $LT\alpha_1\beta_2$ and/or LIGHT (**Figure I.8: A**) [47]. Moreover, the LTβR signaling may trigger host-mediated anti-tumor immune responses both by the expression of pro-inflammatory cytokines and chemokines that chemoattract and activate effector lymphocytes (**Figure I.8: B**) [47,114] or by inducing the differentiation of high endothelial venules (HEVs) that facilitate lymphocyte trafficking to tumors (**Figure I.8: C**) [47,115].

However, more recent studies showed that the LTβR signaling may also trigger pro-oncogenic mechanisms by four distinct processes. Firstly, data by Dhawan and co-workers (2008) [116] indicated that *LTβR* gene upregulation caused constitutive activation of LTβR in melanoma, which was by itself sufficient to promote carcinogenesis. In this setting, LTβR activated the NF-κB signaling pathway, and induced cell proliferation and invasive capacity, all in a ligand-independent manner (**Figure I.8: D**) [47,116].

It was also demonstrated that inhibition of LTβR signaling prevented neoangiogenesis and tumor growth. Some authors have reported that LTβR activation by $LT\alpha_1\beta_2$- or LIGHT-expressing T and B lymphocytes triggered the expression of the angiogenic mediator CXCL2. CXCL2 induction in BFS-1 cells depended on NF-κB activation and contributed for solid tumor growth in vivo (**Figure I.8: E**) [47,117].

It was also found a pro-oncogenic role for LTβR signaling that is mediated by interactions of cancer cells with the tumor microenvironment. Tumor and/or stromal cells respond to injury, infection, and tissue stress by producing cytokines and chemokines that attract immune cells. These cells migrate to the tumor microenvironment where they secrete pro-inflammatory, angiogenic and pro-tumorigenic factors that influence tumor progression and metastasis. Therefore, depending on the tumor microenvironment chemokine milieu, tumor-infiltrating immune cells can stimulate the immune response against tumor cells, as mentioned above, or rather help these to subvert the immune response and promote oncogenesis. In this context, $LT\alpha_1\beta_2$ and LIGHT-expressing lymphocytes induced chemokines in LTβR-expressing cancer cells, promoting the pro-inflammatory oncogenic microenvironment (**Figure I.8: F**) [115,118].

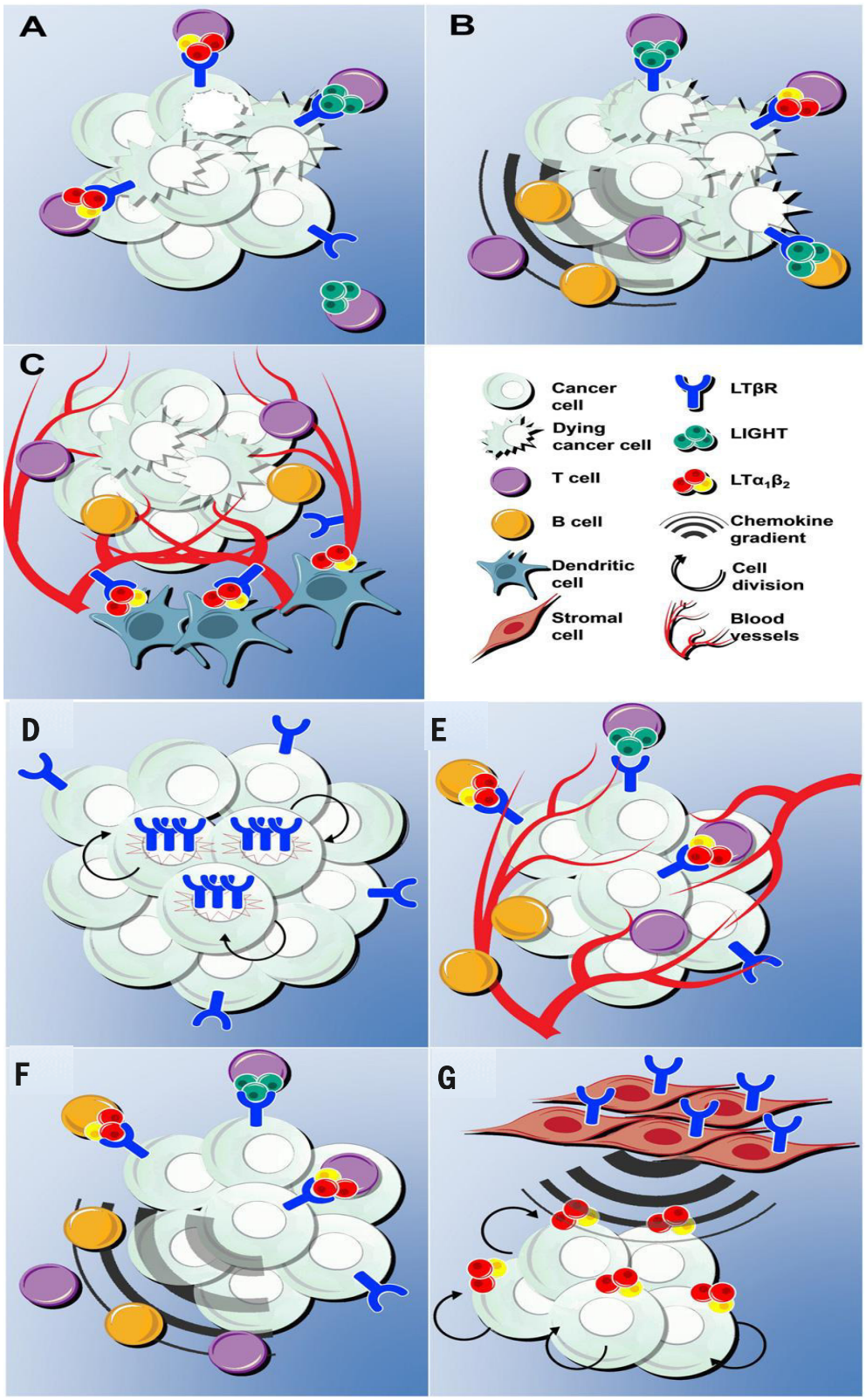


Figure I.8: LTβR role in cancer. The activation of LTβR leads to anti-oncogenic effects due to three main mechanisms. (**A**) Death of LTβR-expressing cancer cells likely induced by immune cells expressing $LT\alpha_1\beta_2$ and/or LIGHT. (**B**) Recruitment of anti-cancer $LT\alpha_1\beta_2$- and/or LIGHT-expressing immune cells mediated by LTβR-

expressing cancer or stromal cell chemokine production. (**C**) Increased anti-tumor immune response linked to high endothelial venule neogenesis triggered by LTβR stimulation of endothelial cells by $LT\alpha_1\beta_2$-expressing DCs. On the other hand, activation of LTβR signaling favors oncogenesis due to four main mechanisms. (**D**) Genetic alterations in the LTBR gene leading either to its overexpression or the expression of alternative forms, result in ligand-independent LTβR activation, which supports cancer cell proliferation and/or survival. (**E**) LTα1β2- and LIGHT expressing lymphocytes induce pro-angiogenic factors in LTβR-expressing cancer cells and induce angiogenesis. (**F**) $LT\alpha_1\beta_2$- and LIGHT-expressing lymphocytes induce chemokines in LTβR-expressing cancer cells, thus development a pro-oncogenic inflammatory microenvironment. (**G**) $LT\alpha_1\beta_2$-expressing cancer cells persuade production of chemokines and pro-survival factors in LTβR-expressing tumor stromal cells, thus triggering cancer cell migration, and favoring tumor progression [47].

Finally, it was also described the induction of pro-tumorigenic niches supported by LTβR-expressing stromal cells, where cancer cells can generate a pro-tumorigenic microenvironment in consequence of increased lymphotoxin expression and LTβR activation in stromal cells (**Figure I.8: G**) [47,119].

In conclusion, despite early evidence for anti-tumor properties of the LTβR signaling pathway, several reports identified a pro-oncogenic role for LT signaling in both solid and hematological malignancies [47,120].

I.4.3. Role of the LTβR signaling in T-ALL

Different reports indicate that hematologic malignancies are caused or helped by LTβR signaling, taking place either intrinsically to cancer cells or indirectly through microenvironmental cells (**Table I.3**). However, the LTβR role in the T-ALL setting is still largely undefined.

Supporting a role for LTβR in T-ALL, high expression levels of LTα and LTβ-encoding genes has been identified in primary human T-ALL samples expressing TAL or LMO oncogenes (TAL/LMO molecular subtype) and in the TEL-JAK2 transgenic mouse model of cortical/mature T-ALL [46]. TEL-JAK2 mice spontaneously develop T-ALL from thymocytes [63] and therefore are ideal for studying the malignant transformation of these cells in their natural microenvironment. As recently reported [46], by detecting aberrant $CD25^+$ $CD8^+$ T cells in thymic, but not in other organs of young asymptomatic mice, our group confirmed that T-ALL originates in the thymus of TEL-JAK2 mice.

Fernandes and collaborators [46] found also that leukemic cells of these TEL-JAK2 mice expressed high levels of *LTA* and *LTB* genes in the initial phase of disease, and importantly both early appearance of malignant cells in the thymus and disease-free mouse survival were delayed in the absence of stromal LTβR. Since specific stromal cells types depend on RelB expression for their function, and it has been shown that RelB is involved in mouse leukemogenesis [79], these recent studies support the notion that LTβR activation in stromal cells promotes T-cells leukemogenesis through NF-κB activation [46,47], and suggest that lymphotoxin mediates the molecular crosstalk between malignant and non-malignant cells, thus facilitating leukemogenesis [46].

To understand how T-ALL arises from thymocytes and to determine which microenvironmental signals play a role in progression, maintenance and chemoresistance of this malignancy [46].

Table I.3: LTβR-induced pro-oncogenic effects in hematological cancers [47].

	Cell types expressing LTβR or its ligands				
Cancer type	LTαβ	LIGHT	LTβR	Biological Context	Cellular effects
Hematological cancers					
Multiple myeloma	-	-	MM cells	Human myeloma cell lines and primary samples	LTβR amplification activating NF-κB and myelomagenesis
B-cell lymphoma	Malignant B cells	n.d.	FRCs	Eμ-Myc transgenic mouse model	Promotion of a pro-carcinogenic niche
B-CLL	Malignant B cells	n.d.	FDCs	Eμ-Tcl1 transgenic mouse model	Promotion of a pro-carcinogenic niche
T-ALL/LBL	Malignant T cells	n.d.	Thymic stromal cells	EμSRα-TEL-JAK2 transgenic mouse model	Promotion of a pro-carcinogenic niche

I.5. T-ALL THERAPY AND POTENTIAL NEW TARGETS

Acute lymphoblastic leukemia (ALL) is the most common malignant neoplasm that affects children, comprising about 20% of cancers occurring before the age of 19 years. The clinical outcome for pediatric ALL has dramatically improved over the last 50 years due to the advent of multidrug risk-adjusted chemotherapy regimens, improved CNS prophylaxis and recognition of clinical, biological and treatment response characteristics that identify patients at risk for treatment failure. Cure rates can now reach about 90%, yet, the prognosis for the 10-20% of children who relapse has not improved in the last two decades [121].

Despite efforts on finding new strategies, patients are still treated with high doses of chemotherapy followed by an allogeneic hematopoietic stem cell transplantation. Only a subset do relapse. Even so, this treatment is extremely aggressive and is associated with severe acute toxicity and long-term side effects, including the increase risk of development of secondary tumors later in life [121–124]. Therefore, one of the current challenges is to develop efficient targeted therapeutic strategies for T-ALL minimizing the emergence of resistant cases and the detrimental side-effects associated with conventional chemotherapy regimens. Thus, we should try to identify and understand the genomic lesions that are at the origin of T-ALL and/or contribute to leukemia expansion, spreading and resistance to therapy. In addition, it will be important to study the influence of the bidirectional molecular crosstalk between the tumor microenvironment and cancer cells on the acquisition of resistance to traditional therapies [125]

Then, when considering a therapeutic strategy for T-ALL, several players in disease development may constitute targets for rational therapy. For example, the common oncogenic transcription factors, like the TAL1 or LMO transcriptional regulators. In some cases, targeting the initiating event could be enough to eliminate leukemic cells since the secondary events are unlikely to replace their function. One caveat is that several proteins (e.g. transcription factors) are very difficult to target.

Also, signaling pathways aberrantly activated due to mutations or upstream activating mechanisms constitute key events in the pathogenesis of T-ALL and are therefore an excellent opportunity for molecular targeting. The potential targets are integrative members of signaling pathways such as the PI3K/Akt/mTOR [126], Notch [127], NF-κB [88,128], and Ras pathway [129,130], Wnt [131], and JAK/STAT pathways [132].

Furthermore, inadequate regulation of epigenetic factors is perturbed during malignant transformation and therefore these are possible therapeutic targets [133,134]. In addition, the receptors responsible for survival signals in T-ALL cells or the tumor stroma itself should be considered for targeted therapy. One argument favoring the latter approach is that unlike malignant cells, the stromal cells that compose the tumor microenvironment are genetically stable and thus potentially less likely to develop resistance and lead to tumor recurrence [125].

Finally, it should be considered that different tumors will differ not only in their cell-intrinsic genetic lesions but also in their dependence on the microenvironment. For this reason, patients may respond distinctly to the same treatment [125].

I.6. T-ALL MOUSE MODELS

Since there is a heterogeneous range of genetic alterations and targeted genes/pathways, several mouse models have been used to study T-cell leukemia/lymphoma, using different approaches, including carcinogen-induced, viral-induced, radiation-induced, transgenic, mosaic, knockout and xenograft models [135]. The, majority of the transgenic or knockout mouse models developed were based on naturally occurring genetic alterations identified in human T-ALL. In addition, one mouse model of T-ALL widely used was developed by transduction of BM cells with activated intracellular NOTCH1 (ICN1) and transplantation to lethally irradiated mice [136,137]. BM reconstituted mice develop aggressive leukemia, showing NOTCH1 oncogenicity when constitutively activated. Other studies, using transgenic murine models, targeted the overexpression of T-ALL oncogenes such as TAL1, TLX1, LMO2 and LYL1 to the T-cell lineage [138–141]. These mice develop T-ALL that models the pathogenesis of specific human molecular subgroups. Transgenic mice overexpressing oncogenes develop T-ALL with involvement of the thymus but leukemogenesis is somewhat delayed and often incompletely penetrant.

However, all the different models have contributed to the understanding of development and progression of T-cell leukemia [142].

I.6.1. TEL-JAK2 transgenic mice

In pediatric T-ALL, the juxtaposition between the *JAK2* gene and the Ets family member, *TEL/ETV6*, results in chromosomal translocation t (9; 12) (p24; p13) and gives rise to a constitutively active chimeric TEL-JAK2 protein [63,143].

TEL-JAK2 mice exhibit a selective expansion of CD8-positive aberrant T cells in blood, lymph nodes, spleen, thymus, BM and present non-hematopoietic organ invasion by leukemic T cells, which depends on the expression of TCR [63,143]. Direct expression of the TEL-JAK2 in the lymphoid lineage induces fast and fatal T-cell leukemia in mice (between 4 and 22 weeks of age).

However, in TJ2-Tg mice, the expression of the TEL-JAK2 fusion protein functions as the initial genetic "hit" in T-ALL initiation. Nevertheless, for these mice to develop T-ALL they need secondary genetic events [63,143].

This model is considered highly relevant to study human T-ALL because although JAK2 gene fusions, like TEL-JAK2 and PCM1-JAK2, are rare in T-ALL/LBL [144], the TJ2-Tg mice model T-ALL with JAK-STAT activation, which occurs across different molecular subgroups [145].

I.7. OBJECTIVES

The aim of this project is to determine the role of lymphotoxin signaling in the crosstalk between leukemic and microenvironmental cells. Since leukemic T cells express lymphotoxin proteins and inactivation of their receptor in mice delayed leukemogenesis, our lab wishes to understand how LTβR activation in stromal cells can facilitate T-cell leukemogenesis. To this end, I tested the hypothesis that lymphotoxin-expressing leukemic cells activate LTβR signaling in stromal cells.

To test whether LT-expressing leukemic cells can indeed activate LTβR in stromal cells we use an *in vitro* co-culture system. Our group has previously shown that primary leukemic T cells derived from TEL-JAK2 transgenic mice interacted *in vitro* with co-cultured LTβR-expressing fibroblasts [46] or stromal cells. Since the main signaling pathway activated by LTβR is that leading to NF-κB transcription factor activation [47], we aimed to verify whether fibroblasts or other mesenchymal cells display increased NF-κB activity upon contact with TEL-JAK2 leukemic cells. Thus, the specific aims of this work were:

i) Express in cell lines a reporter gene linked to an NF-κB promoter: The NIH3T3 fibroblast and MS-5 stromal cell lines were infected with a lentiviral NF-κB promoter reporter plasmid and then selected by fluorescent protein positivity and flow cytometry cell sorting.

ii) Validate the NF-κB reporter system: Functional activity of stable NIH3T3/NF-κB-Luc cells and MS-5/NF-κB-Luc cells was assessed by performing luciferase luminescence assays upon treatment with NF-κB inducers (LPS) and agonistic LTβR antibody as positive controls.

iii) Verify whether mesenchymal cells display increased NF-κB activity upon contact with TEL-JAK2 leukemic cells: Primary leukemic T cells from TEL-JAK2 transgenic mice were co-cultured with NIH3T3 and MS-5/NF-κB-Luc cells before luminescence detection.

iv) Verify whether NF-κB activation in mesenchymal cells occurs due to direct contact with leukemic cells or soluble factors: Harvested conditioned medium from NIH3T3 or MS-5 cell lines and primary leukemic T cells cultures alone or in co-culture were incubated with NIH3T3 or MS-5/NF-κB-Luc cell lines.

v) Validate whether leukemic cells activate NF-κB through LTβR in mesenchymal cells: Primary leukemic T cells from TEL-JAK2 transgenic mice were either co-cultured with

NIH3T3 or MS-5/NF-κB-Luc cell lines together with a soluble LTβR blocker or co-cultured with MEFs deficient or not in LTβR

v) Validate whether leukemic cells activate NF-κB through LTβR in mesenchymal cells: primary leukemic T cells from TEL-JAK2 transgenic mice are co-cultured with MEFs deficient LTβR

CHAPTER II - MATERIALS & METHODS

II.1. MOUSE EXPERIMENTATION

II.1.1. Mouse strains and breeding

Mice were maintained at the i3S Animal Facility under a Specific Pathogen Free (SPF) health status, with 12 h light/dark cycles and food and water *ad libitum*. Microorganism screening was regularly performed at the facility. The presence of *Klebsiella* opportunistic species was detected. All experimental procedures and personnel were authorized by the host institution, covered by *Direção Geral de Agricultura e Veterinária (DGAV)* and performed in tight accordance with recommendations from the European Commission (Directive 2010/63/UE) and Portuguese authorities (*Decreto-Lei n°113/2013*).

All mouse mutant strains were bred on the C57BL/6 genetic background. EμSRα-TEL-JAK2 transgenic (TJ2-Tg) mice, which express the TEL-JAK2 fusion protein specifically in lymphoid cells and develop T-cell leukemia [143], were provided by Dr. Jacques Ghysdael (*Institut Curie*, Orsay, France). Cohorts of TJ2-Tg mice were monitored for the development of TEL-JAK2-induced leukemia.

Ltbr knockout mice, in which the coding exons 1 to 5 of the *Ltbr* gene were targeted for inactivation [146], were provided by Jorge Caamaño (Institute of Biomedical Research, Birmingham, UK). LTβR heterozygotic ($Ltbr^{+/-}$) and knockout ($Ltbr^{-/-}$) embryos were used to prepare mouse embryonic fibroblasts (MEFs) (detailed below).

II.1.2. Monitoring of mice for signs of leukemia

TJ2-Tg mice develop leukemias and lymphomas spontaneously, with a median age of 12-14 weeks. All animals developing leukemia are characterized by marked splenomegaly, with lymphatic hypertrophy in most cases, as well as thymic hypertrophy.

Histologically TJ2-Tg mice with disease present massive infiltration of the thymus, spleen, lymph nodes and BM with leukemic cells. Besides, non-hematopoietic organs including the liver, brain, lungs, kidneys, adrenal gland, salivary gland, and ovaries may also be infiltrated with leukemic cells [143] To detect signs of disease, mice were monitored twice weekly or even daily for evaluation of the disease progression, thus not allowing animals to reach the severe level of the disease. More specifically, mice were monitored for signs of pain/discomfort due

to leukemia, such as weight loss, backwardly positioned ears, loss of tail tonicity, abdominal swelling (due to spleen and liver hypertrophy), and changes in respiratory rate/rhythm (dyspnea). In addition, by palpation or visual inspection, lymph node enlargement (lymphadenopathy) could be detected.

II.1.3. Euthanasia, necropsy

All animals manifesting signs of disease were euthanized by CO_2 inhalation. After death, the organs were dissected and collected for later analysis.

Collected organs at necropsy were held in cold phosphate-buffered saline solution (PBS) till weighing and sample processing. TJ2-Tg leukemic T cells were extracted from lymphoid organs (thymus, spleen or lymph nodes) using a mechanical method, namely by dissociation of the tissue through gentle compression against 70µm cell strainer (BD Biosciences) with a plunger and washing with cold PBS.

II.1.4. Embryo DNA collection

For DNA extraction, each mouse embryo's head we digested with 100µg/ml proteinase K (Fermentas) in 50µl of lysis buffer (200 mM NaCl [BDH Prolabo], 100 mM Tris-HCl, pH 8.3, 5 mM EDTA, 1% Triton X-100 [Sigma-Aldrich]), and incubating at 55ºC for 5 hours. Subsequently, the suspension was mixed by vortex, and the proteinase K was inactivated by 10 min incubation at 90ºC. The supernatant collected from 15 min centrifugation a14,000 rpm was transferred to a new microtube, and mixed with 500µl of isopropanol. The suspension was centrifuged for 10 minutes to collect the precipitated DNA. The DNA pellet was washed with 500 µl of 70% ethanol and the microtube was inverted several times to remove isopropanol from the DNA. Then, after 5 min centrifugation, the supernatant was discarded and the pellet was allowed to dry at room temperature. Lastly, DNA was resuspended in TE buffer, pH 7.4 (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8) and stored at 4ºC.

II.1.4.1. Genotyping by polymerase chain reaction

Polymerase chain reaction (PCR) on the extracted DNA was achieved in 25 µl volumes of the mixture containing 2 µl of DNA solution, GoTaq Flexi Buffer 1x, 1.5 µl of 50 mM $MgCl_2$, 0.5 µl of 10 mM PCR nucleotide mix, 0.7 µl of each primer (**Table II.4 and Table II.5**), 0.1 µl of 0.5 U GoTaq DNA polymerase (Promega) and UltraPure DNase/RNase-Free Distilled Water (Lifes Technologies). PCR amplification was performed on a Thermal Cycler (Bio-Rad), under the following conditions: initial denaturation at 94ºC for 5 min; 30 cycles of denaturation at 94ºC for 30 sec; annealing at 63ºC for 30 sec; extension at 72ºC for 1 min; a final extension step at 72ºC for 8 min was also performed. PCR products were analyzed by electrophoresis in a Greensafe Premium (NZYTech)-stained 1.5% agarose (Lonza) gel prepared in TBE buffer 1x (Life Science) and visualized under UV light (Chemidoc, Bio-Rad).

Table II.4: PCR primers used for embryo genotyping.

Target	Primer	Primer Sequence	Final Concentration for PCR reaction (µM)	TM (ºC)
LTβR	**LTβR 4**	5'CTG GTA TGG GGT TGA CAG CG 3'	0.35	63
	LTβR 7	5'TGT CAG CCG GGG ATG TCC TG 3'	0.35	63
	HSV-TK	5'ATT CGC CAA TGA CAA GAC GCT GG 3'	0.20	63

Table II.5: Expected PCR amplification products.

Allele	Primers	Expected band length (bp)
Wild-type	LTβR 4 + LTβR 7	640
Knockout LTβR	LTβR 4 + HSV-TK	200

II.2. CELL CULTURE

Culture of cell lines (MS-5, NIH3T3, HEK293T), primary TJ2-Tg leukemic T cells and primary mouse embryonic fibroblast (MEFs) was performed in a laminar flow cabinet (Airstream Class II Biohazard Safety Cabinet) under aseptic conditions. Cells were maintained in an incubator (Hera Cell, ThermoFisher Scientific) at 37ºC under a humidified atmosphere with 5% CO_2. The growth of cell cultures was monitored visually on an inverted light microscope (Leica) and by cell counting either on a Counter cell (Bio-Rad) or in a Neubauer chamber (Marienfeld).

II.2.1. Cell lines

The MS-5 murine BM stromal cell line [147], provided by Dr. Françoise Pflumio (CEA, Fontenay-aux-Roses, France), was cultivated in minimum essential medium alpha (αMEM medium; Gibco), supplemented with 10% heat-inactivated fetal bovine serum premium (FBS; Biowest), 2 mM L-glutamine (Lonza), 100 U/ml penicillin plus 100 µg/ml streptomycin (Alfagene) and 100 U/ml penicillin/streptomycin and 100 mM sodium pyruvate (Lonza). The NIH3T3 mouse embryo fibroblast cell line, human embryonic kidney cells 293 T (HEK293T), both provided by Jacques Ghysdael, were maintained in Dulbecco's modified eagle medium (DMEM), supplemented as described before. The cells were split and diluted 1/3 as they reached 70% confluency.

II.2.1.2. Cell cryopreservation and thawing

For cryopreservation of adherent MS-5, NIH3T3 and HEK293T cell lines, cultured cells were first rinsed with PBS, then detached from culture dishes by incubation in 0.05% Trypsin (Sigma-Aldrich) solution, and finally resuspended in supplemented medium in order to stop trypsin action. Then, 10 µl of cell suspensions were transferred to a Counter cell slide (Bio-Rad), and 0.8-1x10^6 cells were centrifuged at 300 g for 5 min. The cell pellet was resuspended in a freezing solution composed of 90% FBS and 10% DMSO (Merck) and preserved in 2 ml cryogenic vials (Fisher Scientific). These were placed in a freezing container (Thermo Scientific) at -80ºC for 24 h, and then transferred to a liquid nitrogen tank for long-term storage.

For cell thawing, all cell lines were subjected to the same protocol, described as follows: a cryotube containing frozen cells was quickly thawed at 37°C in a water bath. Then, the cell suspension was transferred to a 15 ml conical tube containing preheated complete culture medium and centrifuged at 300 g for 5 min (to discard the DMSO-containing supernatant) and plated in fresh medium. The resultant culture plates were incubated at 37ºC with 5% CO2.

II.2.2. Primary cultures

II.2.2.1. Primary TEL-JAK2 leukemic T cells

Primary TJ2-Tg cell suspension were collected as previously described (section **II.1.3.**). In the case of preparation of leukemic cell suspensions from the spleen, the red blood cells were lysed. Briefly, cells from dissociated spleens were transferred to 15 ml conical tubes and centrifuged at 300 *g* for 5 min at 4°C. After supernatant discard, the pellet was resuspended in 5 ml of red blood cell (RBC) lysis solution (composition in **Table II.8.**), and incubated for 5 min at room temperature (RT). Then, the samples were centrifuged at 300 *g* for 5 min at 4°C, the supernatant was discarded, and the cell pellets were resuspended in cold PBS. TJ2-Tg cells diluted in Trypan blue (Gibco) where counted using Neubauer chamber. Viable TJ2-Tg cells at $5x10^6$ cells/ml were cultured in isolation for a maximum of 3 days in RPMI medium supplemented with 10% FBS (Biowest), 2 mM L-glutamine (Lonza), 100 U/ml penicillin plus 100µg/ml streptomycin (Alfagene). Cell suspensions of $5x10^6$ TJ2-Tg were centrifuged (300 *g*, 5 min), and cryopreserved as described above.

II.2.2.2. Mouse embryonic fibroblast generation and culture

For the isolation of primary mouse embryonic fibroblast (MEFs), *Ltbr*$^{+/-}$ and *Ltbr*$^{-/-}$ embryos were collected from a pregnant female mouse on the embryonic day 13.5. Briefly, pregnant females were sacrificed by CO_2 inhalation, the ventral surface was cleaned with 70% ethanol, and a ventral incision was made. The uterine horns were removed from the peritoneal cavity, and the embryos were removed from the uterine horns. The placenta was removed and the embryos were transferred to a sterile culture dish with PBS. Each embryo was handled separately Internal organs were discarded. The head was removed and stored for genotyping by

PCR. The carcass was then transferred to a clean culture dish and washed five times with PBS, working in a laminar flow hood. Afterwards, the carcasses were transferred to sterile 10 cm diameter cell culture dishes with 5 ml of 0.25% Trypsin/0.53 mM EDTA (Gibco) and cut finely with a razor blade. The resulting tissue fragment suspension was homogenized by pipetting up and down six to seven times in order to break up the tissue, and then incubated at 37ºC for 15 min. Next, 5 ml of Trypsin/EDTA was added to the plate and repeatedly pipetted up and down followed by another 10 min incubation. Following homogenization, the cellular suspension was passed through a sterile 100 µm nylon cell strainer (BD Biosciences) to remove large pieces of undigested tissue. The cell strainer was rinsed with 5 ml of PBS and the permeated suspension was collected in the same plate. The cellular suspension was transferred to a 50 ml conical tube and centrifuged at 200 g for 8 min at room temperature. The supernatant was discarded and the cell pellet resuspended in MEF culture medium (complete DMEM media plus 0.1 mM 2-mercaptoethanol (Sigma Aldrich). The cellular content of each embryo was plated and incubated at 37ºC with 5% CO2. The medium was changed after six hours and after one day. The medium was changed every two days, and when a confluence of about 70% was reached, MEFs were either cryopreserved or subcultured. Primary MEFs were resuspended in freezing solution (80% FBS, 20% DMSO) at 2/9 cells per ml, and cryopreserved as described above.

II.2.2.3. Co-cultures

The culture of adherent cells (MS-5 cells, NIH3T3 or MEFs) together with cells cultured in suspension (primary T cell leukemic cells) are hereafter designated as co-cultures. Stromal cells, as MS-5, NIH3T3, MEFs or lentivirally-transduced derived cells were seeded at $5x10^5$ cells. About 18 h later, TJ2-Tg leukemic T cells ($1x10^6$ cells/ml) were added and co-cultured for 24 hours in triplicate, in fresh complete RPMI medium (Lonza) with or without heat-inactivated FBS for different conditions of conditioned medium.

II.2.2.4. Preparation of conditioned medium

The conditioned media were obtained from different sources, namely, from stromal luciferase reporter cell lines, TJ2-Tg primary cultures or from co-culturing both cell types. The stromal luciferase reporter cell lines were co-cultured with leukemic cells as described above.

Conditioned media was collected after either of two incubation periods, 6 and 24 h. As controls, conditioned media from luciferase reporter cell lines alone or TJ2-Tg primary cultures alone were also obtained. After each incubation period, the supernatant was collected and filtered through a 0.22 μm sterile filter and stored at -80°C. Six hour conditioned media were obtained from cells cultured either with FBS-free RPMI medium (designated as "6 h without FBS") or with complete RPMI medium (designated as "6 h"). Regarding conditioned media from 24 h of incubation, half of it was filtered and stored as described before and it was designated as "24h"; whereas the other half was subjected to a denaturation process, 10 min in boiling water, then filtered and stored (designated as "24 h-protein denaturation").

II.3. GENERATION OF CELL LINES WITH A STABLE REPORTER GENE

To test whether LT-expressing leukemic cells can activate LTβR in stromal cells we used an *in vitro* co-culture system by induction of a reporter gene linked to an NF-κB promoter [148]. The NIH3T3, MS-5 or MEF (described in section **II.2.2.2.**) cells were infected with lentiviral NF-κB promoter reporter plasmids as described below.

II.3.1. Production of lentiviral particles

The lentiviral vector system consists of: a transfer vector (pHAGE NF-kB-TA-LUC-UBC-eGFP-W (hereafter designated as NF-κB-Luc-eGFP) or NF-κB-TA-LUC-UBC-dTomato-W (hereafter designated as NF-κB-Luc-dTom), and three additional packaging plasmids pMDL-RRE, pREV and pMD2.G (VSV-G envelope expressing plasmid). The lentiviral plasmids NF-κB-Luc-eGFP) and NF-κB-Luc-dTom contain four copies of the NF-κB-binding consensus sequence. To allow tracking of transduced cells by flow cytometry, the plasmids constitutively express enhanced green fluorescent protein (eGFP) or dTomato (dTom) fluorescent proteins (**Figure II.9**) and were a gift from Darrell Kotton (Addgene plasmids # 49343 and #49335, respectively). Plasmid pRRE encodes a Gag-Pol precursor protein that is eventually processed into an integrase, reverse transcriptase, and structural proteins. Rev interacts with a cis-acting element (RRE) in the transfer vector enhancing export of unspliced, full-length genomic transcripts. The presence of VSV-G in the viral envelope membrane confers the viral particle with the ability to transduce a broad range of cell types. These plasmids were a gift from Didier

Trono (Addgene plasmid #12251, #12253, #12259). The plasmid CEP4-tat was used for increasing the efficient production and concentration of lentiviral vectors, this plasmid was a gift from Sergey Kasparov (Addgene plasmid # 22502). For production of lentiviral particles carrying NF-κB promoter reporter plasmids we followed the protocol of Tiscornia *et al.,* 2006 [149]. In detail, HEK293T cells were seeded $1x10^6$ cells/ml in complete DMEM as described above and incubated at 37°C overnight. On the day of transfection, the culture media was replaced 3 h prior to transfection and plasmid-mix was added with 2.5 M of $CaCl_2$. This mixture was composed by the following plasmids at the indicated concentrations (**Table II.8**):

Table II.6: Plasmids used to produce lentiviral particles carrying NF-κB promoter reporter.

Plasmid Name	Type vector	Bacterial Resistance	Reference	Final Concentration (µg)
pHAGE NF-κB-TA-LUC-UBC-eGFP-W	Lentiviral	Ampicillin	[148]	10
pHAGE NF-κB-TA-LUC-UBC-dTomato-W	Lentiviral	Ampicillin	[148]	10
pRRE (gag-pol)	Mammalian Expression, Lentiviral; Packaging	Ampicillin	[149]	5
pRev- (Rev)	Mammalian Expression, Lentiviral; Packaging	Ampicillin	[149]	1.5
pCEP4-tat (Tat)	Mammalian Expression	Ampicillin	[150]	1.5
pMD2.G(VSV-G envelope expressing plasmid)	Mammalian Expression, Lentiviral; Envelope	Ampicillin	[149]	2

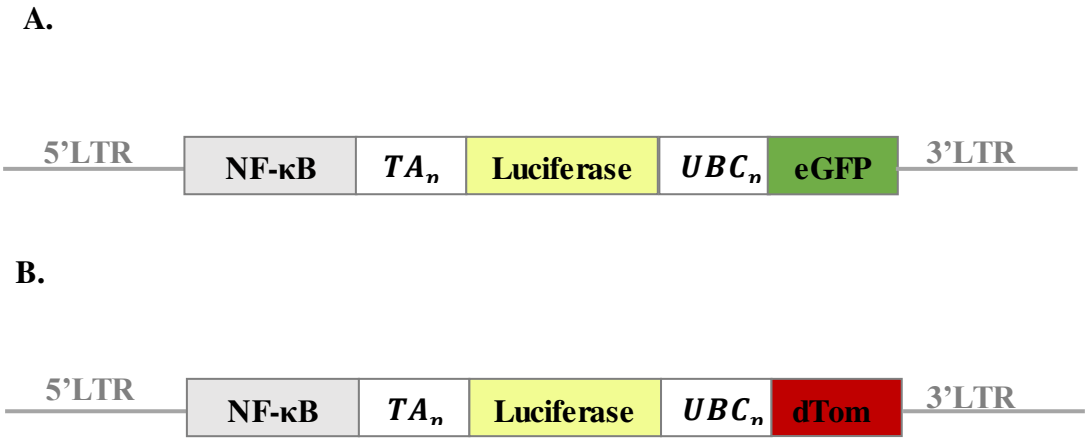


Figure II.9: Lentiviral NF-κB reporter vector. The NF-κB consensus sequence precede the minimal thymidine kinase promoter (TAp) of the herpes simplex virus. (**A.**)- Schematic of lenti-NF-κB-Luc-eGFP. (**B.**)- Schematic of lenti-NF-κB-Luc-dTom [148].

The plasmids were mixed by repeated pipetting, and then added drop-by-drop to a volume of 2x HEBS solution equal to the final volume of the mix and quenched for 10 min to allow the formation of a precipitate. Finally, this mix was added drop-by-drop to the cells. Cells were incubated at 37ºC. Twenty-four hours after transfection, the medium was replaced by fresh complete DMEM and the cells were incubated for another day. Forty-eight hours after transfection, the first viral supernatant was harvested and filtered, first through a 0.45µm sterile filter and then through a 0.22µm filter. The collected viral supernatant was stored at 4ºC. A second harvest was performed 72 h after transfection and filtered as described above. First and second collection supernatants were mixed and rapidly frozen in liquid nitrogen for storage at -80ºC.

II.3.1.1. Titration of lentiviral vectors and multiplicity of infection

The definition of a functional vector titer is the number of vector particles required to infect a cell [151]. The vector titers are usually determined by fluorescence activated cell sorting (FACS) analysis [151]. To determine the vector titers, we performed a ten-fold serial dilutions [from undiluted to a dilution of 10^{-3} (100, 10, 1 and 0.1µl) of the lentiviral preparation in DMEM culture medium. Then, 20 µl of each lentiviral dilution was added to each well containing previously plated 1 x10^5 NIH3T3 cells, and the cells were incubated at 37ºC. After 48 h, the percentage of fluorescent positive cells was detected by FACS. The biological titer (BT =

TU/ml, transduction units) was calculated according to the following formula: TU/µl = (P×N/100×V)×1/DF, where P = % eGFP positive cells or dTOM positive cells, N = number of cells at transduction time = 10^5, V = dilution volume added to each well = 20 µl and D = dilution factor =1 (undiluted), 10^{-1} (diluted 1/10), 10^{-2} (diluted 1/100), and 10^{-3} (diluted 1/1000) [149]. Additionally, the Multiplicity of Infection (MOI) is the number of transducing lentiviral particles per cell and this was calculated: (total number of cells per well) x (desired MOI) = total transducing units needed (TU) (total TU needed) / (TU/ml) = total ml of lentiviral particles to add to each well.

For each cell line, 2.5 x 10^5 cells were plated and incubated overnight at 37°C, the day before. The cells were transduced with lenti-NF-κB-luc-eGFP or lenti-NF-κB-luc-dTom with a MOI of 100 for NIH3T3 and MS-5, and MOI of 500 for MEFs.

II.3.2. Transduced NIH3T3 and MS-5 cells

The transduced NIH3T3 and MS-5 cells were subjected to flow cytometry cell sorting using FACS Aria II cell sorter (BD Biosystems) to select eGFP-positive or dTom-positive depending from the lentiviral vector used for transduction. In brief, 3 days after transduction, after removal of the medium and washing the cultured cells, 1 ml of 0.05% Trypsin (or Trypsin/EDTA 1x) solution was added to the cells, gently shaken, and incubated at 37ºC for 3-10 min until cell detachment. Next, cells were resuspended in complete culture medium to stop the trypsin action. Cells were centrifuged at 300 *g* for 5 min. The supernatant was carefully removed, the pellet resuspended in 1 ml of PBS + 1% FBS and placed on ice. Cells were immediately analyzed and sorted in the FACS Aria II. After the FACS sorting, the tube containing the sorted cells was centrifuged at 300 *g* for 5 min, the supernatant removed and the pellet was resuspended in complete αMEM medium. The cells were then plated, incubated at 37°C and 5% CO_2.

II.3.2. Transduced MEFs

To determine the percentage of transduced eGFP-positive MEFs prior to each co-culture experiment, the lentivirally infected MEFs were subjected to flow cytometry analysis. Upon subcultivation, a small volume of medium with MEFs was collected for flow cytometry. The

infected MEFs were centrifuged at 300 *g* for 5 min. The pellet was resuspended in 300 µl of 10 mM NaN_3 in PBS solution and filtered through a 35 µm nylon filter into a 5 ml round bottom polystyrene tube (BD Falcon). The nonviable cells were excluded from analyses by propidium iodide (PI; Sigma-Aldrich) staining.

II.4. CELL-CULTURE TREATMENTS

To evaluate the functional activity of the reporter constructs in stably transduced cell lines, treatments with inducers and inhibitors were performed. Thus, 5 x10^4 cells/well of each cell line (MS-5, MS-5/NF-κB-Luc-eGFP, NIH3T3, NIH3T3/NF-κB-Luc-eGFP, *Ltbr*$^{-/-}$MEFs (KO *Ltbr*), *Ltbr*$^{+/-}$MEFs, *Ltbr*$^{-/-}$MEFs/NF-κB-Luc-eGFP or *Ltbr*$^{+/-}$MEFs/NF-κB-Luc-eGFP) were plated and incubated at 37ºC overnight. The cells were treated in triplicate with either PBS (control), 1 mg/ml agonistic anti-mouse LTβR antibody (5G11; Serotec Bio-Rad), 1 mg/ml anti-IgG 2 (Biolegend) and 50 mg/ml LPS for 10 h. MEFs were only treated with 50 mg/ml LPS for 24 h. The samples were measured by luciferase assay (see section **II.5.**).

II.4.1. Co-cultures and LTβR ligand blocking

TJ2-Tg leukemic T cells (1x10^6 cells/ml) were co-cultured for 24 h in triplicate with confluent MS-5, MS-5/NF-κB-Luc-eGFP, NIH3T3, NIH3T3/NF-κB-Luc-eGFP or *Ltbr*$^{-/-}$ and *Ltbr*$^{+/-}$ MEFs (5x10^4 cells/well) in complete RPMI medium as previously described. Co-culture images were obtained using a Zoe microscope (Bio-Rad). To block LTβR signaling *in vitro*, 1 µg/ml of murine LTβR-hIgG (mLTβR-Fc) was added to co-cultures. The mLTβR-Fc fusion protein was provided by Biogen Idec (USA). The samples were measured by luciferase assays as described below (see section **II.5.**).

II.4.2. Cell line cultures with conditioned medium

Fifty thousand (5 x10^4) MS-5/NF-κB-luc-eGFP and NIH3T3/NF-κB-luc-eGFP cells were plated in αMEM or DMEM culture medium, respectively, and incubated at 37°C. One volume of conditioned medium, as prepared in **II.2.2.4**., was diluted with 3 volumes of complete RPMI

medium and added to the cultured stromal cells described above. After 24 h, cultures were harvested and samples submitted to luciferase assays.

II.5. LUCIFERASE REPORTER ASSAYS

Functional NF-κB activity in stable NIH3T3/NF-κB-Luc or MS-5/NF-κB-Luc cells was assessed by performing luciferase luminescence assays upon treatment with an NF-κB pathway activator (lipopolysaccharide; LPS-B5 Ultrapure, InvivoGen) or agonistic LTβR antibody (AC.H6 from Biogen Idec). Also, luciferase luminescence assays after co-cultures were performed to verify whether leukemic cells could activate the NF-κB reporter in NIH3T3 fibroblasts. For this, the culture medium was removed, the cells were washed with PBS, and lyzed after addition of 100 µl of lysis buffer to each well of 96-well. The cells were lyzed for 15 min at room temperature on a shaker and the lysates were subsequently stored at -20°C.

For luciferase measurements, 100 µl of luciferase buffer (composition in Table I.8.) was placed in each well. Then, 50 µl of luciferin were added. As a blank control, luminescence was measured at this stage. Then, 15 µl of each cell lysate was added to each well and the firefly luciferase luminescence activity was measured on a Synergy™ HT (Biotek) multiplate reader with BioTek's software (Gen5).

II. 6. FLOW CYTOMETRY IMMUNOSTAINING

II.6.1. Fluorescent protein detection in luciferase reporter-expressing cell lines

After transduction with lentiviral particles, the sorted cells maintained in culture were regularly (about every 3 weeks) subjected to eGFP detection by flow cytometry to verify whether the lentiviral NF-κB-Luc-eGFP incorporation was still maintained throughout cell passaging. A small volume, between 1 and 2 ml, of MS-5, MS-5/NF-κB-Luc-eGFP, NIH3T3 or NIH3T3/NF-κB-Luc-eGFP cells were collected and centrifuged for 300 g for 5 min at 4ºC. The supernatant was discarded and the pellet was resuspended in 300 µl of 10 mM NaN_3 in PBS solution and filtered through a 35 µm nylon filter into a 5 ml round bottom polystyrene tube (BD Falcon).

The cells were analyzed in a FACS Calibur Flow Cytometer (BD Biosciences) for fluorescent eGFP (channel FL1).

II.6.2. Detection of cell surface LTβR protein

LTβR was detected using a purified anti-mouse LTβR monoclonal antibody (clone 5G11; Biolegend). One x 10^6 cells collected from stromal cell cultures were centrifuged at 300 g for 5 min at 4ºC and were resuspended in cold FACS buffer (composition in **Table II.8**.). The single-cell suspension was again centrifuged at the same conditions and the supernatant was eliminated. Afterwards, for staining of the cell pellet, LTβR antibody previously diluted was added and incubated for 1 h, on ice in the dark. Thereafter, to stop incubation and remove unbound antibody, 1 ml of FACs buffer was added and cells centrifuged. The cell pellet was washed with FACs buffer. Subsequently, the cell pellet was resuspended in FACS buffer with secondary antibody, which was also used alone as a negative control. In cells which lentiviral reporter contain fluorescence eGFP, LTβR antibody was detected by secondary PE anti-rat IgGa, whereas in cells which lentiviral reporter contain fluorescence dTomato, LTβR antibody was detected by Donkey anti-rat IgG (H+L) secondary antibody, conjugated to Alexa 488 (ThermoFisher) (Table II.7.). Then, the cells were incubated for 1 h, on ice in the dark. The cells were washed twice and the supernatant was discarded. Finally, the cell pellet was resuspended in 1 ml of PBS/10 mM NaN_3 and the cells were analyzed in a Flow Cytometer. The data were analyzed using FlowJo version 10 software.

II.6.3. Thymocyte/T-cell marker analysis

One x 10^6 single-cell suspension from collected organs prepared by dissociation were resuspended in cold FACS buffer. Single-cell suspensions were centrifuged at 300 *g* for 5 min at 4ºC. The cells were stained with fluorochrome-labeled antibodies, in 50 µl of staining buffer, and incubated for 45 min on ice, in the dark. Fluorescein isothiocyanate (FITC)-, R-phycoerythrin (PE-) or PE-cyanine 5 (PE-Cy5)-conjugated antibodies specific for CD25 (PC61), CD4 (GK1.5) and CD8 (53-6.7) (BioLegend) were used (**Table II.7**). Then, cells were washed twice with 1 ml of cold FACs buffer and resuspended in 1 ml of 10 mM NaN_3 in PBS solution, and analyzed in a FACSCalibur flow cytometer (BD Biosciences). Isotype negative

controls and cells stained with each antibody individually were also prepared to adjust the cytometer settings and fluorescence compensations. To gate viable cells for analysis, staining with PI was used. Analyses were performed on FlowJo software version 10.

II.6.4. Detection of cell surface LTαβ protein

For leukemic T cells, the membrane-bound LTβR ligands were detected using the murine LTβR-hIgG (mLTβR-Fc), and visualized using PE-conjugated AffiniPure F(ab')2 fragment donkey anti-human IgG (H+L) (Jackson ImmunoResearch Laboratories) secondary antibody (**Table II.7.**), which was also used alone as a negative control. As negative control we also analyzed cells without antibody staining. We applied an adapted immunostaining protocol as the one described by Ansel and coworkers [152]. Briefly, a single-cell suspension containing 1×10^6 TJ2-Tg cells was transferred to each 5 ml round-bottom polystyrene tube (BD Falcon) and washed with 1 ml of ice-cold FACS solution. After washing, cells were centrifuged at 300 *g* for 5 min at 4ºC, the supernatant was discarded carefully and 100 µl of Fc-block (TruStain FCX, anti-CD16/32, Biolegend) in FACS buffer 1 µg/1x10^6 cells was added for 10 min on ice. Afterwards, 25 µl of mLTβR-hIgG was added directly to the Fc-block. After 45 min incubation on ice, the cells were washed twice with FACS solution. Then, the cells were resuspended in 50 µl of 2.5 µg/ml donkey anti-human secondary antibody and incubated for 30 min on ice, in the dark. All antibodies were prepared in 2% normal mouse serum (NMS; Jackson Immunoresearch Laboratories) in PBS to prevent non-specific staining. To stop incubation, 1 ml of FACS buffer was added and cells were centrifuged at 300 *g* for 5 min at 4ºC. Supernatant was discarded. Then, the cells were washed twice with FACS solution and resuspended in 1 ml of 10 mM NaN3 in PBS solution. For PI staining, the cells were washed with FACS buffer and 1 ml of PBS/10 mM NaN_3 and 2.5 µl of 1 mg/ml PI solution were added. Finally, the tubes were agitated and the cells analyzed in a flow cytometer. Negative controls and cells stained with each antibody individually were also prepared to adjust the FACS instrument settings and fluorochrome compensation.

Table II.7: Antibodies used for flow cytometry analyses of T-cell markers and LTβR.

Mouse Antigen	Target species	Antibody	Clone	Fluorochrome	Concentration (mg/ml)	Dilution
Purified anti-mouse LTβR	Mu	Rat IgG2a, k	5G11	--	1	1/200
CD4	Mu	Rat IgG2b, κ	GK1.5	PE	0.5	1/200
CD8	Mu	Rat IgG2b, κ	53-6.7	PE/Cy5	0.2	1/600
CD25	Mu	Rat IgG1, λ	PC61	FITC	0.5	1/400
Secondary Antibody						
Donkey anti-Rat IgG (H+L)	Rat	IgG	Polyclonal	Alexa 488	0.1	1/1000
Anti-rat IgG2a	Rat	Mouse IgG	MRG2a-83	PE	0.2	1/200
Donkey anti-human IgG	Human	IgG (H+L)	Polyclonal	R-PE	0.25	1/200

II.7. STATISTICS

Statistical analyses were performed using GraphPad Prism 6 software. Statistical tests were used as indicated in figure legends. Samples were assumed to have Gaussian distribution and parametric unpaired or paired t-test were applied, as described in figure legends. A *P*-value below 0.05 was considered statistically significant and the asterisk (*) denotes the p-value, as follows: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; **** $P \leq 0.0001$.

Table II.8:The solutions used in this work.

PB sample treatment	**RBC Lysis Solution pH7.2**	160 mM NH_4Cl	Sigma-Aldrich
		170 mM Tris-HCl, pH 7.4	Sigma -Aldrich
		Mix 1 volume of 170mM Tris-HCl with 9 volumes of 160 mM NH_4Cl	
Flow Cytometry	**FACs Solution**	PBS	Sigma-Aldrich
		3% FBS	PAA
		10 mM NaN_3	Sigma-Aldrich
Luciferase Reporter Assay	**Passive lysis buffer, 5x**		Promega
	0.3 mg/ml D-Luciferin		Promega
	Luciferase assay buffer	1 M $MgSO_4$	Sigma-Aldrich
		1 M KPO_4 pH 7.8	Sigma-Aldrich
		0.1 M EGTA pH 7.8	Sigma-Aldrich
		ATP 100 mM	Sigma-Aldrich
		DTT 1M	Sigma-Aldrich
Agarose Electrophoresis	**TBE Buffer, pH 8.0**	0.089 M Tris Base	
		0.089 M Borate	VWR
		0.002 M de EDTA	

CHAPTER III - RESULTS

III.1. GENERATION OF STROMAL CELL LINES EXPRESSING A LUCIFERASE REPORTER GENE LINKED AN NF-κB PROMOTER

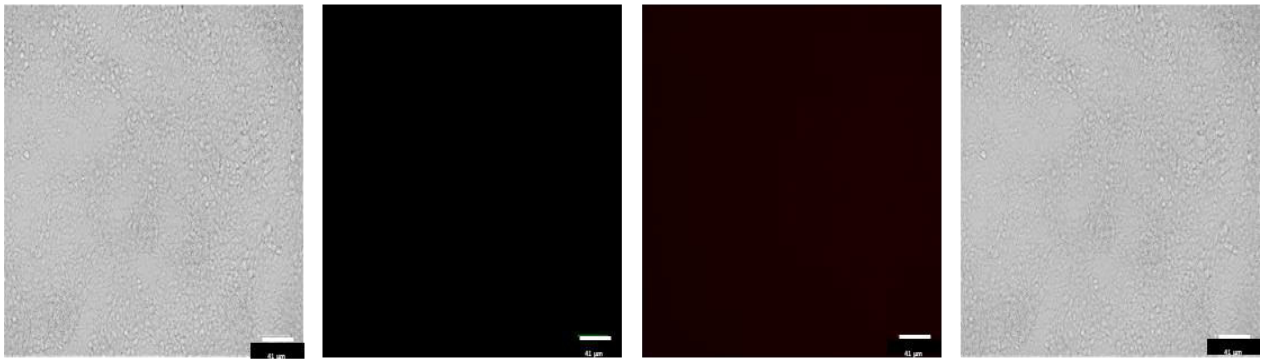
III.1.1. NF-κB promoter reporter plasmid lentiviral transduction of NIH3T3 and MS-5 cell lines

To test whether LT-expressing leukemic cells can indeed activate LTβR in stromal cells, we decided to use an *in vitro* co-culture system. Instead of assessing directly LTβR activation, we assessed NF-κB activation, which occurs downstream of LTβR [89]. In order to detect NF-κB activation, we transduced adherent cell lines with a lentiviral NF-κB -Luc promoter reporter plasmid [148]. The reporter vectors we used are characterized by the presence of an NF-κB-inducible promoter fused to a minimal thymidine kinase promoter (TAp) driving expression of the firefly luciferase reporter gene. This construct enables quantification of NF-κB activation in cells by measurement of luciferase activity. In addition, the plasmids contain a constitutively active promoter, the ubiquitin C (UBC) promoter, driving the expression of genes encoding fluorescent proteins, either eGFP or dTomato. These sequences allow the tracking and sorting of transduced cells by flow cytometry.

Lentiviral particles carrying the NF-κB promoter reporter plasmids were obtained through transfection of the HEK293T cell line (**Figure III.10: A. and D.**). This cell line was transfected as described in Materials and methods. Efficiency of transfection of: pHAGE NF-κB-Luc-eGFP plasmid (**Figure III.10: B. and E.**) or pHAGE NF-κB-Luc-dTomato plasmid (**Figure III.10: C. and F.**), as assessed by the proportion of cells expressing eGFP or dTomato fluorescence proteins, was high. Cell media containing lentiviral particles was used to infect the NIH3T3 and MS-5 cell lines. After lentiviral infection, between 25% a 35% of transduced NIH3T3 and MS-5 cell lines constitutively expressed eGFP or dTomato, accordinly to the fluorescent protein encoded in the lentiviral vector (data not shown). Fluorescent cells were then sorted by flow cytometry and cultured to maintain only transfected cells.

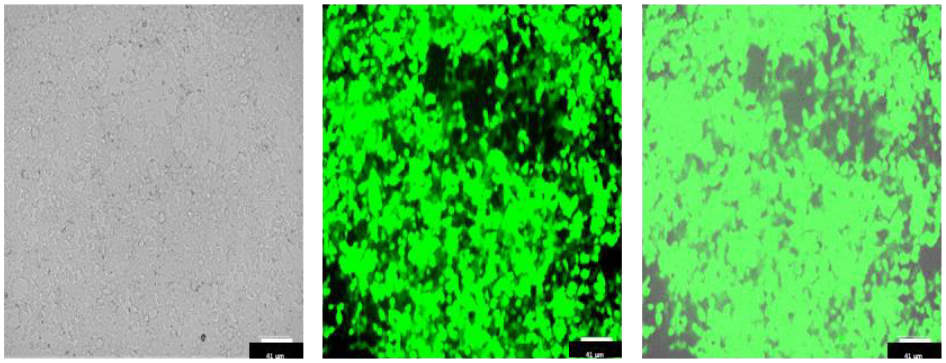
I. Harvest 1st viral supernatant

A. Negative Control – only HEK 293 T cells



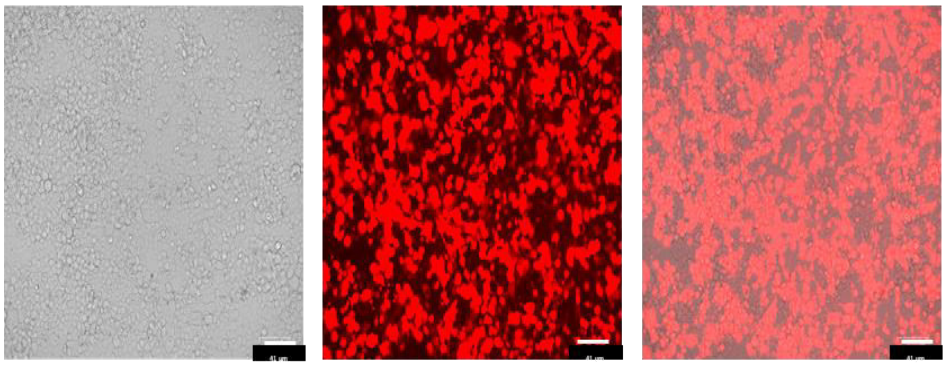
Brightfield Green Red Merge

B. HEK 293 T cells transfected with lentiviral -NF-κB-luc-eGFP



Brightfield Green Merge

C. HEK 293 T cells transfected with lentiviral -NF-κB-Luc-dTom



Brightfield Red Merge

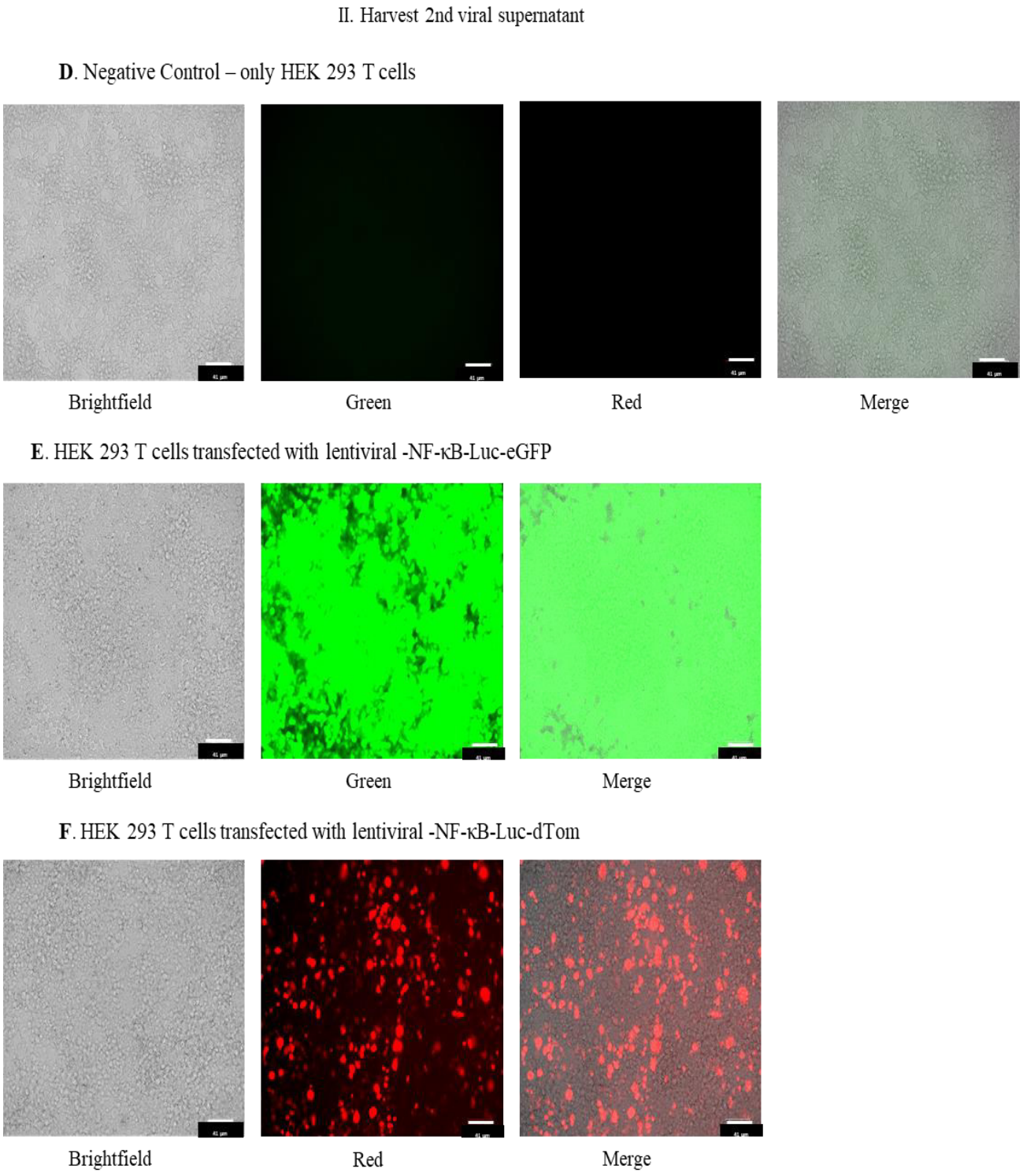


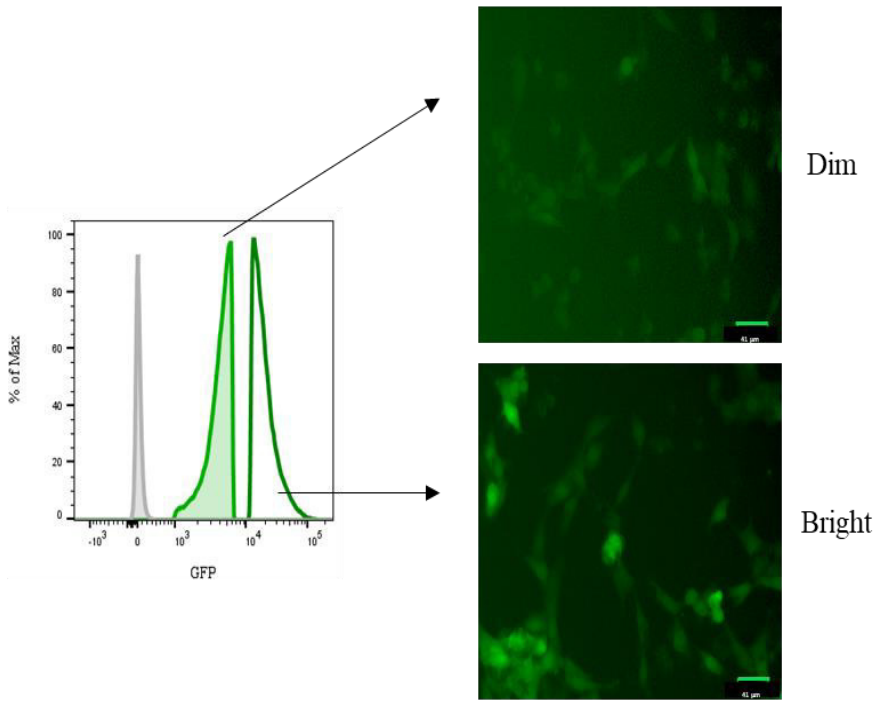
Figure III.10: Production of lentiviral vector NF-κB-Luc in HEK293T. The HEK293T cell line was infected with NF-κB-Luc-eGFP reporter (channel- **Green**) or NF-κB-Luc-dTom reporter (channel- **Red**). Microphotograph from harvests first viral supernatant (**I.- A. -C.**) or second viral supernatant (**II.- D.** - **F.**).

The NIH3T3 cell line after pHAGE-NF-κB-Luc-eGFP lentiviral transduction was characterized by 2 subpopulations with different levels of eGFP expression (data not shown). After sorting

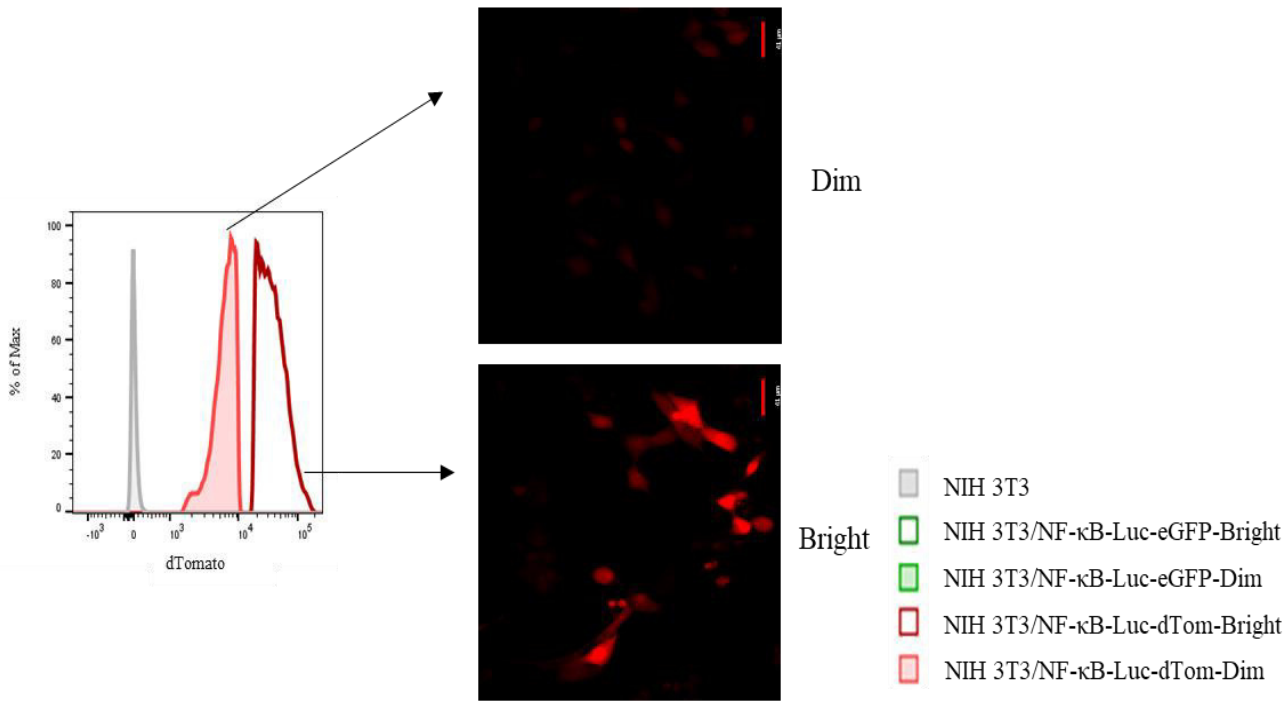
by flow cytometry, we obtained a population expressing high levels of eGFP fluorescence, being designated as NIH3T3/NF-κB-Luc-eGFP-Bright, and a population with lower fluorescence levels and designated as NIH3T3/NF-κB-Luc-eGFP-Dim (**Figure III.11: A.**). Similarly, NIH3T3 populations derived from transduction with pHAGE-NF-κB-Luc-dTomato lentiviruses were sorted by fluorescence intensity and designated as NIH3T3/NF-κB-Luc-dTom-Bright or NIH3T3/NF-κB-Luc-dTom-Dim (**Figure III.11: B.**). Each cell population was cultured as independent cell lines.

Regarding the MS-5 cell line, only an eGFP-positive population was observed, which upon sorting was designated as MS-5/NF-κB-Luc-eGFP (**Figure III.10: C.**). Likewise, a single positive MS-5 population for the dTomato fluorescent protein was observed, which upon cell sorting, was consequently designated as MS-5/NF-κB-Luc-dTom **Figure III.11: D.**).In sum, with these experiments we obtained different cell lines with stable expression of NF-κB reporter genes.

A. NIH 3T3/NF-κB-Luc-eGFP



B. NIH 3T3/NF-κB-Luc-dTom



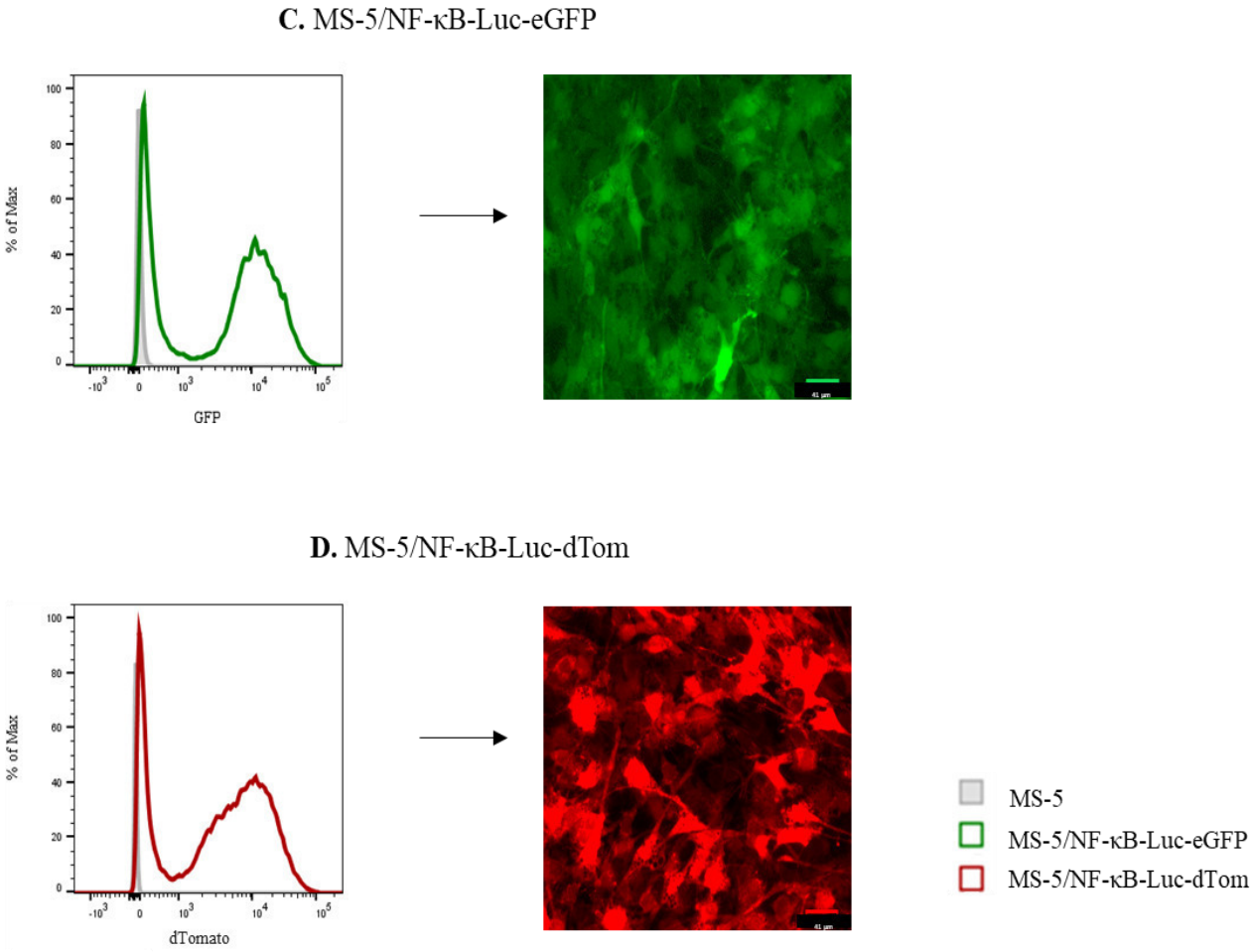


Figure III.11: Generation of stable NF-κB-Luc reporter stroma cell lines. The graphics refer to sorted cells in different populations depending of fluorescent level – Dim (lower fluorescent) or Bright (high fluorescent). Microphotograph from sorted fluorescent positive cells, generated from NIH3T3 cells (**A.** and **B.**) or MS-5 (**C.** and **D.**), previously transduced with a lentiviral vector - NF-κB-Luc-eGFP (**A.** and **C.**) or NF-κB-Luc-dTom (**B.** and **D.**)

III.1.2. Lentiviral transduction and cell sorting did not affect LTβR expression on NIH3T3 and MS-5 cell line

MS-5 and NIH3T3 cells normally express LTβR (see below), so we evaluated whether lentiviral vector transduction and cell sorting did not affect LTBR expression on the generated NF-κB reporter cell lines. LTβR was detected using a purified anti-mouse LTβR monoclonal antibody conjugated with PE anti-rat IgG2a secondary antibody. As negative control we analyzed only cells or cells with addition of this antibody. For optimization of this protocol, different dilutions of either the primary antibody (anti-mouse LTβR) or the secondary antibody were tested. Thus,

the anti-mouse LTβR was tested for a 1/50, 1/100 and 1/200 dilution using NIH3T3/NF-κB-Luc-dTOM-Dim and NIH3T3/NF-κB-Luc-dTOM-Bright cells. The negative control refers to only cells (no antibody). In this case, the secondary antibody was used at a dilution of 1/1000. We found that even at the most diluted concentration (1/200) the primary antibody was efficient in detecting LTβR on the surface of NIH3T3 cells (**Figure III.12: A.** and **B.**), so this dilution was chosen for the following experiments. The best dilution (1/100, 1/200, 1/500 or 1/1000) for the secondary antibody was tested using the NIH3T3/NF-κB-Luc-eGFP-Bright cell line. It was found that for lower concentrations (dilution 1/500 and 1/1000) a lower fluorescence level was detected, almost superimposed to the negative control (**Figure III.12: C**). The level of detection was improved with dilution of 1/200 and 1/100. The 1/200 dilution was chosen for the next experiments.

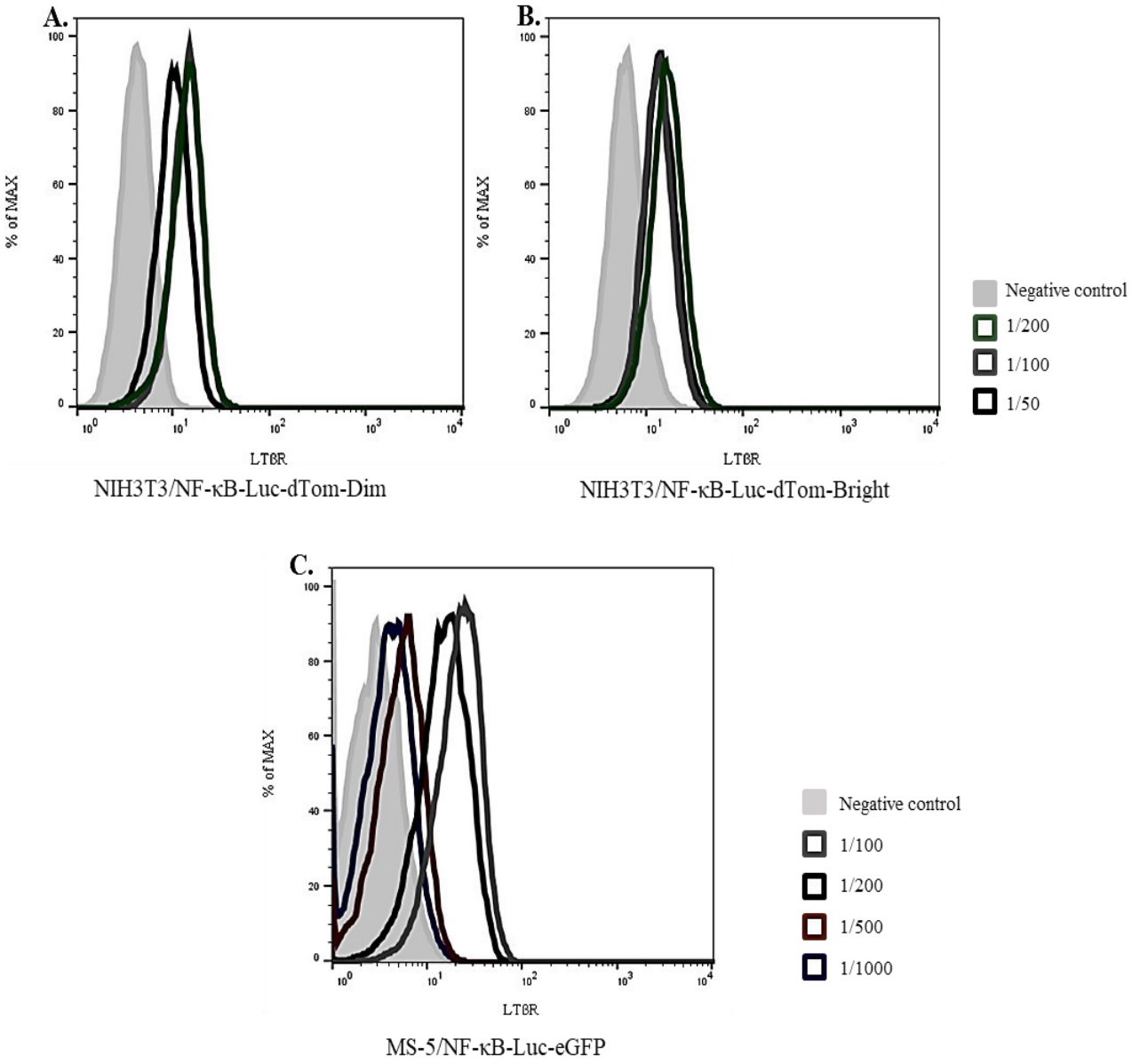


Figure III.12: Optimization of primary and secondary antibody dilution. The NIH3T3/NF-κB-Luc-dTom-Dim (**A.**) or NIH3T3/NF-κB-Luc-dTom-Bright (**B.**) were stained with anti-mouse LTβR and Donkey anti-rat IgG (H+L) secondary antibody, Alexa Fluor 488 at 1/1000. The anti-mouse LTβR was tested for different concentrations: dilution of 1/50 (black), dilution of 1/100 (dark gray) and dilution of 1/200 (dark green). (**C.**) MS-5/NF-κB-Luc-eGFP-Bright was stained with anti-mouse LTβR 5G11 (1/200) and PE-conjugated PE anti-rat IgG2a with different dilutions: 1/100 (dark green), 1/200 (black), 1/500 (dark red) or 1/1000 (dark blue). The negative control refers to only cells - no antibody (shading).

Then, to confirm that the lentiviral vector infection, flow cytometry sorting and culture maintenance did not affect LTβR expression, the following cell lines were immunolabeled with anti-mouse LTβR at a dilution 1/200 conjugated to the secondary antibody PE anti-rat IgG2a also at a final dilution of 1/200: NIH3T3/NF-κB-Luc-dTom-Bright (**Figure III.13: A.**),

NIH3T3/NF-κB-Luc-dTom-Dim (**Figure III.13: B.**), parental MS-5 (**Figure III.13: C.**) and MS-5/NF-κB-Luc-eGFP-Bright (**Figure III.13: D.**). The negative control refers to only cells (no antibody). Flow cytometry analysis showed similar expression levels among all cell lines, thus indicating that the lentiviral transduction and sorting of NIH3T3 and MS-5 cell lines did not affect LTβR expression.

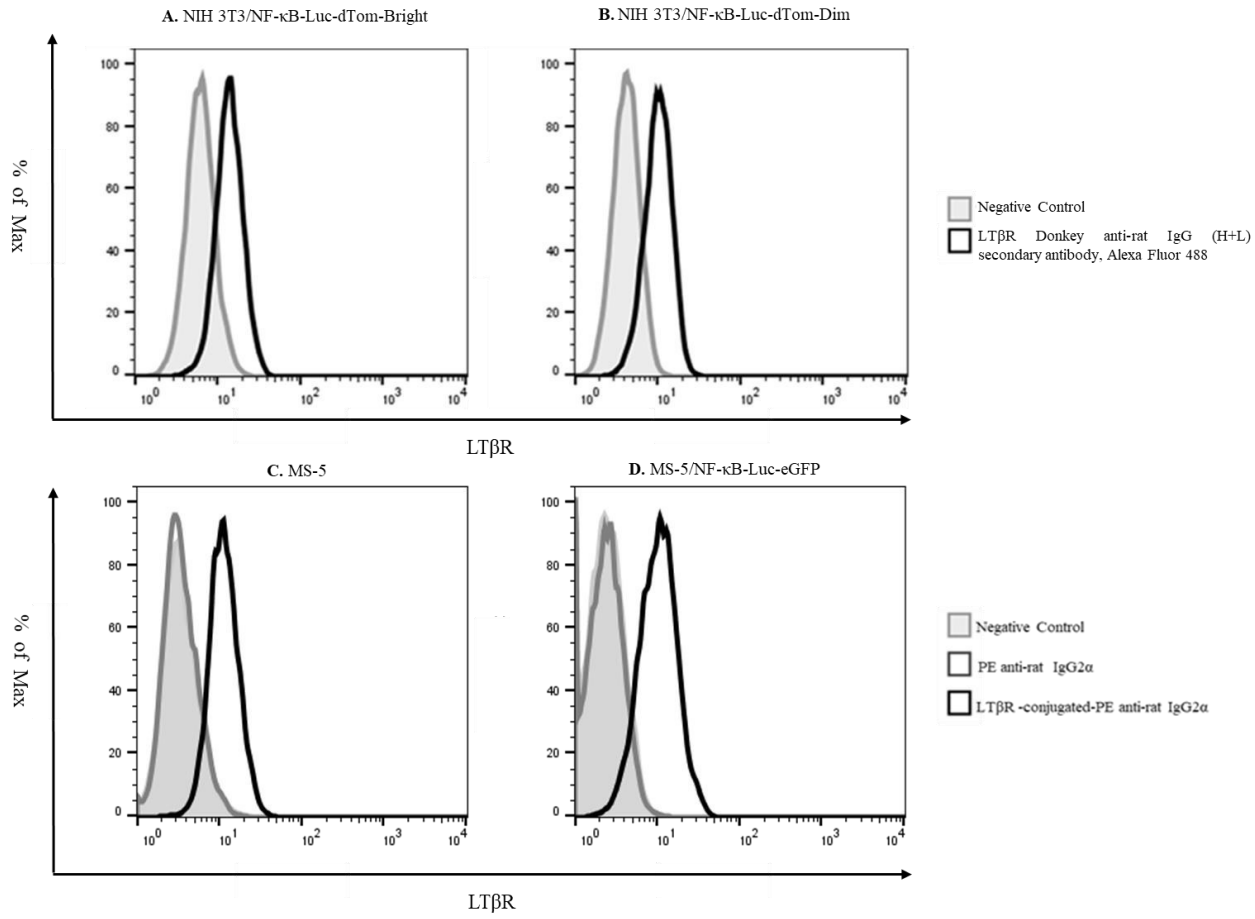


Figure III.13:Reporter cell lines express normal levels of surface LTβR. NIH3T3/NF-κB-Luc-dTom-Bright (**A.**) and NIH3T3/NF-κB-Luc-dTom-Dim (**B.**) express surface LTβR. Fibroblasts cells stained with anti-mouse LTβR 5G11 (1/200) conjugated with Donkey anti-rat IgG (H+L) secondary antibody, Alexa Fluor 488 (Thermos Fisher) at 1/1000. (**D.**) MS-5/NF-κB-Luc-eGFP express surface LTβR. This expression was not affected by transduction of lentiviral NF-κB-Luc-eGFP, comparing the LTβR of the cells transfected with MS-5 (**C.**), respectively. Stromal cells stained with anti-mouse LTβR 5G11 (1/200) and PE-conjugated PE anti-rat IgG2a (1/200). The negative control refers to only cells – no antibody (shading) or cells with only PE-conjugated PE anti-rat IgG2a secondary antibody (dark gray).

In sum, these results show that after transduction and cell sorting the obtained reporter cell lines maintained normal expression levels of our receptor-of-interest (LTβR), and that reporter constructs were maintained stably throughout our experiments.

III.1.3. Validation of the *in vitro* NF-κB reporter system by LPS stimulation

In our transduced cell lines, luciferase activity is a reporter system to monitor the activation of NF-κB transcription factors. To test if luciferase activity could be induced in the stable cell lines, we stimulated them with LPS. Cell stimulation by LPS was tested under different conditions: different concentrations (with 0.5, 5 and 50 µg/ml), duration of stimulation (6, 8 and 10 h) and different concentrations of FBS (0.1 or 10%). However, there was no significant activation of the NF-kB reporter for less than 10 h of stimulation and concentrations lower than 50 µg/ml (data not shown). When we performed a treatment for 10 h with a concentration of 50 µg/ml of LPS, relative luminescence increased 4.9-fold (4.923 ± 0.8300, n=2) in comparison to cells without treatment (1.000± 0.1290, n=2) (**Figure III.14: A.)**. This difference was statistically significant increase of the activity of NF-κB (*P*=0.0011). All the experiments of 50 µg/ml of LPS stimulation for 10 hours in NIH3T3/NF-κB-Luc-dTom-Bright cell line are summarized in **Figure III.14: B**. With this result, we conclude that our NF-κB reporter *in vitro* system was functional, and could be used for further experiments.

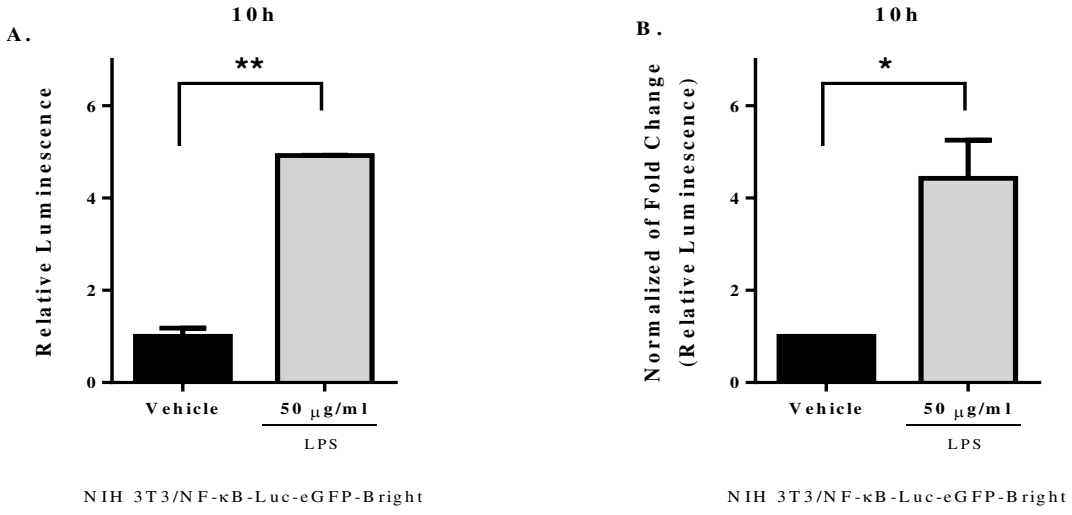


Figure III.14: Lipopolysaccharide activates NF-κB on NIH3T3/NF-κB-Luc-eGFP cells. Relative luminescence measurements on NIH3T3/NF-κB-Luc-eGFP-Bright reporter cell line not treated (vehicle -black) or treated with 50 µg/ml of LPS (gray) in complete medium for 10 hours. (**A.**): The graphic on the left represent

the relative luminescence of an each representative experiment, each performed in duplicate (standard error and mean represented by bar). The results were statistically significant (**) *P* value=0.011≤0.05. (Unpaired *t*-test performed experiment). (**B.**): The graphic on the right represent the luciferase activity of all experiments performed (4 independent experiments). The luciferase activity values were normalized, and fold change calculated in comparison to untreated cells (vehicle). The results were statistically significant (*) *p* value = 0.0258 ≤0.01. (Paired *t*-test. Standard error and mean represented by bar).

Since we had generated different NIH3T3 NF-κB reporter cell lines, we also tested whether all would respond to LPS stimulation.

Four different NIH3T3 reporter cell lines (NIH3T3/NF-κB-Luc-eGFP-Dim, -eGFP-Bright; -dTom-Dim and -dTom-Bright) were subjected to 50 µg/ml LPS stimulation for 10 h. All transfected NIH3T3 cells stimulated by LPS activated the NF-κB promoter reporter (**Figure III.14**). Therefore, for further experiments we used the NIH3T3/NF-κB-Luc-eGFP-Bright cell line.

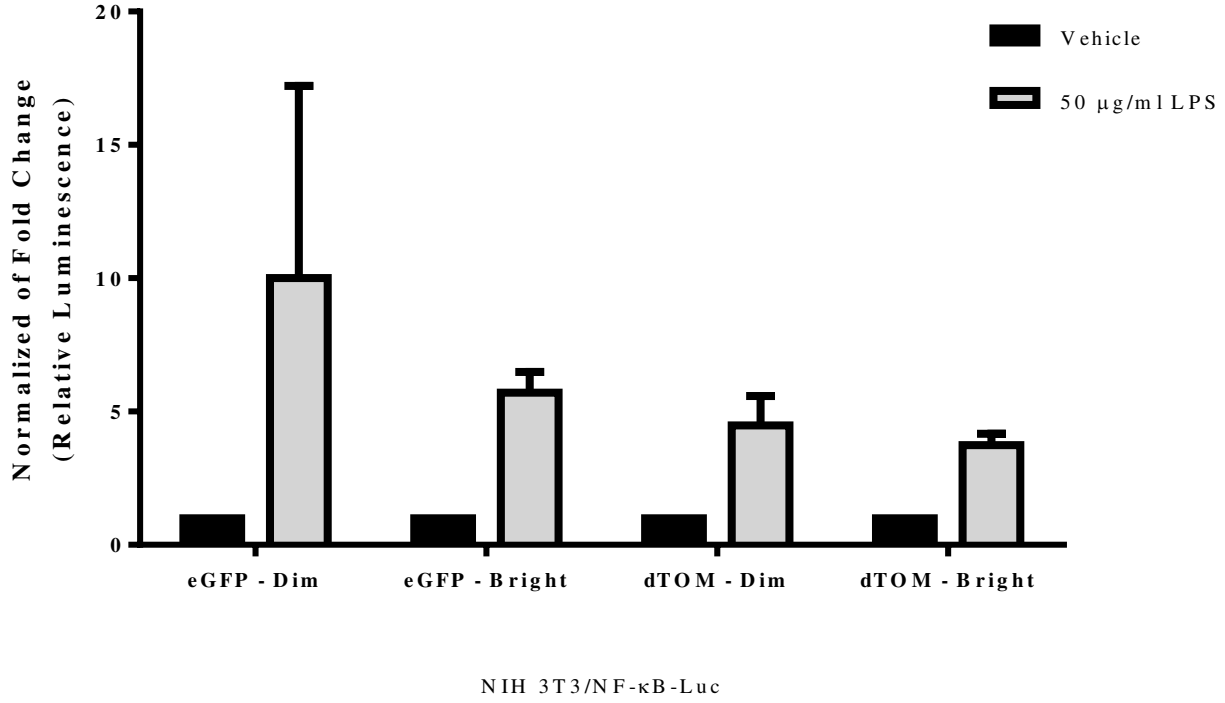


Figure III.15: Relative luminescence measurements on different reporter cells line. NIH3T3/NF-κB-Luc-eGFP-Dim, -eGFP-Bright, -dTOM-Dim or -dTOM-Bright cells were treated with 50 µg/ml of LPS for 10 h. Vehicle refer to untreated cells (n=2 independent experiments; standard error of mean represented by bar).

III.1.4. Activation of the *in vitro* NF-κB reporter system by agonist LTβR antibody

Since both NIH3T3 and MS-5 cell lines express LTβR, we set out to determine whether LTβR stimulation led to activation of the NF-κB reporter. Thus, we stimulated cells with an agonistic LTβR antibody that mimics ligand binding and receptor aggregation [153,154]. We performed several agonist LTβR antibody stimulation experiments, (three technical replicates per experiment), and then measured the induction of luciferase activity (**Figure III.16: A.**). In short, we found that in three out of five experiments the NF-κB pathway was activated in NIH3T3/NF-κB-Luc-eGFP-Bright cells (**Figure III.16: B.**). In contrast, NF-κB activation was detected in only one out of four experiments performed with MS-5/NF-κB-Luc-eGFP cells (**Figure III.16: C. and D.,** respectively). All experiments show, either with MS-5 or NIH3T3, were performed with same conditions: same medium culture for each cell line, antibody's incubation time and same luciferase assay. Therefore, although NF- κB could be activated in NIH3T3 derived-cells by direct LTβR stimulation, the results with MS-5/NF-κB-Luc-eGFP cells were not conclusive. Further assays are required to understand the differences in cell line.

To formally confirm whether the increase in the relative luminescence induced by the agonist LTβR antibody treatment was due to activation of the NF-κB signaling pathway, we used BMS-344501, an IKK kinase inhibitor. We would expect the inhibitor to prevent the phosphorylation of IkBα and consequently the NF-κB signaling pathway, but no or counterintuitive effects were observed (data not shown).

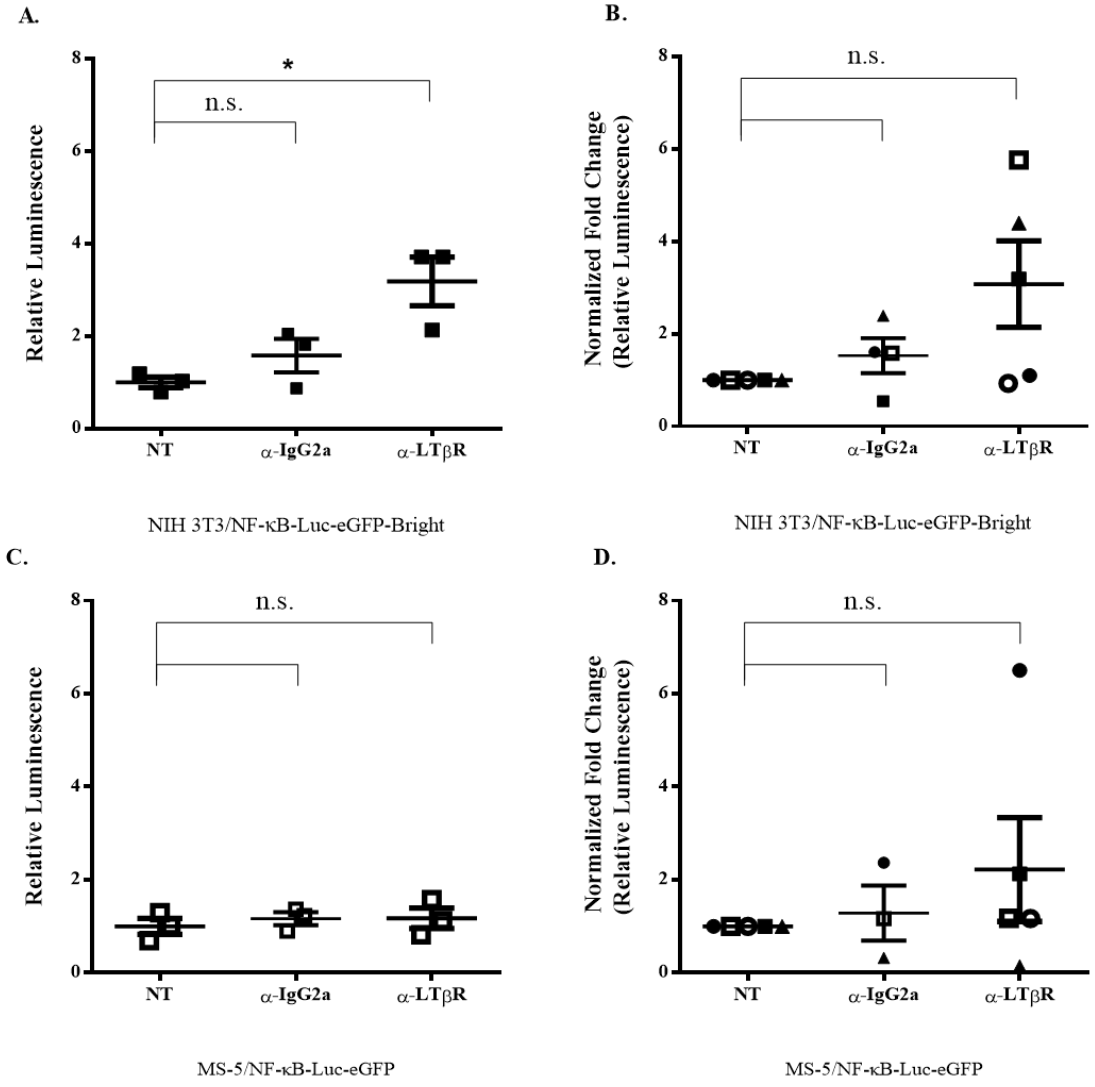


Figure III.16: Stimulation of NIH3T3 and MS-5 derived cells with agonist LTβR antibody. NIH3T3/NF-κB-Luc-eGFP-Bright or MS-5/NF-κB-Luc-eGFP cells were treated with 1 μg/ml of agonist LTβR antibody for 16 h, left untreated or treated with control isotypic antibody (1μg/ml of α-IgG2a). The graphics on the left, (**A.**) and (**C.**), respectively NIH3T3/NF-κB-Luc-eGFP-Bright or MS-5/NF-κB-Luc-eGFP, represent the relative luminescence of each triplicate of only independent experiment. The results in graph **A.** were statistically significant (*) p value = 0.0154 $\leq$ 0.05, and in graph **C.** the results were not statistically significant (n.s.) (Unpaired t-test performed all, standard error of mean represented by bar). The graphics on the right, (**B.**) and (**D.**), represent the luciferase activity all experiments, n=5 or n=4 independent experiments were performed respectively. The luciferase activity values were normalized, and fold change calculated in comparison to untreated cells (NT). The results in graphics (**B.**) and (**D**.) were not statistically significant (n.s.) (Paired t-test performed all, standard error of mean represented by bar).

III.2. LEUKEMIC T CELLS ACTIVATE NF-KB ON STROMAL CELL LINES

III.2.1. Leukemic T cells from TEL-JAK2 transgenic mice express LTβR ligands

LTβR ligands have been previously shown to be expressed in leukemic cells from TJ2-Tg mice [46]. Hence, we decided to confirm that TJ2-Tg leukemic cells expressed LTα1β2. To this end, we used LTβR-Fc, a fusion protein that binds both LTα1β2 and LIGHT. Fernandes *et al.,* 2015 [46] showed that surface expression of LTα1β2 was only detectable in ex vivo culture or upon stimulation with PMA+Iono. Phorbol myristate acetate (PMA) acts at the level of protein kinase C (PKC) which activate the NF-κB pathway, and ionomycin (Iono) acts at the level of the calcium cascade activating the calcineurin/NFAT pathway [155,156]. Then, we stimulated leukemic cells with PMA plus Iono for 24 hours, followed by analysis of LTβR ligands expression by flow cytometry.

Our results confirmed the results obtained by Fernandes *et al.,* 2015 [46], that under culture conditions (ex vivo) leukemic cells express the LTβR ligands (**Figure III.17: A.**). Expression of LTβR ligands was also detected in leukemic cells stimulated with PMA plus Iono, but without much increase as compared to non-stimulated cultured cells (**Figure III.17: B.**). Nevertheless, we confirmed that TJ2-Tg leukemic cells expressed LTβR ligands (note that the previously reported data Fernandes *et al.,* 2015 [46], were obtained in another research institution), so those cells can present them to stromal cells expressing the receptor and potentially activate it.

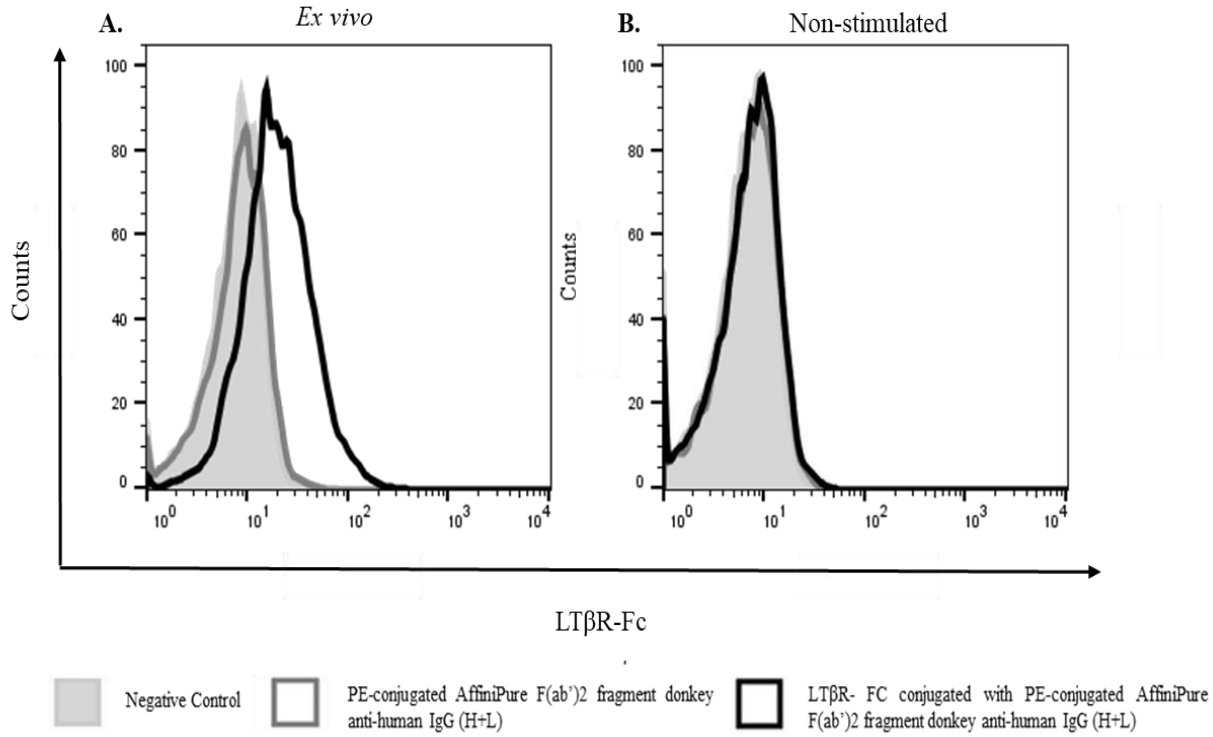
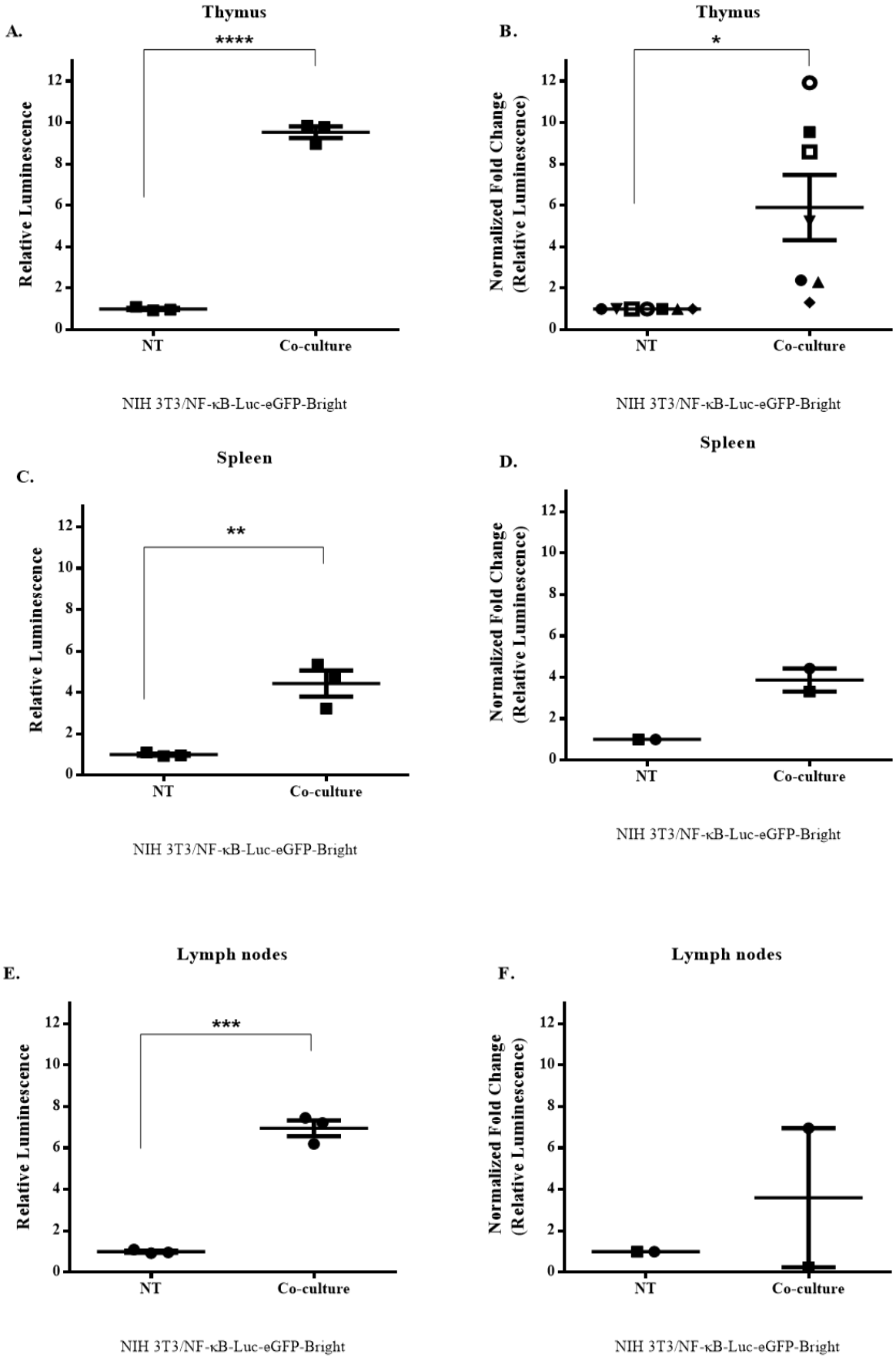


Figure III.17: LTβR ligand expression at the surface of TJ2-Tg leukemic cells. Flow cytometry immunostaining of LTβR ligands(black) on representative TJ2-Tg leukemic cells with LTβR-Fc protein on cultured ex vivo for 24 h (**A.**), (B.) non-stimulated cultured cells. The only cells, no antibody (gray shading) and secondary antibody alone (PE-conjugated AffiniPure F(ab')2 fragment donkey anti-human IgG (H+L) (dark gray) were used as negative controls for LTβR-Fc.

III.2.2. Activation of the NF-κB luciferase reporter in stromal cell lines co-cultured with leukemic cells

Our group has previously shown that primary leukemic T cells derived from TEL-JAK2 transgenic mice lose the LTα1β2 surface expression when in contact with LTβR-expressing fibroblasts, but not with LTβR-deficient fibroblast [46]. These data indicated that LT expressed in leukemic cells interacts with LTβR in other cells, but did not show that this resulted in receptor activation. Since the NF-κB signaling pathway is activated by LTβR [47], we first aimed to verify whether stromal cells displayed increased NF-κB activity upon contact with TEL-JAK2 leukemic cells. Thus, primary leukemic T cells collected from different affected organs of diseased TEL-JAK2 transgenic mice were co-cultured with NIH3T3/NF-κB-Luc-eGFP-Bright (**Figure III.18:** from **A.** to **F.**) or MS-5/NF-κB-Luc-eGFP (**Figure III.18:** from **G.** to **J.**) for 24 h. Luciferase activity assays were then performed to verify whether leukemic cells could activate the NF-κB reporter in stromal cells. In both cells lines, which express both LTβR and NF-κB-Luc reporter (see above), a considerably significant increase in luciferase activity

was found when in contact with leukemic cells collected from either the thymus (**Figure III.18: A. and B.; G. and H.**), spleen (**Figure III.18: C. and D.; I. and J.**), or lymph nodes (**Figure III.18: E. and F.**). NF-κB activation was observed in most experiments and suggest that the primary leukemic T cells activated the NF-κB pathway in contacting stromal cell lines.


A.
Thymus
****
Relative Luminescence
12
10
8
6
4
2
0
NT
Co-culture
NIH 3T3/NF-κB-Luc-eGFP-Bright
B.
Thymus
*
Normalized Fold Change (Relative Luminescence)
12
10
8
6
4
2
0
NT
Co-culture
NIH 3T3/NF-κB-Luc-eGFP-Bright
C.
Spleen
**
Relative Luminescence
12
10
8
6
4
2
0
NT
Co-culture
NIH 3T3/NF-κB-Luc-eGFP-Bright
D.
Spleen
Normalized Fold Change (Relative Luminescence)
12
10
8
6
4
2
0
NT
Co-culture
NIH 3T3/NF-κB-Luc-eGFP-Bright
E.
Lymph nodes
***
Relative Luminescence
12
10
8
6
4
2
0
NT
Co-culture
NIH 3T3/NF-κB-Luc-eGFP-Bright
F.
Lymph nodes
Normalized Fold Change (Relative Luminescence)
12
10
8
6
4
2
0
NT
Co-culture
NIH 3T3/NF-κB-Luc-eGFP-Bright

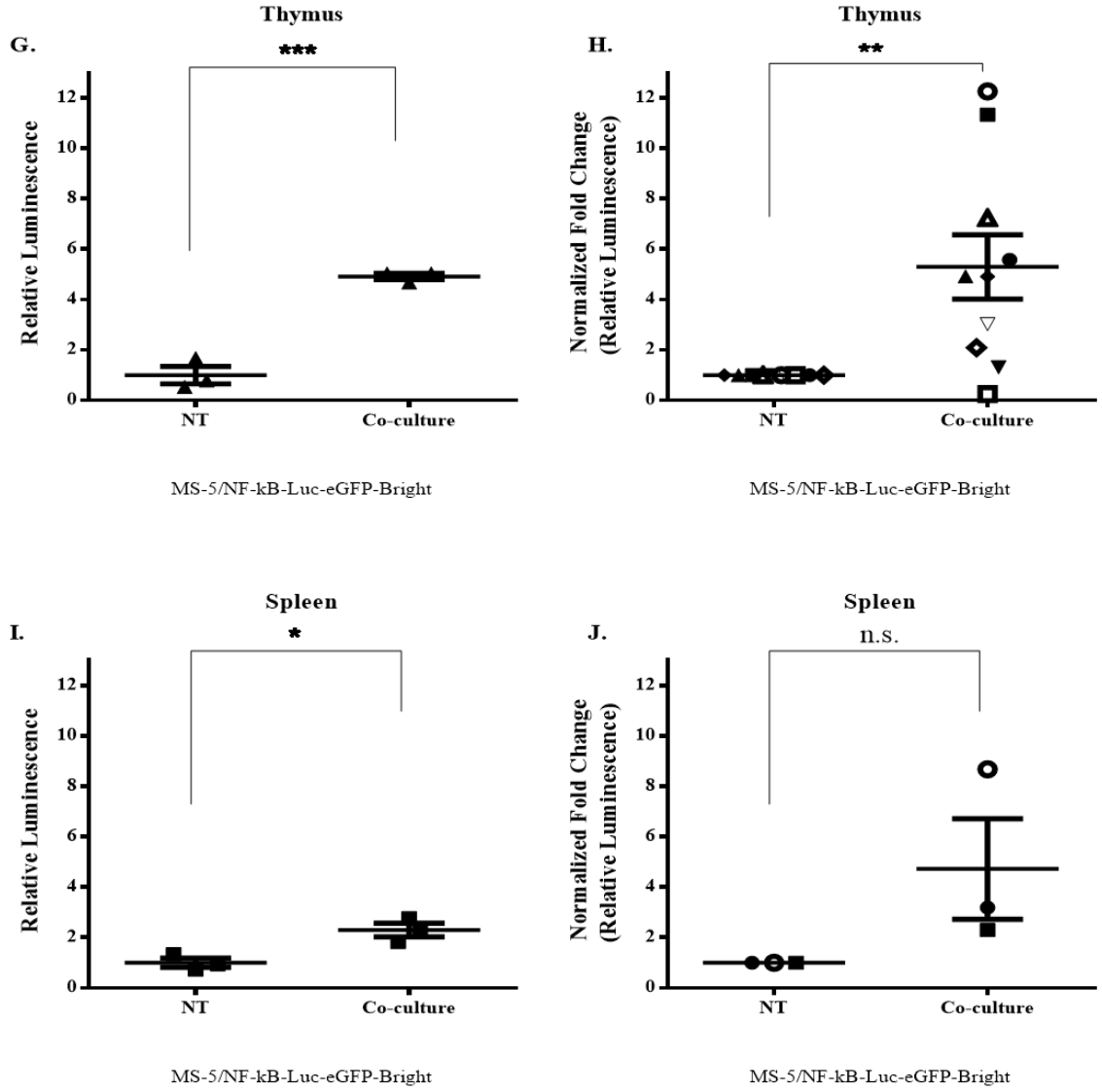
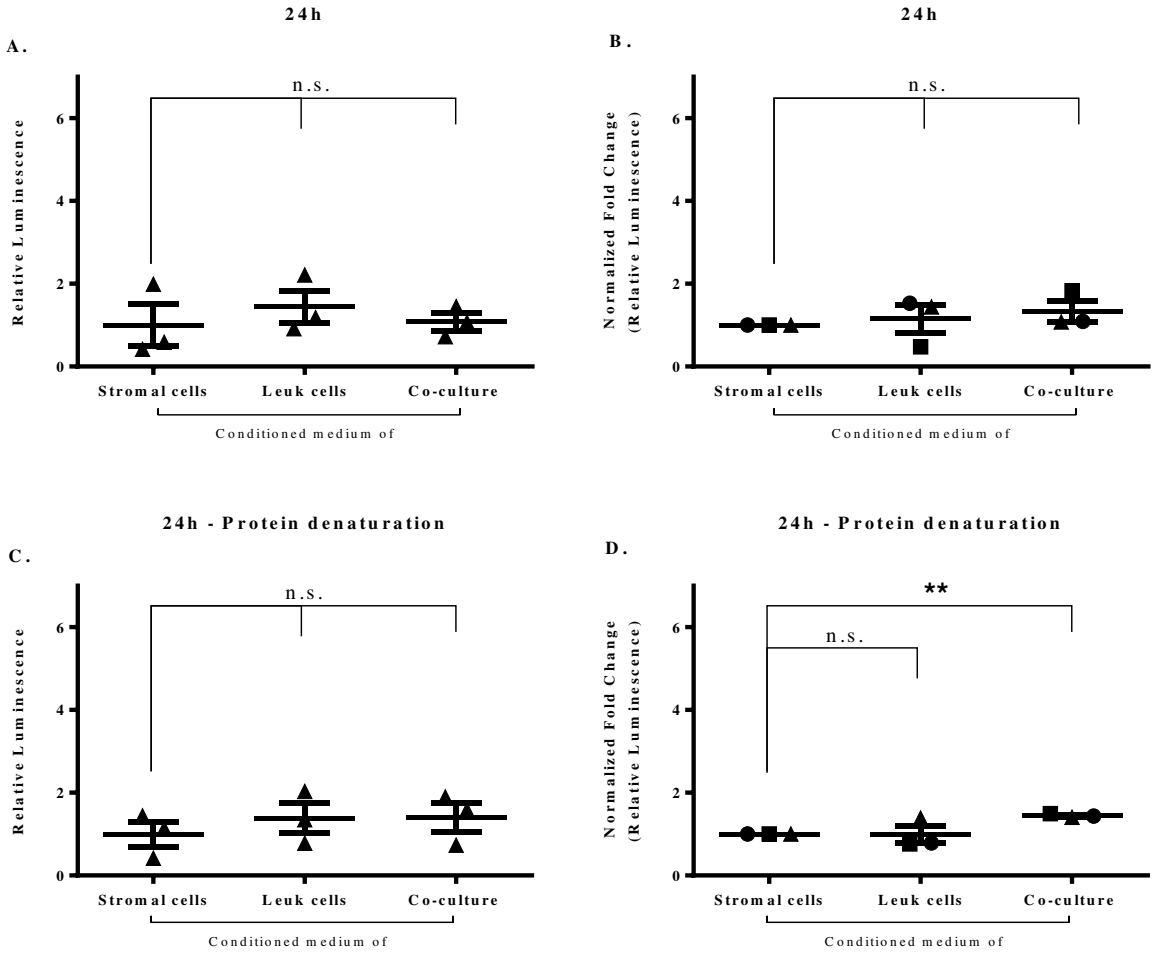


Figure III.18: Leukemic cells activate the NF-κB reporter on NIH3T3 or MS-5-derived cells. Five x 10^4 cells per well of NIH3T3/NF-κB-Luc-eGFP-Bright or MS-5/NF-κB-Luc-eGFP were co-cultured with 1 x 10^6 cells/ml of primary leukemic T cells derived from TEL-JAK2 transgenic mice for 24 hours. The graphics on the left represent the relative luminescence for a representative experiment, each performed in triplicates. The results were statistically significant in graphics (**A.**), (**C.**), (**E.**), (**G.**), (**I.**). (Unpaired *t*-test performed all experiments: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.001$. Standard error and mean represented by bar). The graphics on the right represent the luciferase activity for all experiments performed (between 2 and 10 independent experiments depending on the organ of origin and stromal cell line). The luciferase activity values were normalized, and fold change calculated in comparison to non-co-cultured cells (NT). The results were statistically significant in graphics (**B.**) and (**H.**). (Paired t-test performed all experiments - *, $p \leq 0.05$; ***, $p \leq 0.001$. Each symbol represents an average of each experiment). (**A.**) and (**B.**) refer to primary leukemic T cells from thymus; (**C.**) and (**D.**) refer to primary leukemic T cells from spleen; (**E.**) and (**F.**) refer to primary leukemic T cells from lymph nodes when co-cultivated with NIH3T3/NF-κB-Luc-eGFP-Bright. Graph from (**G.**) to (**J.**) are the equivalent to graph (**A.**) to (**D.**) but instead referring to the MS-5/NF-κB-Luc-eGFP cell line.

III.2.3. The NF-κB reporter in stromal cells is not activated by secreted factors

Following the above results, we asked whether leukemic cells activated NF-kB on the co-cultured cell lines through direct contact or through secreted molecules. To address the second question, we assayed whether the NF-κB reporter in stromal cells could be activated by soluble factors secreted by leukemic cells into the culture medium. We obtained conditioned medium from cultures of only primary leukemic T cells or these cells co-cultured with the NIH3T3/NF-κB-Luc-eGFP-Bright (**Figure III.19: I.**) or MS-5/NF-κB-Luc-eGFP (**Figure III.19: II.**) cell lines. In addition, as a negative control we also collected conditioned medium from only stromal cell lines. After 6 or 24 h of culture or co-culture of NIH3T3/NF-κB-Luc-eGFP-Bright, the conditioned medium from these cells was added to culture dishes containing the same reporter stromal cell lines. The same procedure was applied for MS-5/NF-κB-Luc-eGFP cell lines. After 24 h incubation, luminescence was measured. Conditioned medium of 6 h cultures with or without FBS was studied to determine if the presence of the proteins of the FBS could induce the expression of the NF-κB reporter, but activation of the reporter was not observed in either case (data not shown). The incubation of NF-κB reporter cells lines with conditioned medium from 24 h cultures indicated that any soluble factors secreted by leukemic cells cultured alone or co-cultured with MS-5-derived cells were not capable of activating significantly the NF-κB reporter. However, when denature 24 h conditioned medium was cultured with NIH3T3-derived cells, there was an apparent activation of the NF-κB reporter (**Figure III.19: I. D.**). These results is unexpected, because the same medium without denaturation did not activated the NF-κB reporter. Indirectly, these results indicated that cell-cell contact is required for NF-κB activation.

I. NIH 3T3/NF-κB-Luc-eGFP-Bright



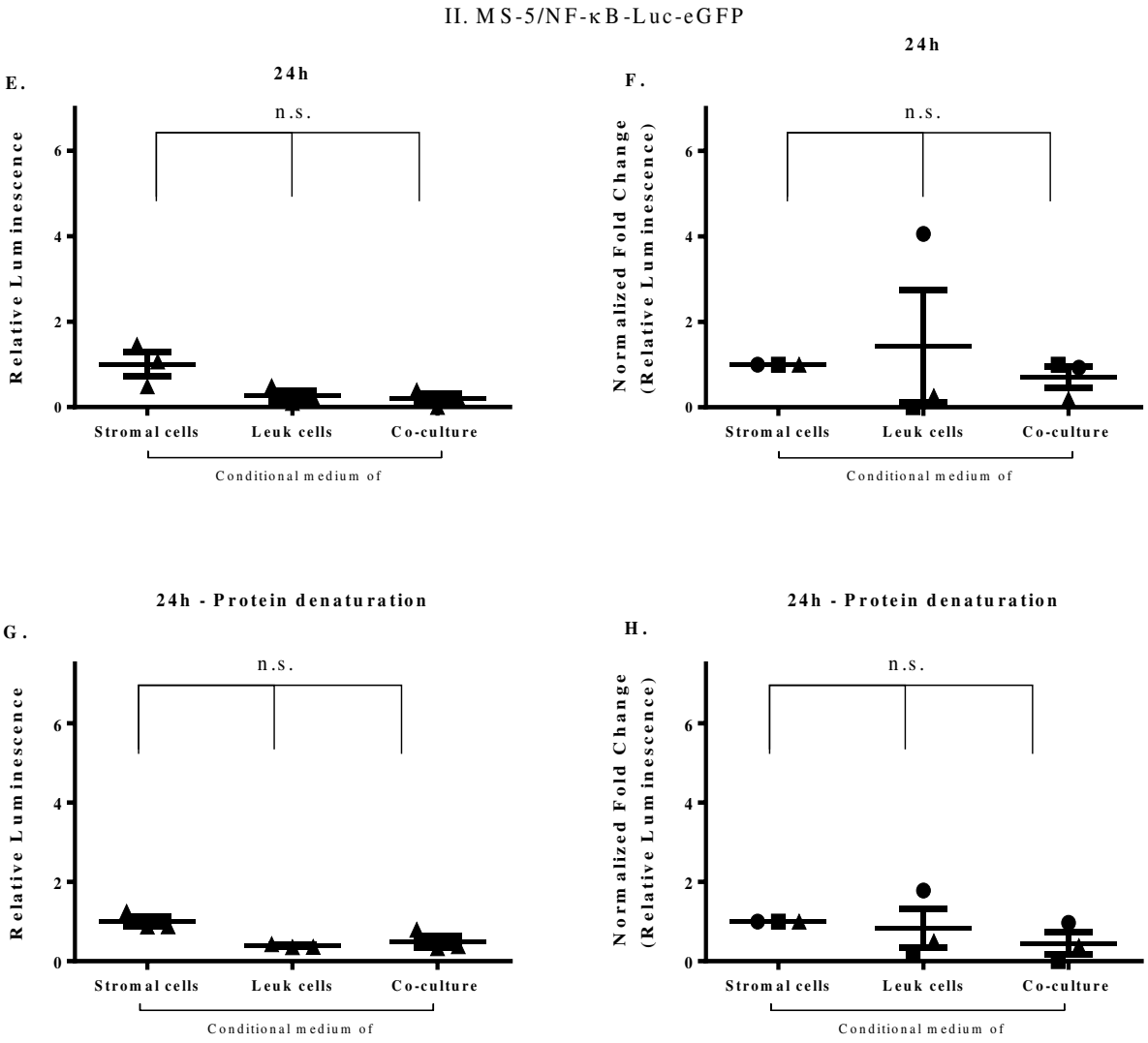


Figure III.19: Secreted soluble factors do not activate of NF-κB in co-cultures The conditioned medium was obtained from culture 0.5-1.5 x 10^6 of: stromal cells alone (represented as Stromal cells); 5x10^6 leukemic cells/ml alone (represented as Leuk cells); or the co-culture of both cell types (represented as Co-culture), in medium supplemented with 10% FBS for 24 h (represented as 24 h), being 1/2 of this conditioned medium boiled for 10 min for protein denaturation (represented as 24 h- Protein denaturation). (**A.**) from to (**D.**) represents the relative luminescence of NIH3T3 cell line and (**E.**) from to (**H.**) represents the relative luminescence of MS-5 cell line. The graphics on the left represent the relative luminescence for a representative experiment, each performed in triplicates. The results were not statistically significant (n.s.). (Unpaired *t*-test performed all experiments. Standard error and mean represented by bar). The graphics on the right represent the luciferase activity for 3 independent experiments performed. The luciferase activity values were normalized, and fold change calculated in comparison to culture of stromal cells alone. The result in graph (**D.**), when compared culture of stromal cells with co-culture with leukemic T cells, was statically significant (**) p value=0.0034 $\leq$ 0.01. (Paired *t*-test performed all experiments. Each symbol represents an average of each experiment).

III.3. LTβR MEDIATES NF-κB ACTIVATION IN STROMAL CELLS INDUCED BY CO-CULTURED LEUKEMIC T CELLS

III.3.1. LTβR blockade hampers activation of the NF-κB reporter in stromal cell lines by co-culture with leukemic cells.

To demonstrate that NF-κB activation upon contact with leukemic cells was due to stimulation of LTβR present on the stromal cell lines, we blocked LTβR ligands with an excess of soluble LTβR-Fc fusion protein in co-cultures. As previously reported [46], LTβR-Fc binding to the surface of leukemic cells masks both the lymphotoxin ligands (LTα1β2) and consequently blocks interaction with their receptor. This should prevent LTβR-mediated activation of the NF-κB reporter in the co-culture system. LTβR-Fc binding to the surface of leukemic cells masks the lymphotoxin ligands (LTα1β2) and consequently blocks interaction with their receptor. This should prevent LTβR-mediated activation of the NF-κB reporter in the co-culture system.

Thus, co-cultures between primary leukemic T cells and stromal cells were treated with different concentrations of LTβR-Fc: 10 ηg/ml, 100 ηg/ml and 1 μg/ml, and after 24 h cells were harvested and luciferase measured. Both NIH3T3/NF-κB-Luc-eGFP-Bright (**Figure III.20: A.** and **B.**) and MS-5/NF-κB-Luc-eGFP cell line (**Figure III.20: C.** and **D.**) co-cultures with leukemic cells showed a decrease in NF-κB activity after addition of LTβR-Fc. So, for the highest LTβR-Fc concentration (1 μg/ml) (2.382 ± 0.3057, n=3) for the MS-5/NF-κB-Luc-eGFP (**Figure III.20: C.**), the values of relative luminescence were statistically significant when compared with the co-culture alone (4.911± 0.177, n=3). The results for NIH3T3/NF-κB-Luc-eGFP-Bright were not statistically significant. However, this allows us to conclude that in MS-5 cell lines, leukemic cell-induced activation of NF-κB was indeed mediated, at least partially, by LTβR stimulation.

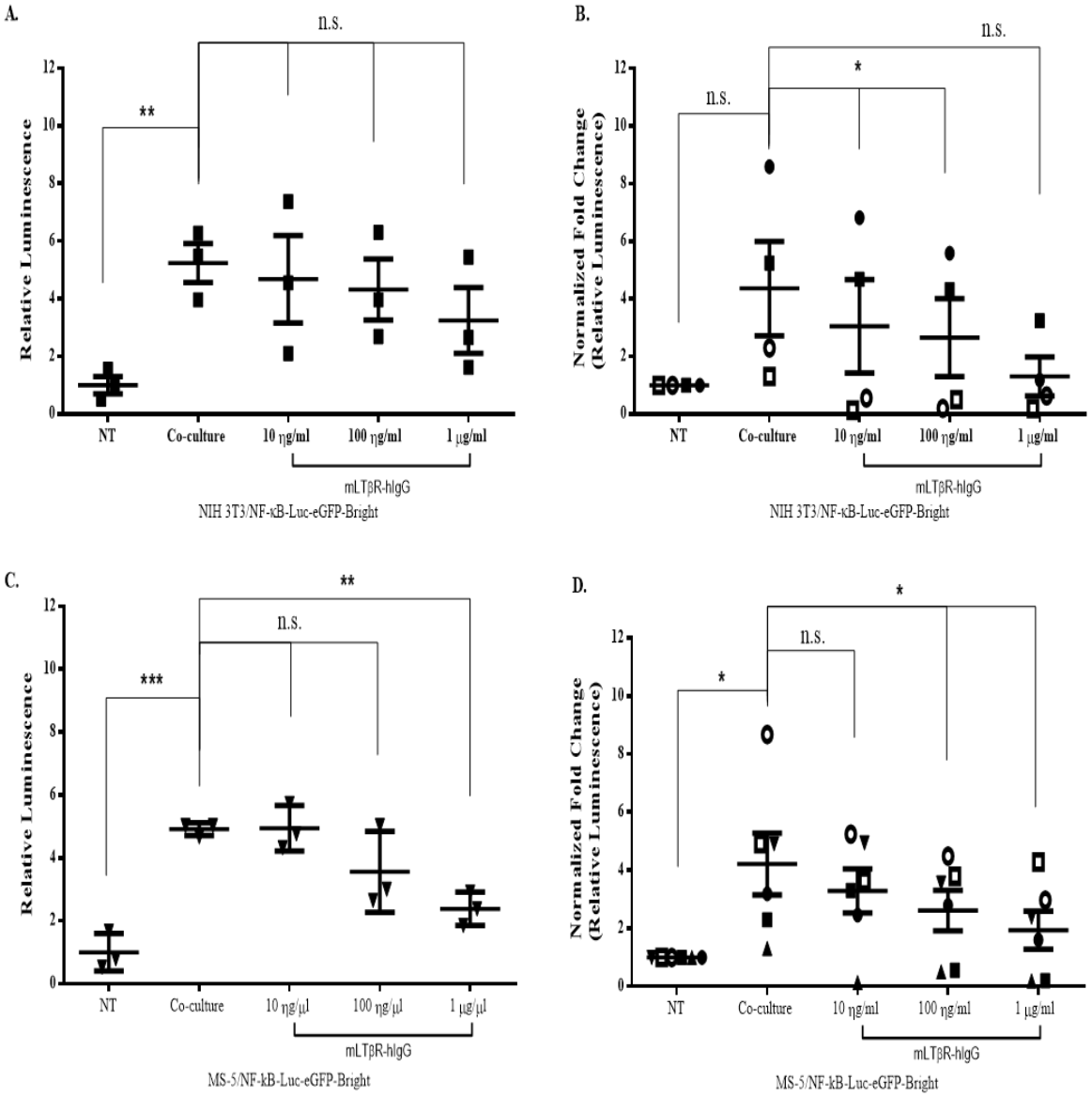


Figure III.20: LTβR blockade also blocks activation of the NF-κB reporter in stromal cell lines co-cultured with leukemic cells. Five x 10^4 cells per well of NIH3T3/NF-κB-Luc-eGFP-Bright or MS-5/NF-κB-Luc-eGFP were co-cultured with 1 x 10^6 cells/ml of primary leukemic T cells derived from TEL-JAK2 transgenic mice and these co-cultures were treated with 10 ηg/ml, 100 ηg/ml or 1 μg/ml of LTβR-Fc (mLTβR-hIgG) for 24 hours. (**A.**) and (**B.**) refer to co-culture of primary leukemic T cells with NIH3T3/NF-κB-Luc-eGFP-Bright, and (**C.**) and (**D.**) refer to MS-5/NF-κB-Luc-eGFP. The graphics on the left (**A.** and **C.**) represent the relative luminescence of a representative experiment, performed in triplicate. The result in graph (**C.**), at the highest concentration, was statistically significant (**) P value= 0.0258 $\leq$ 0.01. (Unpaired t-test performed all experiments. Standard error and mean represented by bar). The graphics on the right (**B.** and **D.**) represent the luciferase activity of all experiments performed (between 4 and 6 independent experiments). The luciferase activity values were normalized, and fold change calculated in comparison to mono-cultured. The result in graph (**D.**), at the highest concentration, was statistically significant (*) p value=0.0274 $\leq$ 0.05. (Paired t-test performed all experiments. Each symbol represents an average of each experiment). n.s.-not statistically significantly.

III.3.2 LTβR genetic inactivation in fibroblasts hampers activation of the NF-KB reporter by co-cultured leukemic cells

In order to complement the previous results based on LTβR signaling inhibition using a soluble LT decoy protein, we also used a genetic approach, by co-culturing leukemic T cells with either *Ltbr*$^{-/-}$ or *Ltbr*$^{+/-}$ mouse embryonic fibroblasts (MEFs). To generate MEFs, we cultured fibroblasts from individual embryos. Genotyping for the *Ltbr* gene was performed for each embryo (**Figure III.21**), and one MEF culture from each genotype, embryos 1 (*Ltbr*$^{-/-}$) and 4 (*Ltbr*$^{+/-}$), was selected for further experiments.

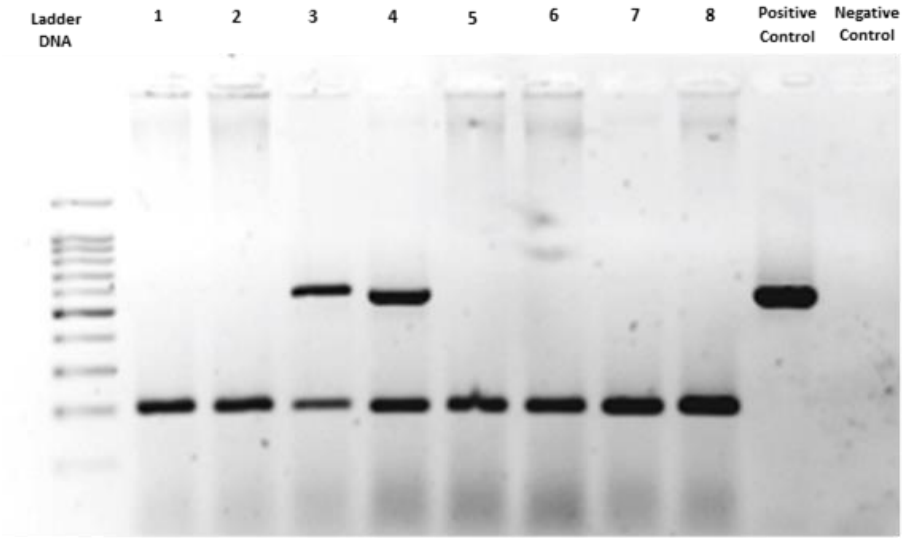


Figure III.21: PCR genotyping of the *Ltbr* gene. The embryos 1, 2, 5, 6, 7 and 8 were KO for *Ltbr*, presenting only a PCR band of 200 bp while embryos 3 and 4 were heterozygotes for the *Ltbr* having 200 and 600 bp bands (allele KO *Ltbr* and allele with expression of LTβR).

Like for NIH3T3 and MS-5 cell lines, we generated MEF NF-κB reporter cells by infecting both *Ltbr*$^{-/-}$ and *Ltbr*$^{+/-}$ MEFs with lentiviral particles containing the NF-κB-Luc-eGFP construct (**Figure III.22: A. and B.**). Although we aimed to sort and purify efficiently transduced (eGFP+) cells, we were unable to do so due to lack of cell viability. Thus, we decided to perform MEF co-culture experiments in unsorted infected cells. To confirm the efficiency of each infection and NF-κB reporter integration, the percentage of GFP-positive transduced MEFs was determined by flow cytometry prior to each co-culture experiment. The transduction rate was of about 25%. For example, for one transduction experiment, after 7 days of lentiviral infection, 23.3% of *Ltbr*$^{-/-}$MEF-1/NF-κB-Luc-eGFP cells were positive for eGFP (**Figure III.22: D.**) while 27.6% of *Ltbr*$^{+/-}$MEF-4/NF-κB-Luc-eGFP cells were positive (**Figure III. 22: E.**). Non-transduced MEFs were negative for eGFP (**Figure III.22: C.**).

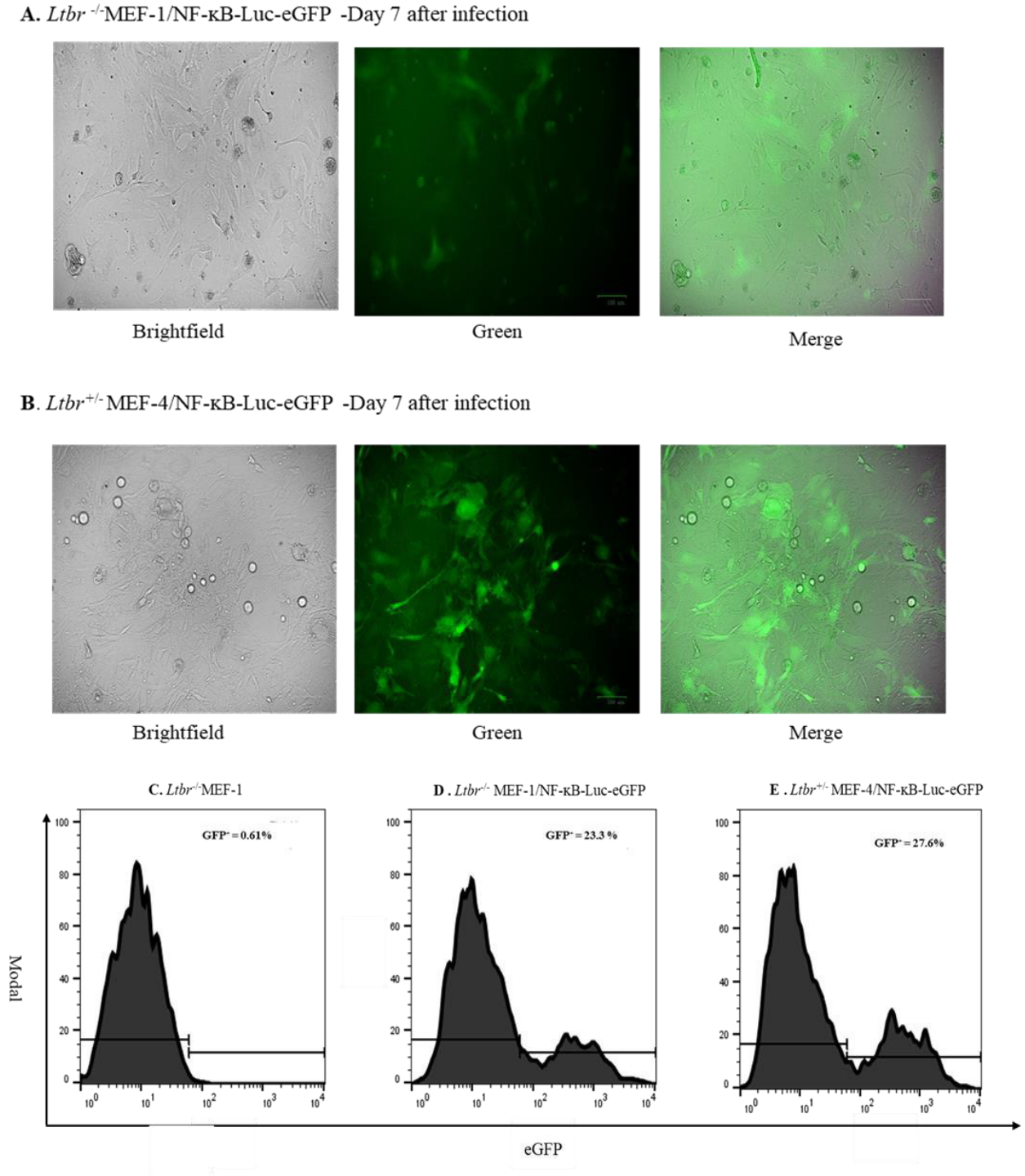


Figure III.22: Infection of MEFs with NF-κB reporter lentiviral vector. Microphotograph from sorted eGFP positive cells (Channel green), generated from as *Ltbr*$^{-/-}$MEF-1/NF-κB-Luc-eGFP cells (**A.**) or *Ltbr*$^{+/-}$MEF-4/NF-κB-Luc-eGFP (**B.**), after 7 days the transduction with a lentiviral vector - NF-κB-Luc-eGFP. The graphs **C.**, **D.**, **E.** represent the confirmation of transduction by flow cytometry and the percentage of *Ltbr*$^{-/-}$MEFs-1 (negative control), or *Ltbr*$^{-/-}$MEF-1/NF-κB-Luc-eGFP or *Ltbr*$^{+/-}$MEF-4/NF-κB-Luc-eGFP eGFP positive cells, respectively.

To make sure we were using MEFs of correct genotype and expected phenotype for co-culture experiments, we assessed surface LTβR expression by flow cytometry. Indeed *Ltbr*$^{-/-}$MEF-

1/NF-κB-Luc-eGFP **(Figure III.23: B.)** cells did not express LTβR, whereas the *Ltbr*$^{+/-}$MEF-4/NF-κB-Luc-eGFP **(Figure III.23: D.)** cells presented low but clear surface LTβR expression.

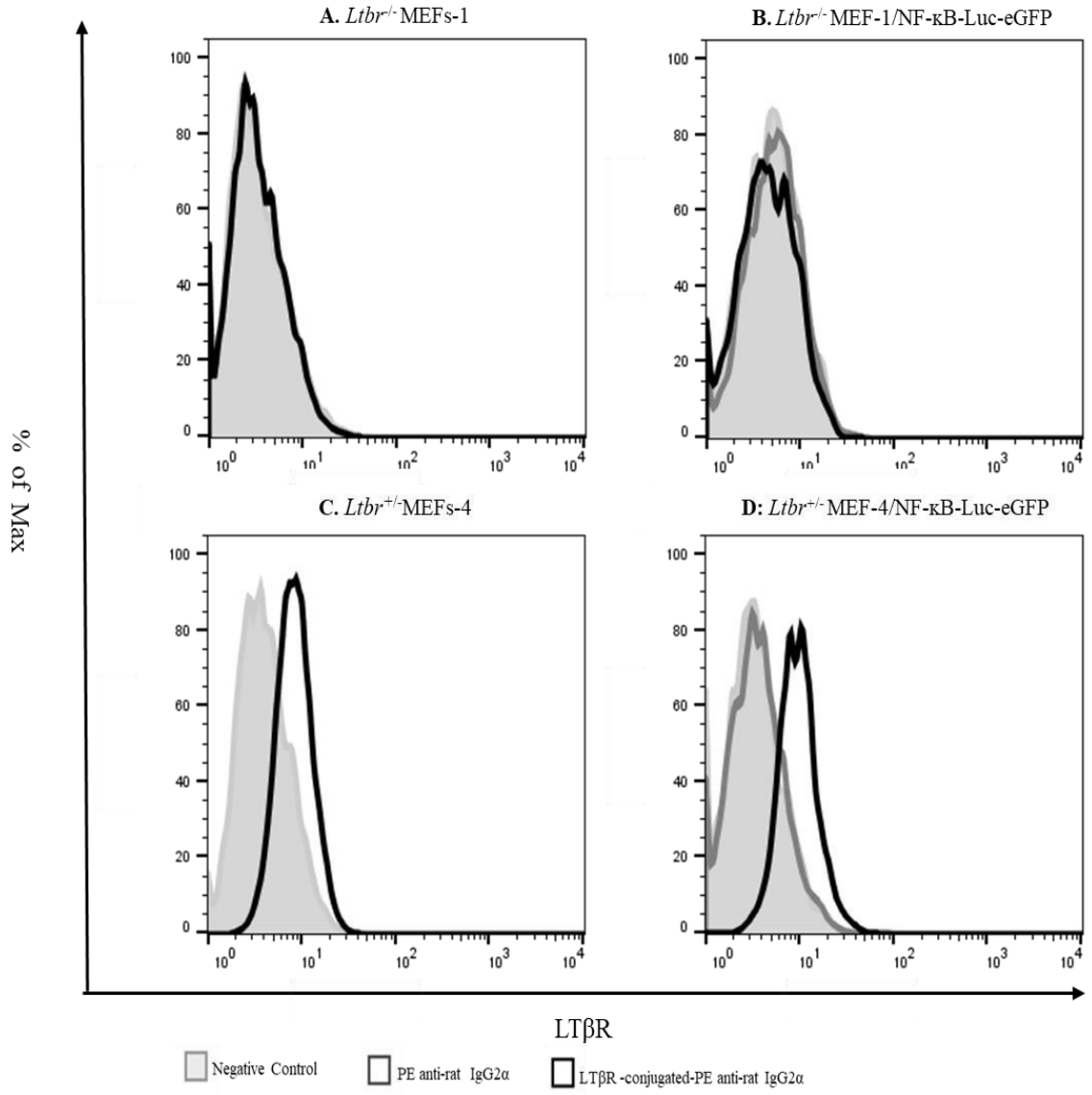


Figure III.23: LTβR surface expression reflects the genotype of primary mouse embryonic fibroblasts. *Ltbr*$^{-/-}$MEF-1/NF-κB-Luc-eGFP **(B.)** not express surface lymphotoxin β, whereas *Ltbr*$^{+/-}$MEF-4/NF-κB-Luc-eGFP (**D.**) express surface LTβR, but in residual level. LTβR expression in *Ltbr*$^{+/-}$MEF-4 was not affected by lentiviral transduction, (**C.**) and (**D.**). Primary MEFs stained with anti-mouse LTβR 5G11 (1/200) and conjugated PE anti-rat IgG2a (1/200) and as negative control only cells – no antibody (shading) or cells with only PE-conjugated PE anti-rat IgG2a (dark gray).

Likewise, when the level of surface LTβR expression was compared between *Ltbr*$^{+/-}$MEF-4 and the derivative *Ltbr*$^{+/-}$MEF-4/NF-κB-Luc-eGFP cells, no significant alteration at the expression level was detected. From these results, we conclude that LTβR surface expression in *Ltbr*$^{+/-}$ MEFs containing the NF-κB reporter remains stable.

Next, to assess whether the integrated reporter construct was functional in primary MEF cultures, we stimulated MEFs with 50 μg/ml of LPS. As a negative control for LPS treatment we performed the luciferase assays with non-transduced *Ltbr*$^{-/-}$MEF-1 cells. As expected, no luciferase activity was detected in these cells (**Figure III.24: A.**). In contrast, low levels of luciferase activity were detected in lentivirally transduced MEFs, but those were increased by LPS stimulation, independently of *Ltbr* genotype (**Figure III.24: B.** and **C.**). These results indicate that our *in vitro* system was functional in transduced primary MEFs.

Next, we co-cultured MEFs of each genotype with primary leukemic T cells to assess whether NF-κB was activated in MEFs through LTβR mediation. By co-culturing leukemic cells with *Ltbr*$^{+/-}$reporter MEFs, we found that NF-κB-dependent luciferase activity increased on average about 4-fold (**Figure III.24: F.** and **G.**). In contrast, luciferase relative activity in co-cultured *Ltbr*-deficient MEFs was increased only about 2-fold on average (**Figure III.24: D.** and **E.**). These results indicate that in LTβR-deficient MEFs, leukemic cell-induced NF-κB activation was impaired but not totally abrogated. Although showing a clear role for LTβR, these data suggest that other leukemic cell-derived factors also induce NF-κB activation in fibroblasts.

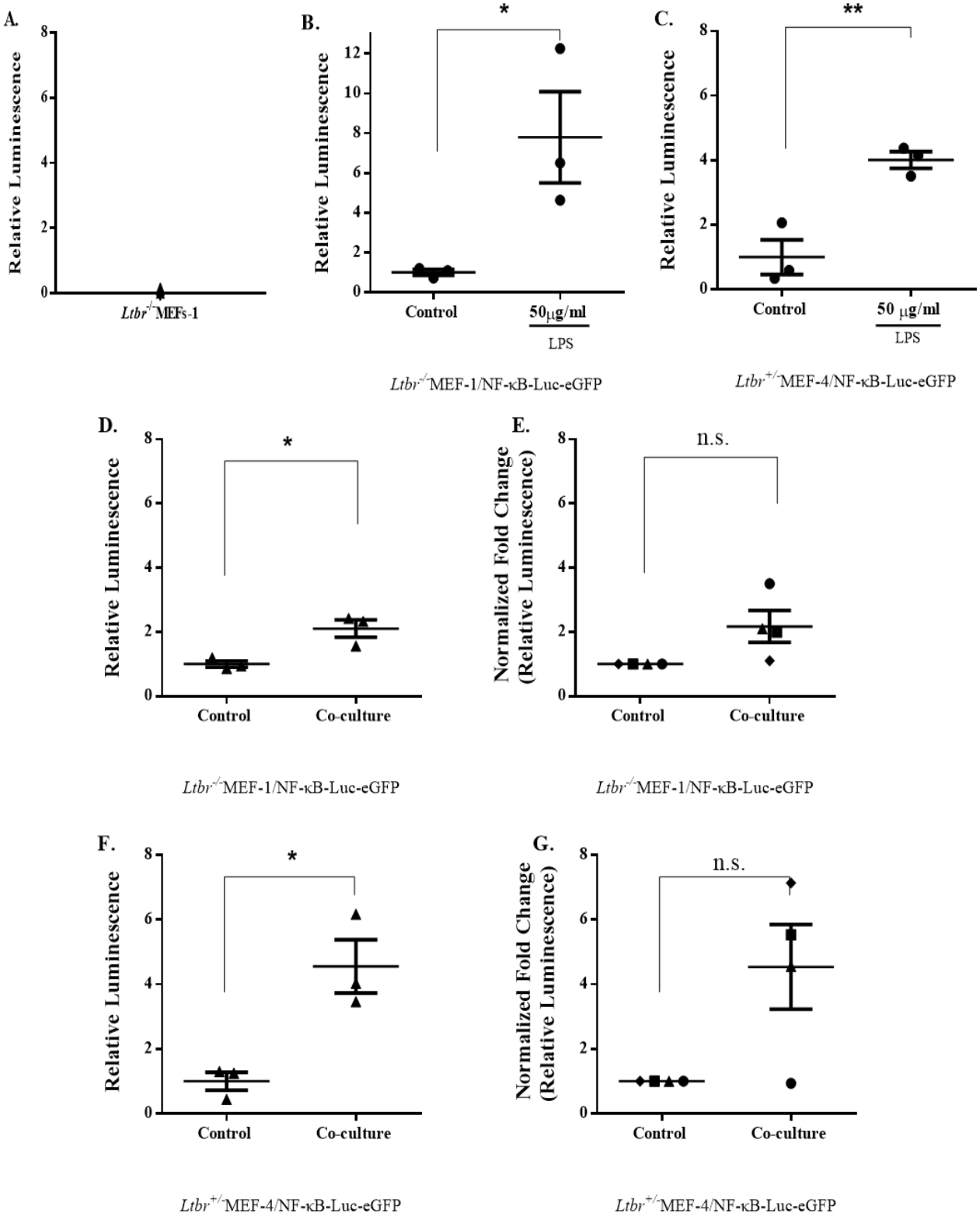


Figure III.24: NF-κB activation in co-cultured leukemic cells was impaired in LTβR-deficient MEFs. Five x10^4 cells per well of *Ltbr*$^{-/-}$MEF-1/NF-κB-Luc-eGFP or *Ltbr*$^{+/-}$MEF-4/NF-κB-Luc-eGFP were co-cultured with 1 x10^6 cells/ml of primary leukemic T cells derived from TEL-JAK2 transgenic mice. The *Ltbr*$^{-/-}$MEFs-1 (**A.**), *Ltbr*$^{-/-}$MEF-1/NF-κB-Luc-eGFP (**B.**) or Ltbr$^{+/-}$MEF-4/NF-κB-Luc-eGFP (**C.**) were stimulated with 50 µg/ml of LPS.

These graphics represent the relative luminescence of each triplicate of a single independent experiment. The results in graphics (**B.**) and (**C.**) were statistically significant: (*) *p* value=0.0417 ≤ 0.05; (**) *p* value= 0.0073≤ 0.01, respectively. (Unpaired *t*-test performed all experiments. Standard error and mean represented by bar). From (**D.**) and (**G.**) refer to co-culture of MEFs with primary leukemic T cells. The graphics on the left (**D.** and **F.**) also represent the relative luminescence of representative experiment each triplicates of a single independent experiment. The results were statistically significant (*): *p* value= 0.0188 and *p* value= 0.0150 ≤0.05, respectively. (Unpaired *t*-test performed all experiments. Standard error and mean represented by bar). The graphics on the right (**E.** and **G.**) represent the of luciferase activity of all experiments performed 4 independent experiments. The results were not statistically significant (n.s.). (Paired *t*-test performed all experiments. Each symbol represents an average of each experiment).

CHAPTER IV - DISCUSSION & FUTURE PERSPECTIVES

IV. DISCUSSION & FUTURE PERSPECTIVES

IV. 1. AN NF-κB REPORTER CELL SYSTEM WAS USED A TOOL TO DETECT LTβR ACTIVATION

Our group previously showed that primary leukemic T cells derived from transgenic TEL-JAK2 mice interacted *in vitro* with co-cultured fibroblasts through interaction with LTβR expressed by these cells [46]. However, these results did not show that LTβR was activated. The major purpose of this thesis was to verify whether leukemic cells could activate LTβR signaling on fibroblasts (or mesenchymal cells). Since the main signaling pathway activated by this receptor is that leading to the activation of NF-κB transcription factors [41,46,157], we started by creating an *in vitro* system to evaluate NF-κB activation in NIH3T3 fibroblasts or MS-5 stromal cells. These cell lines, which were known and were confirmed to express LTβR [41,46,157] were transduced with a lentiviral construct expressing the luciferase reporter gene under control of an NF-κB-responsive transcriptional promoter. With this luciferase reporter we were able to quantify the activation of NF-κB. These constructs also included an eGFP gene controlled by an ubiquitous promoter, allowing us to verify that the stromal cells or fibroblasts expressed our reporter gene. In addition, we confirmed that the LTβR expression levels were not affected by our cell manipulations, i.e. lentiviral transduction, cell sorting, and successive culture passaging.

To verify that our *in vitro* system was functional, we first stimulated cells containing the NF-κB-Luc reporter with known NF-κB activators. It has been previously reported that stimulation by the bacterial product lipopolysaccharide can lead to NF-κB signaling pathway activation through an TLR4-dependent pathway [93,96,148,155]. Indeed, an increased NF-κB activity at 10 h following LPS stimulation was observed. Therefore, the reporter system was functional and LPS stimulation could be used as a positive control for further experiments.

At the same time, we tested whether the reporter plasmid could be stimulated by an anti-LTβR agonist antibody. LTβR-dependent downstream signaling may be initiated artificially by anti-LTβR agonistic antibodies that induce receptor aggregation [47]. Thus, it was found in NIH3T3-derived cells that NF-κB could be activated by direct LTβR stimulation. In contrast, in MS-5-derived stromal cells NF-κB was not consistently activated by anti-LTβR. In that case, we need further studies to understand the reason. Nevertheless, we confirmed that the MS-5 reporter cell line expressed surface LTβR and that the NF-κB reporter was functional, because it responded to LPS stimulation and co-cultured leukemic T cells. More importantly, using a decoy LTβR-

Fc protein we found a decreased NF-κB activation in these co-cultures. Together these data show that LTβR can be activated in MS-5 cells. It is unclear if the anti-LTβR agonist antibody we used to be not capable of NF-κB activation in MS-5 cells. We could test other LTβR antibody. For example Macho-Fernandez *et al.,* 2015 [158] reported LTβR stimulation in CMT-93 epithelial cells with the agonistic (ACH6 anti-LTβR antibody) for 6 h or 24 h.

Although, we show that the system created for NF-κB activation reporter system is suitable for the study of $LT\alpha_1\beta_2$-LTβR signaling pathway, we could use a different approach to validate this observation. One possibility would be the detection of p65/RelA and RelB nuclear translocation by immunofluorescence after the LTβR activation cascade.

Taken together, however, the results of LPS stimulation ascertained that our *in vitro* system is functional and that we can detect the activation of NF-κB in stromal cells by measuring luciferase activity.

IV.2. LEUKEMIC CELLS ACTIVATE NF-KB ON NEIGHBORING CELLS

The process by which LTβR signaling contributes to the development of T-ALL is still not well understood. LTβR is expressed in stromal cells, including cells from the thymic microenvironment, and expression of lymphotoxin by leukemic T cells may promote leukemogenesis by activating LTβR in those microenvironmental cells [46,47]. The activation of the LTβR may favor carcinogenesis either by regulating the development and organization of supportive tumor microenvironmental cell networks [35,159], or by induction of LTβR target genes in stromal cells that potentially stimulate tumor cell growth and survival [46,119], such as cytokines or chemokines [41,160].

Thus, our future aim will be to understand how $LT\alpha_1\beta_2$ expression in emerging malignant thymocytes could activate LTβR in stromal cells to activate a transcription program in thymic microenvironmental cells that promote T-cell leukemogenesis. Following previous studies, we confirmed that primary cells obtained from TEL-JAK2 transgenic mice, including $CD8^+CD25^+$ populations, infiltrated the thymus, spleen, BM and lymph nodes. In addition, we confirmed that leukemic cells expressed surface lymphotoxin when cultured *ex vivo* or stimulated by PMA and ionomycin. Primary leukemic T cells collected from diseased TEL-JAK2 transgenic mice were co-cultured with NIH3T3/NF-κB-Luc fibroblasts or MS-5/NF-κB-Luc bone marrow stromal cells and it was observed that leukemic cells activated the NF-κB reporter in fibroblasts

or MS-5 cells. This likely occurred through direct contact, because NF-κB activation was not detected when reporter cell lines were cultured in conditioned medium previously obtained from primary leukemic cell cultures, stromal cell line cultures, or co-cultures of these two cell types. Thus, we conclude that cytokines or other secreted proteins or molecules are not sufficient to activate NF-κB. The NF-κB pathway can be activated by multiple stimuli [88], so different proteins produced by leukemic cell could potentially activate NF-κB in the reporter cell lines. To test the role of LTβR signaling in this process, we blocked lymphotoxin ligands by addition of an LTβR-Fc decoy protein to the co-cultures and observed diminished luciferase activity. Reduction of luciferase activity was correlated with increasing LTβR-Fc concentrations. These results indicate that NF-κB activation in mesenchymal cells was indeed mediated through $LT\alpha_1\beta_2$-LTβR signaling as initially hypothesized.

Further supporting the notion that NF-κB reporter activation was linked to LTβR activation and not activation of other receptors presents in MS-5 cells or fibroblasts, we co-cultured leukemic cells with *Ltbr*-deficient MEFs. By doing this, we verified that in the absence of this receptor there was no or reduced activation of the NF-κB signaling pathway. These findings thus reinforce the notion that the mechanism responsible for the activation of the NF-κB signaling pathway is mediated through $LT\alpha_1\beta_2$-LTβR signaling. Therefore, we confirm our main hypothesis, that leukemic cells stimulate LTβR signaling in microenvironmental cells. The next step will be to restore LTβR expression in *Ltbr* knockout (KO) MEFs, by transduction with the plasmids pBabe-puro-LTβR encoding wild type (wt) or 345-358 mutant, provided by Emmanuel Dejardin[160,161], to confirm that NF-κB activation in MEFs by is mediated by LTβR signaling.

To confirm activation of the LTβR signaling pathway through another approach, we plan to evaluate by RT-qPCR the expression of genes in co-cultured MS-5 bone marrow stromal cells. Several genes have been reported to be induced in fibroblasts or stromal cells by LTβR, like Spi-B, Spi-B1, and IL-18. We be use *NFKB2* expression as a control for NF-κB activation and *GApDH* expression as a RNA normalizing control [75,112,162].

Thus, after verifying that TEL-JAK2 leukemic cells expressing LT contact and trigger the LTβR signaling pathway in stromal cells and enhancing the activity of NF-κB transcription factors, we intend to identify specific anchored or secreted specific proteins that may induce survival or proliferation of malignant cells.

IV.3. FUTURE PERSPECTIVES

MS-5 cells were shown to secrete factors (e.g. IL18) that favor leukemic cell progression [75], so most likely specific membrane-anchored or secreted factors produced by this cell line foster TEL-JAK2 leukemic cell survival and expansion. But how does LTβR/NF-κB signaling in stromal cells promote leukemic cell survival and proliferation? Which soluble factors induced and secreted by NF-κB-dependent LTβR activation are involved in the development of leukemia? Is LTβR signaling important for human T-ALL? Can the identification of these "new" factors have a therapeutic effect on human T-ALL? Many questions remain unanswered.

LTβR signaling can induce the expression of specific secreted proteins [47] that may potentially induce the survival or proliferation of leukemic cells. Thus, we plan to study the impact of LTβR inactivation on leukemic cell survival in MS-5 co-cultures. For this purpose, we will inactivate genetically LTβR in MS-5 cells, by using the Crispr-Cas9 system. LTβR gene knockout will be induced in MS-5 cells and then used in co-culture assays with leukemic cells. The impact of LTβR inactivation on survival and expansion of leukemic cells will then be evaluated.

To identify potential membrane-anchored or secreted ligands dependent on LTβR signaling in MS-5 cells, we will perform gene expression profiling of MS-5/*Ltbr*-WT and MS-5/*Ltbr*-KO cells cultured alone or co-cultured with leukemic cells. Bioinformatic analyses will be used to identify genes which expression is induced by co-cultured leukemic T cells, and which are known to be involved in paracrine or juxtacrine cell signaling. To test the functionality of membrane-anchored or secreted proteins, we will verify whether the cognate receptors are expressed by leukemic T cells and then we will block receptor-ligand interactions in co-cultures to verify whether leukemic cell survival or expansion is prevented.

Upon successful identification of protein(s) involved in molecular communication between leukemic and stromal cells, we can perform more studies on this or these proteins of interest. We can verify for example: if there are differences in disease progression when comparing the development of leukemia in the TEL-JAK2 mouse model with the development of malignancy in knockout mouse models for the protein under study, to study the role of the protein of interest in human T-ALL, and if there are available specific inhibitors for the protein of interest (or pathway under its control) one could test as therapy for the treatment of T-ALL.

IV.4. CONCLUSION

Here we demonstrated that mouse LT-expressing leukemic cells can indeed activate LTβR in stromal cells in an *in vitro* co-culture system. This activation was detected by the enhanced luciferase activity in stromal cells harboring a NF-κB-Luc construct. The NF-κB activation in stromal cells appeared to be via direct contact of TEL-JAK2 leukemic cells expressing LT with LTβR-expressing stromal cells.

This work thus support the model that TEL-JAK2 leukemic cells induce gene expression in stromal cells via a $LT\alpha_1\beta_2$-LTβR signaling pathway (**Figure IV.25**).

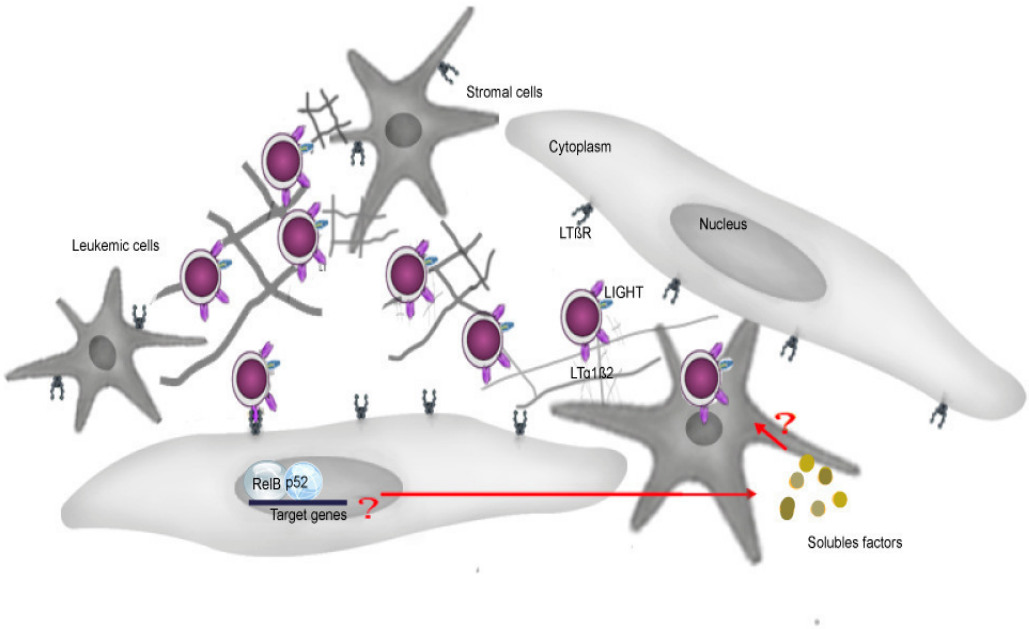


Figure IV.25: Influence of microenvironment cells, through LTβR signaling, in T-ALL. Communication between stromal cells and TJ2-Tg leukemic cells can activate LTβR and subsequently lead to NF-κB signaling. Then, the RelB/p52 are released and translocated to the nucleus, thus activating unknown target genes transcription, and consequently production of soluble factors (such as proteins, cytokines), which can stimulate leukemic T cells proliferation and survival. Therefore, its effect T-ALL development.

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