UNIVERSIDADE DO ALGARVE

Lymphotoxin-beta receptor and RANK signaling in TEL-JAK2-induced T-cell leukemia

Mónica Alexandra Teotónio Fernandes

Tese para obtenção do grau de Doutor em Ciências Biomédicas

Trabalho efetuado sob a orientação de:

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Declaração de autoria de trabalho

Declaro ser a autora deste trabalho, que é original e inédito. Autores e trabalhos consultados estão devidamente citados no texto e constam da listagem de referências incluída.

Dedicated to my family and friends for their unconditional love and support.

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RESUMO

A leucemia linfoblástica aguda de linfócitos T (LLA-T) é uma patologia hematológica maligna que afeta essencialmente crianças e adultos jovens e é fatal na ausência de um diagnóstico precoce e uma terapêutica apropriada. É do conhecimento geral que a LLA-T tem origem em precursores dos linfócitos T, também designados por timócitos, que sofrem um bloqueio da diferenciação e expansão clonal. As células imaturas transformadas disseminam-se pela corrente sanguínea e invadem a medula óssea e vários órgãos como o baço, fígado, gânglios linfáticos e, por vezes, o sistema nervoso central. Apesar das melhorias observadas em termos de prognóstico nas últimas décadas, vários problemas clínicos persistem incluindo o prognóstico reservado nas recidivas e no subgrupo de LLA-T caracterizado pela presença de células leucémicas mais imaturas, o crescente número de casos resistentes à terapêutica e as graves sequelas derivadas da terapêutica agressiva com recurso a quimioterapia em altas doses e/ou radiação. Logo, a identificação de novos alvos terapêuticos para usar possivelmente em terapêutica combinada, o que permitiria reduzir as doses de fármacos quimioterapêuticos, constitui um objetivo prioritário.

Na origem do desenvolvimento da LLA-T encontram-se sucessivas alterações genéticas em timócitos como, por exemplo, translocações cromossómicas que levam à sobre-expressão de fatores de transcrição oncogénicos ou criam genes de fusão aberrantes, deleções, duplicações e mutações específicas em proto-oncogenes ou em genes supressores de tumores. Estas alterações acabam por desregular processos cujo controlo tem uma importância extrema e que acabam por conduzir as células transformadas a um bloqueio da sua diferenciação, a adquirir capacidades de auto-renovação, de subverter os controlos da proliferação e de resistir a sinais pro-apoptóticos.

Apesar da grande maioria dos conhecimentos adquiridos até à data sobre o desenvolvimento da LLA-T referirem-se a alterações genéticas acumuladas nas células leucémicas, a importância dos fatores do microambiente onde estas células se desenvolvem tornou-se evidente nas últimas décadas. Acredita-se que as células neoplásicas podem interagir com células do estroma modificando a composição do microambiente onde se desenvolvem, em termos celulares e expressão de fatores, de modo a favorecer o desenvolvimento das primeiras. Como se pensa que a LLA-T poderá ter origem no timo e este órgão é caracterizado por um microambiente dinâmico, rico em fatores de crescimento, citoquinas e contactos linfo-estromais diretos indispensáveis ao desenvolvimento dos

timócitos e das próprias células do estroma tímico, será razoável acreditar que as células que constituem o estroma do timo poderão intervir de alguma forma no desenvolvimento da doença em questão. Recentemente, estudos realizados utilizando um modelo murino de LLA-T demonstraram que a proteína RelB, pertencente à família de fatores de transcrição NF-κB, quando expressa em células do estroma, é importante para o desenvolvimento desta doença. Esta descoberta apoia a hipótese de que sinais moleculares produzidos por timócitos transformados podem, através da ativação do fator de transcrição RelB em células do estroma tímico, levar à constituição de um microambiente favorável ao desenvolvimento de leucemia. Contudo, a identidade das células do estroma e/ou os sinais provenientes do estroma tímico que favorecem o desenvolvimento da LLA-T constituem ainda uma incógnita.

No timo, o fator de transcrição RelB é ativado em células do estroma por membros da superfamília de recetores TNF como, por exemplo, o recetor da linfotoxina beta (LTβR) ou o recetor ativador do NF-κB (RANK) e, tal como no caso da proteína RelB, a sua ausência em células do estroma tímico leva a defeitos na microestruturais. Estes defeitos, em parte caracterizados por uma diminuição do número e maturação das células epiteliais medulares do timo, são causados pela ausência de comunicação bidirecional entre timócitos que expressam os ligandos do LTβR e RANK (i.e., $LT\alpha_1\beta_2$, LIGHT e RANKL) e as células do estroma que expressam os respetivos recetores. Para além de afetar a homeostasia de células que fazem parte do estroma, a ativação dos referidos recetores induz a ativação das vias clássica e alternativa do NF-κB, conduzindo a diferentes padrões de expressão génica. A ativação dos heterodímeros p50/RelA (via clássica) resulta essencialmente na expressão de citoquinas pro-inflamatórias. Por outro lado, a ativação dos heterodímeros p52/RelB (via alternativa) resulta por exemplo, na expressão de quimiocinas como CCL19, CCL21, CXCL12 ou CXCL13. Destas, as quimiocinas CCL19 e CCL21 e o seu recetor CCR7 que é expresso por timócitos, foram previamente implicadas na disseminação de células leucémicas do tipo LLA-T. Estudos recentes sugeriram também o envolvimento da via de sinalização $LT\alpha_1\beta_2$/LTβR no desenvolvimento de leucemia e linfoma com origem em linfócitos B, nomeadamente na indução de nichos pro-tumorigénicos que suportam a viabilidade das células malignas.

É portanto possível que a interação dos ligandos, expressos em timócitos transformados, com o LTβR em células do estroma tímico promova a ativação do fator de transcrição RelB e consequente expansão das células do estroma que compõem o microambiente do timo e/ou a expressão de genes-alvo que podem eventualmente favorecer o desenvolvimento da LLA-T.

A realização deste trabalho teve portanto como objetivo geral determinar se a via de sinalização do LTβR poderá ser ativada e desempenhar um papel relevante no desenvolvimento da LLA-T. Para este fim, recorreu-se ao murganho transgénico TEL-JAK2 como modelo pois desenvolve LLA-T a partir de timócitos que expressam a referida proteína de fusão. Estes animais desenvolvem LLA-T com características histológicas e patológicas bastante similares à doença em humanos e períodos de latência variáveis que resultam da acumulação de alterações genéticas secundárias e do maior ou menor suporte de fatores do microambiente no qual as células leucémicas se desenvolvem.

Ao estudar a expressão de RNA mensageiro em linfomas tímicos de murganhos transgénicos TEL-JAK2, verificou-se expressão do gene *Ltbr*. Por outro lado, detetou-se expressão aumentada dos genes que codificam os ligandos $LT\alpha_1\beta_2$ (i.e., *Lta* e *Ltb*) e LIGHT nas células T leucémicas quando comparadas com timócitos normais. Recorrendo ao uso de inibidores farmacológicos, identificou-se a via de sinalização NF-κB como sendo a principal responsável pela indução da expressão de *Lta*, *Ltb* e *Light* nas células leucémicas, provavelmente através da ativação dos recetores de células T, i.e., pre-TCR/TCR. Para além disso, a expressão de *Lta* parece ser também induzível pela via JAK/STAT. É portanto possível que a sinalização pelo LTβR esteja ativa em linfomas tímicos de ratinhos TEL-JAK2, tal como acontece no timo normal. Todavia, apenas a expressão de $LT\alpha_1\beta_2$, em detrimento de LIGHT, foi detetada à superfície de células T leucémicas cultivadas *ex vivo* ou estimuladas com mitogénios, i.e., PMA e ionomicina. Além disso, verificou-se diminuição da expressão de $LT\alpha_1\beta_2$ à superfície *in vivo* quando estas células contactam células do estroma que expressam o LTβR, pois aquele ligando é facilmente detetado quando as células permanecem em ambientes desprovidos do recetor. Portanto, as dificuldades encontradas em termos de deteção dos ligandos em microambientes com expressão do LTβR serão consequência de um controlo apertado em termos de expressão superficial do ligando e/ou sinalização continuada seguida por clivagem do mesmo.

De forma a avaliar se o desenvolvimento da leucemia induzida pela proteína de fusão TEL-JAK2 é comprometido na ausência do LTβR, cruzaram-se murganhos transgénicos TEL-JAK2 com murganhos nos quais a expressão do gene *Ltbr* foi geneticamente eliminada, de forma a gerar 2 coortes: TEL-JAK2;$Ltbr^{-/-}$ e TEL-JAK2;$Ltbr^{+/-}$ (controlo). Neste contexto, verificou-se que a inativação do LTβR levou a um atraso significativo no desenvolvimento da leucemia apesar da carga tumoral em órgãos linfóides e o fenótipo celular de superfície das células leucémicas não serem significativamente diferentes entre os dois grupos na fase terminal da doença. Curiosamente, numa fase precoce, quando os animais ainda não

apresentariam sinais de doença, verificou-se uma expansão diminuída de timócitos malignos, caracterizados pela expressão de CD8 e CD25, no timo de murganhos transgénicos TEL-JAK2;*Ltbr*$^{-/-}$ em relação a TEL-JAK2;*Ltbr*$^{+/-}$, o que implica o eixo de sinalização $LT\alpha_1\beta_2$/LTβR na fase inicial do desenvolvimento da leucemia. Além disto, a expressão de RANKL por células T leucémicas e do seu recetor RANK em linfomas tímicos, abrem caminho à possibilidade de os dois receptores, LTβR e RANK, colaborarem na indução do nicho adequado para suportar o desenvolvimento de células leucémicas no murganho transgénico TEL-JAK2.

De forma a verificar se as observações descritas poderão ser transpostas para LLA-T humana, procedeu-se inicialmente ao estudo de linhas celulares humanas. Várias semelhanças foram verificadas no que diz respeito ao padrão de expressão de $LT\alpha_1\beta_2$ e à sua regulação por NF-κB. Para além disso, detetou-se a expressão dos genes que codificam LTα e LTβ em amostras de doentes com LLA-T, encontrando-se significativamente aumentada no subtipo molecular TAL/LMO.

Assim, pode-se concluir que a ativação do recetor LTβR no microambiente por células T leucémicas que expressam o ligando $LT\alpha_1\beta_2$ contribui para o desenvolvimento de LLA-T numa fase precoce da doença. É portanto essencial o desenvolvimento de mais estudos visando clarificar os mecanismos pelos quais a comunicação entre células leucémicas e células do estroma através do eixo de sinalização $LT\alpha_1\beta_2$/LTβR poderá apoiar a LLA-T e determinar a utilidade da inibição desta via como nova estratégia terapêutica.

PALAVRAS-CHAVE: Leucemia linfoblástica aguda de linfócitos T, modelo murino transgénico TEL-JAK2, timo, microambiente tumoral, recetor da linfotoxina beta, fator nuclear κB.

ABSTRACT

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematopoietic malignancy that arises from the combination of genetic and epigenetic alterations in thymic T-cell precursors and extracellular signals provided by the microenvironment. It was previously found that RelB expression in non-hematopoietic stromal cells promoted T-cell leukemogenesis in the EμSRalpha-TEL-JAK2 transgenic (TJ2-Tg) mouse model. In thymic stromal cells, RelB is a transcription mediator of lymphotoxin-beta receptor (LTβR). Lymphotoxin-mediated activation of LTβR has been implicated in physiological crosstalk between T cells and lymphoid organ stromal cells, but also pathological processes, including carcinogenesis. Since its role in T-ALL has remained elusive, we aimed to determine whether LTβR signaling is activated and playing a role in TEL-JAK2-induced leukemogenesis. In TJ2-Tg thymic lymphomas, activation of $LT\alpha_1\beta_2$-LTβR signaling axis was supported by LTβR-encoding gene expression, while the genes encoding its cognate ligand, lymphotoxin (LT)-α and LTβ, were found to be expressed by leukemic T cells, in an NF-κB-dependent manner. $LT\alpha_1\beta_2$ protein was detected at the surface of TJ2-Tg leukemic cells only upon *ex vivo* culture or mitogenic stimulation. Moreover, we found that cell-surface $LT\alpha_1\beta_2$ is downmodulated *in vivo*, indicating ongoing signaling. Further supporting a role for lymphotoxin signaling, LTβR genetic deficiency delayed TEL-JAK2-induced leukemia onset, but the tumor load in lymphoid organs and leukemia cell surface phenotype were comparable in end-stage disease. In accordance, the detection of reduced proportions of malignant thymocytes in TJ2-Tg;*Ltbr*$^{-/-}$ mice with no signs of disease implicated LTβR in early stages of leukemia development. Together, these data indicate that T-ALL-derived lymphotoxin activates LTβR signaling in thymic stromal cells, promoting leukemogenesis. Importantly, lymphotoxin-encoding genes were expressed in T-ALL patient samples, indicating that these may be also involved in human disease. Thus, future studies should provide a better understanding on how cellular crosstalk mediated by the lymphotoxin-LTβR axis supports T-ALL and assess the utility of blocking LTβR signaling as a novel therapeutic approach.

KEYWORDS: T-cell acute lymphoblastic leukemia, TEL-JAK2 transgenic mouse model, thymus, tumor microenvironment, lymphotoxin-beta receptor, nuclear factor kappaB.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

2D – two dimensional
3D – three dimentional
ºC – degree Celsius
×g – times gravity

A

7-AAD – 7-aminoactinomycin D
ABL1 – Abelson murine leukemia viral oncogene homolog 1
ActD – actinomycin D
Aire – autoimmune regulator
ALL – acute lymphoblastic leukemia
AML – acute myeloid leukemia
AP-1 – activator protein 1
AP2 – adaptor protein 2
APC – allophycocyanin
ASK1 - apoptosis signal-regulating kinase 1

B

BAFF – B-cell activating factor
BCL11B – B-cell leukemia/lymphoma 11B
BCR – B-cell receptor
BLC – B lymphocyte chemoattractant
BM – bone marrow
BSA – bovine serum albumin

C

CaP – prostate cancer
CCND2 – cyclin D2
CD (number) – cluster of differentiation
CD – cytoplasmic domain
CDKN2A – cyclin-dependent kinase inhibitor 2A
cDNA – complementary DNA
c-fos – FBJ murine osteosarcoma viral oncogene homolog
Chr – chromosome
CHX – cycloheximide
cIAP1/2 – cellular inhibitor of apoptosis 1/2
CLL – chronic lymphocytic leukemia
CLP – common lymphoid progenitor
CMP – common myeloid progenitor
CMR – cortico-medullary region
CNS – central nervous system
CO_2 – carbon dioxide
CRD – cysteine-rich domain
CREB – cAMP response element-binding protein
c-Src – C-terminal Src kinase

D

DAMP – damage-associated molecular patterns
DC – dendritic cell
DcR3 – decoy receptor 3
DEPC – diethylpyrocarbonate
DLL – delta-like ligand
DMEM– Dulbecco's modified eagle medium
DMSO – dimethyl sulfoxide
DN – double negative
DNA - deoxyribonucleic acid
dNTP - deoxyribonucleotide
DP – double positive

E

ECD – extracellular domain
ECL – enhanced chemoluminescence
EDTA – ethylenediamine tetraacetic acid
EGIL – European Group for Immunological Characterization of Leukemias
Egr-1 – early growth response protein 1
ELC – EBl1-ligand chemokine
ETP – early T-cell precursor
Ets – E-twenty six
EZH2 – enhancer of zeste homolog 2

F

FAA – formaldehyde-acetic acid-alcohol
FACS – fluorescence-activated cell sorting
FBS – fetal bovine serum
FBXW7 – F-box/WD domain-containing 7
FDC – follicular dendritic cell
FITC – fluorescein isothiocyanate
FLT3 – Fms-like tyrosine kinase 3
FRC – fibroblastic reticular cell
FVB mice – Friend virus B-type

G

GALT – gut-associated lymphoid tissues
GAPDH – glyceraldehyde 3-phosphate dehydrogenase

H

h – hour
HBV – hepatitis B virus
HCC – hepatocellular carcinoma
HCV- hepatitis C virus
HEV – high endothelial venules
HMGB1 – high-mobility group protein B1
HOX – homeobox gene
HPRT1 – hypoxanthine phosphoribosyltransferase 1
HPV – human papilloma virus
HRP – horseradish peroxidase
HSC – hematopoietic stem cell
HVEM – herpes virus entry mediator

I

ICAM-1 – intercellular adhesion molecule 1
ICD – intracellular domain
ICN1 – intracellular NOTCH1
IFNγ – interferon gamma
IGF-1 – insulin-like growth factor 1
IKK – IkappaB kinase
IL – interleukin
Inr – initiator element
Iono – ionomycin

J

JAK – Janus kinase
JNK – c-Jun N-terminal kinase

K

KLH – keyhole limpet hemocyanin
Krt – keratin

L

LBL – lymphoblastic lymphoma
LCK – lymphocyte-specific tyrosine kinase
LEF1 – lymphoid enhancer-binding factor 1
LFA-1 – lymphocyte function-associated antigen
LIC – leukemia-initiating cell
LIGHT – lymphotoxins, exhibits inducible expression, and competes with HSV glycoprotein D for HVEM, a receptor expressed by T lymphocytes
LMO – LIM domain only
LN – lymph node
LSC – leukemic stem cell
LT – lymphotoxin
LTβR – lymphotoxin-beta receptor
LYL1 – lymphoblastic leukemia-derived sequence 1

M

MAPK – mitogen-activated protein kinase
mBD14 – mouse beta-defensin 14
M-CSF – macrophage colony-stimulating factor
MEF – mouse embryonic fibroblast
MEF2C – myocyte-specific enhancer factor 2C
MEMα – minimum essential medium alpha
MHC – major histocompatibility complex
MIP – macrophage inflammatory protein
MITF – microphthalmia-associated transcription factor
MKK – MAP kinase kinase
MLL – mixed-lineage leukemia
MMP – matrix metalloproteinase
MPP – multipotent progenitor
mRNA – messenger RNA
miRNA – micro RNA
mTOR – mammalian target of rapamycin
MYB – myeloblastosis oncogene
MYC – myelocytomatosis oncogene
MyoD – myogenic differentiation

N

NaN3 – sodium azide
NEC – non-enzyme control
NF1 – nuclear factor 1
NFAT – nuclear factor of activated T cells
NF-κB – nuclear factor-kappa B
NIK – NF-κB-inducing kinase
NK – natural killer
NKX2-1 – NK2 homeobox 1
NMS – normal mouse serum
NPC – nasopharyngeal carcinoma
NS5B – nonctructural protein 5B
NTC – non-template control
NUP214 – nucleoporin 214

O

Oct-1 – octamer-binding transcription factor
ODF – osteoclast differentiation factor
OPG – osteoprotegerin

P

PAGE – polyacrylamide gel electrophoresis
PB – peripheral blood
PBMC – peripheral blood mononuclear cell
PBS – phosphate-buffered saline
PCR – polymerase chain reaction
PDC – pancreatic ductal carcinoma
PE - phycoerythrin
PE-Cy5 – phycoerythrin-cyanine 5
PGE2 – prostaglandin E2
PHF6 – PHD finger protein 6
PI – propidium iodide
PICALM – phosphatidylinositol binding clathrin assembly protein
PI3K – phosphoinositide 3-kinase
PMA – phorbol myristate acetate
PTEN – phosphatase and tensin homolog

Q

qPCR – quantitative PCR

R

Rag2 – recombination activating gene 2
RAS – rat sarcoma gene
RasGRP1 – Ras activator guanine nucleotide exchange factor
RANK – receptor activator of NF-κB
RANKL – RANK ligand
RB – retinoblastoma gene
RBC – red blood cell
RelA – v-Rel reticuloendotheliosis viral oncogene homolog A
RelB – v-Rel reticuloendotheliosis viral oncogene homolog B
RIPA – radioimmunoprecipitation assay
RNA – ribonucleic acid
ROS – reactive oxygen species
rRNA – ribosomal RNA
RT – reverse transcription
RUNX1 – runt-related transcription factor 1

S

SCF – stem cell factor
SCL – stem cell leukemia
SDS – sodium dodecyl sulfate
SLC – secondary lymphoid tissue chemokine
Socs2 – suppressor of cytokine signaling 2
SP – single positive
Sp1 – stimulating protein 1
SRE – skeletal-related effects
STAT – signal transducer and activator of transcription

T

TAB1 – TAK1-binding protein
TACE – TNF alpha-converting enzyme
TAE – Tris-acetate
TAK1 – TGF-beta activated kinase
TAL1 – T-cell acute lymphoblastic leukemia protein 1
TCR – T-cell receptor
TEC – thymic epithelial cell
TEL – translocated ets leukemia
TET1 – ten-eleven translocation-1
TJ2-Tg – TEL-JAK2 transgenic
TLX – T-cell leukemia homeobox
TMD – transmembrane domain
TNFα – tumor necrosis factor alpha
TNFSF – TNF superfamily
TNFRSF – TNF receptor superfamily
TRANCE – TNF-related activation-induced cytokine
TSC – thymic stromal cell
TRA – tissue-restricted antigen
TRAF – TNF receptor-associated factor
TRAMP – transgenic adenocarcinoma mouse prostate

U

UTR – untranslated region

V

VCAM-1 - vascular-cell adhesion molecule 1

W

WBC – white blood cell
WT1 – Wilms tumor-1

CHAPTER 1

INTRODUCTION

"To study the abnormal is the best way to understanding the normal."

Although this famous statement from William James (American philosopher and psychologist) addressed character studies, it may be applied in the context of malignancy to state that there is no sharp line drawn between healthy/good or unhealthy/bad players in cancer development. Also, studying the normal context where a specific type of cancer develops may reveal hints on how cells become malignant/abnormal. In the specific case of T-cell acute lymphoblastic leukemia (T-ALL), several studies support the notion that in order to become transformed, T-ALL cells undergo cell-intrinsinc alterations and evolve by taking advantage of normal developmental mechanisms.

In this first chapter, a review of the literature on normal T-cell development and the determinants of transformation leading to T-ALL is presented. Possible therapeutic targeting approaches and experimental models used currently to study T-ALL are also revisited. In addition, important notions on signaling and functions attributed to two TNF superfamily receptors, lymphotoxin-beta receptor (LTβR) and receptor activator of NF-κB (RANK) are provided.

1.1. HEMATOPOIESIS AND T LYMPHOCYTE DEVELOPMENT

All mature blood cell types are derived from bone marrow (BM) resident hematopoietic stem cells (HSCs), through a developmental process called hematopoiesis. Throughout this process, HSCs experience progressive loss of self-renewal properties as they differentiate to multipotent progenitors (MPPs), which lack the ability to self-renew. In addition, HSC multilineage differentiation capabilities are also progressively restrained while the cell commits to a particular lineage (Lai and Kondo, 2008).

Two conflicting lines of evidence defend that the first step towards lineage restriction occurs in different phases of hematopoiesis. The so-called classic model states that MPPs originate symmetrically either a common myeloid progenitor (CMP) or a common lymphoid progenitor (CLP) (Akashi et al., 2000; Kondo et al., 1997). These common progenitors have

differentiation potentials restricted to all cell types within their respective lineage: megakaryocytes, erythrocytes, granulocytes, and macrophages are originated by CMPs, while T, B and NK lymphocytes derive from CLPs. Challenging this model, an alternative version in which common progenitors are generated asymmetrically from MPPs was put forward. Accordingly, myeloid cells were shown to potentially diverge from different subsets of MPPs, while lymphoid differentiation appeared to surpass multiple obligatory lineage restriction steps in such a way that CLPs are generated when the cells lose all abilities to generate myeloid cells (Adolfsson et al., 2005; Lai and Kondo, 2006). Furthermore, the existence of CLPs in the BM is also controversial. In fact, some studies demonstrated that the B lineage branch is segregated from the T cell pathway before a branch point for the T versus myeloid lineage (Bell and Bhandoola, 2008; Porritt et al., 2004; Wada et al., 2008). In spite of these debatable issues concerning hematopoiesis, the notion that in normal conditions BM-derived progenitors have to migrate to the thymus in order to originate self-tolerant, functional T lymphocytes (also known as T cells) is widely accepted (Sitnicka, 2009).

Like the BM, the thymus is a primary lymphoid organ but its only known function is to support the development of T lymphocytes from T-cell progenitors (Heinonen and Perreault, 2008). In humans, the thymus is located in the upper anterior mediastinum and lower part of the neck and is most active during childhood, reaching a peak weight at puberty, after which it undergoes slow involution (Gray et al., 2008). It is composed by two lobes and invested externally by a loose collagenous capsule. Each lobe is further divided in lobules by incomplete septa, which contain a peripheral dark cortical area densely populated by lymphoid cells, and a light medullary area composed of many voluminous pale cells and less abundant lymphoid cells (Milićević et al., 2008; Rezzani et al., 2008). A transitional area rich in blood vessels, known as cortico-medullary region (CMR) separates the thymic cortex and medulla (Figure 1.1). This is the entry site for early T cell precursors (ETPs), which derive from progenitors that emigrate from the BM to the thymus (Lind et al., 2001), and also the exit site for the end-product of thymopoiesis, naïve mature T lymphocytes (Jin et al., 2006). T-cell precursors developing in the thymus, also known as thymocytes, make up approximately 90% of the thymic cellular compartment and form a heterogeneous population composed of cells in different stages of differentiation. According to the cell phenotypes defined by expression of CD4 and CD8 coreceptors, thymocytes can be divided into four major subsets that represent consecutive steps in development namely, $CD4^-CD8^-$ double negative (DN), $CD4^+CD8^+$ double positive (DP), $CD4^+CD8^-$ single positive (CD4 SP), and $CD4^-CD8^+$ single positive (CD8 SP). Mouse DN thymocytes are further classified from DN1

to DN4 based on different profiles of CD117 (also known as c-kit), CD25 and CD44 expression (Godfrey et al., 1993) (Figure 1.2).

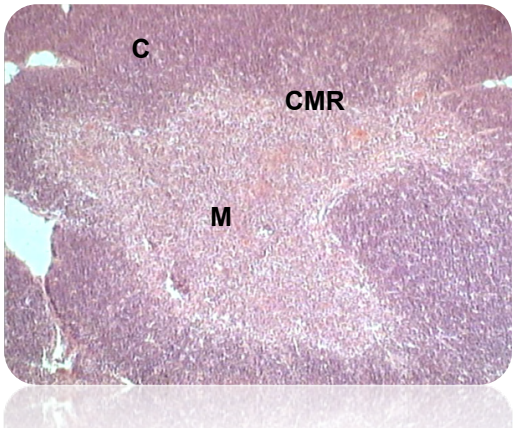
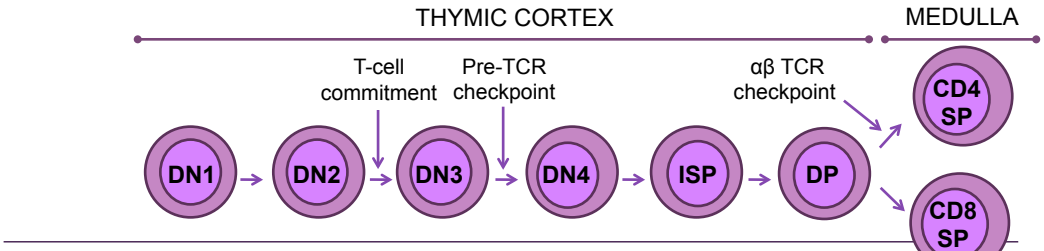


Figure 1.1. Thymus Structure. Hematoxilin-eosin staining of a thymus section showing the thymic structure, composed of an external region, the cortex (C), an internal region, the medulla (M), separated by the cortico-medullary region (CMR).



	Immunophenotype							
	CD4	-	-	-	-	+ (Human)	+	+ CD4 SP
	CD8	-	-	-	-	+ (Mouse)	+	+ CD8 SP
	sCD3	-	-	-	-	-	-/+	+
MURINE	CD117	+	+	-	-	-	-	-
MURINE	CD25	-	+	+	-	-	-	-
MURINE	CD44	+	+	-	-	-	-	-
MURINE	CD24	+	+	+	+	+	+/-	-
MURINE	CD69	-	-	-	-	-	-/+	+
HUMAN	TdT	+	+	+	+	+	+	-
HUMAN	CD34	+	+	+	+	+/-	-	-
HUMAN	CD38	-	+	+	+	+	+	+
HUMAN	CD1a	-	-/+	+	+	+	+/-	-

Figure 1.2. Scheme depicting T-cell developmental stages in the thymus, and murine and human thymocyte markers. The different thymocyte subpopulations defined by CD4/CD8 expression are shown along with the thymic region where they are located, their immunophenotypes, and the main checkpoints found during T-cell development. Differences between murine and human markers are shown.

In the thymus, T cell precursors interact with stromal cells, mainly dendritic cells (DC), fibroblasts, macrophages, endothelial cells, and epithelial cells, which make up a three-dimensional network and distinct microenvironments characterized by the production of diverse sets of cytokines, growth factors, and adhesion molecules (Petrie and Zúñiga-Pflücker, 2007; Takahama, 2006). Recent thymic immigrants acquire T-lineage potential and progress through distinct developmental stages by migrating through diverse inductive microenvironments of the thymus guided by factors produced by stromal cells. In such locations, they interact with stromal cells or are simply exposed to factors that control defined steps in T-cell development, such as proliferation, differentiation, T-cell receptor (TCR) gene rearrangements, and selection (Takahama, 2006).

For the initial TCR-independent proliferation and differentiation until the DN3 stage, thymocytes are exposed to signals derived from cortical stromal cells as they migrate to the subcapsular region and begin to rearrange their *Tcr* loci (Capone et al., 1998; Lind et al., 2001). The complementary expression patterns of both cytokines and their receptors determine their function at every stage of T-cell maturation. For example, gradients of chemokines like chemokine (C-X-C motif) ligand 12 (CXCL12), chemokine (C-C motif) ligand 25 (CCL25), CCL19 or CCL21 were shown to attract and mediate migration of thymocytes expressing their cognate receptors (i.e., CXCR4, CCR9 and CCR7, respectively) (Fu and Chen, 2004; Sitnicka, 2009; Takahama, 2006). Furthermore, expansion and proliferation of immature progenitors are dependent on stem cell factor (SCF) binding to c-kit in thymocytes and on signals delivered by interleukin-7 (IL-7) (Peschon et al., 1994; Rodewald et al., 1997), whereas Notch-mediated signals through binding of Delta-like 1 (DLL1) and Delta-like 4 (DLL4) ligands are also required for T lineage specification at the expense of B lineage (Pui et al., 1999; Radtke et al., 1999; Wilson et al., 2001). After T lineage commitment, DN thymocytes rearrange the genes encoding β, γ, and δ TCR chains. A small proportion of thymocytes productively rearrange γ and δ chains, express TCR γδ at their surface, and are named accordingly as γδ T cells. These cells do not differentiate further in the thymus and have functions related to innate immunity. On the other hand, the vast majority of thymocytes rearrange the TCRβ loci and express a functional TCRβ chain that associates with pre-TCRα (pTα), generating the pre-TCR complex at the cell surface. Through the pre-TCR complex, thymocytes receive specific survival and proliferation signals required for further differentiation to immature $CD4^+$ single positive (ISP) ($CD8^+$ISP in mice) and DP thymocytes (Figure 1.2). In addition, DP thymocytes that rearrange the TCRα loci to

form the TCRαβ complex, undergo TCR-mediated positive and negative selection through interaction with major histocompatibility (MHC) complexes expressed by cortical TECs (cTECs) and DCs (Goldrath and Bevan, 1999). Low-affinity recognition of self-MHC, promotes thymocyte survival and CCR7 expression, which drives migration to CCL19 and CCL21 produced by medullary TECs (mTECs) (Ueno et al., 2004), and determines commitment to either the CD4 or CD8 SP lineage. In the medulla, SP thymocytes that are reactive to tissue-specific antigens promiscuously presented by mTECs are deleted, a process termed negative selection, which is essential for the establishment of central tolerance (Chen, 2004; Takahama, 2006). Finally, the egress of mature T cells from the thymus was also shown to rely on thymic stromal-derived elements either by chemorepulsion or loss of responsiveness to thymic retention signals. In addition, a third mechanism was also described involving chemoattraction to peripheral signals (Jin et al., 2006).

In addition to the multiple roles attributed to stromal cells in thymocyte development, SP thymocytes reciprocally support mTEC organization and differentiation mainly through the action of members of the tumor necrosis factor (TNF) superfamily of ligands and receptors, such as $LT\alpha_1\beta_2$/LTβR, RANKL/RANK, and CD40L/CD40 (Boehm et al., 2003; van Ewijk et al., 2000; Hikosaka et al., 2008; Klug et al., 1998). In general, these lympho-stromal bidirectional exchanges are referred to as thymic crosstalk. In normal conditions, hematopoiesis is tightly regulated by an exquisite balance between expression and extinction of transcription factor action in various combinations and their associated chromatin remodeling factors. Furthermore, it is controlled by the diverse array of factors that mediate complex bidirectional interactions between the microenvironment and hematopoietic stem cells or progenitors. Importantly, these two cell-intrinsic and microenvironmental factors are closely linked since certain transcription factors are inducible by microenvironmental cues (Kittipatarin and Khaled, 2007; Mohtashami et al., 2013; Peschon et al., 1994; Schmitt et al., 2004). When the regulation of proliferation, differentiation and/or survival fails, hematopoietic malignancies such as leukemia and lymphoma may arise. These are designated after the affected cell lineage as myeloid or lymphoid and, when untreated, can range from being rapidly fatal (acute) to slowly growing (chronic).

1.2. T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

The predominant types of pediatric cancers are leukemia, lymphoma, and cancers of the brain and central nervous system (CNS). Acute lymphoblastic leukemia (ALL) is the most common childhood malignancy and represents about 80% of all leukemia cases in children and 56% in adolescents. This percentage is significantly lower in adults (American Cancer Society, 2015). ALL consists of a group of malignancies of lymphoid cells that morphologically and immunophenotypically resemble B-lineage (B-ALL) and T-lineage precursor cells (T-ALL). These neoplasms may present predominantly with primary involvement of the bone marrow and peripheral blood (ALL) or may be limited to tissue infiltration, with absent or only limited bone marrow involvement, being in this case designated as lymphoblastic lymphomas (LBL). A combination of the two presentations often coexists. In this case, the disease is classified as leukemia when 25% or more lymphoblasts are detected in the bone marrow, even in the presence of tumor masses in organs such as lymph nodes or thymus (Vardiman et al., 2009).

T-cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematologic malignancy that originates from T-cell precursors. In comparison with B-ALL, T-ALL is relatively rare and, importantly, is overall characterized by an inferior treatment outcome (Pui et al., 1990). This disease affects mainly children and adolescents but also adults and accounts for about 15% and 25% of ALL in pediatric and adult cohorts, respectively (Pui et al., 2004). T-ALL is characterized by high white blood cell counts, higher or lower replacement of the bone marrow cellular population with the malignant clone, which interferes with normal development of blood cells, and variable infiltration of organs like the lymph nodes, spleen, liver and the CNS. In some cases, a mediastinal mass is present with or without pleural effusions, which may lead to respiratory distress (Longo, 2012; Pui et al., 1990). Therefore, although this neoplastic disorder originates either in the BM or in the thymus, leukemic cells metastasize throughout the body and it is rapidly fatal without appropriate therapy. It is though a heterogeneous disease regarding clinical presentation.

The precise cell-of-origin is still debated but T-ALL cells share numerous cellular, immunophenotypic, and molecular properties with thymocytes at different stages of differentiation. Therefore, transformation events most likely occur in crucial stages of T-cell development, and thymocytes are considered the normal counterparts of T-ALL (Asnafi et

al., 2003; Crist et al., 1988; Ferrando and Look, 2003; Reinherz et al., 1980). Furthermore, it was previously shown that altering the cell-of-origin in mice produces leukemic cells that model the intrinsic genetic heterogeneity of human T-ALL (Berquam-Vrieze et al., 2011). Due to the heterogeneity found in leukemic cells from different patients, T-ALL is normally classified in different subgroups relying either on the immunophenotype, or on the gene expression profile. Each subgroup has in turn been associated with a particular disease prognosis, yet not sufficiently compelling to justify its use to determine the use of different treatment protocols (Ferrando et al., 2002; Homminga et al., 2011; Soulier et al., 2005).

Three main classifications segregate T-ALL in different subtypes based on the presence or absence of immunophenotypic markers. The European Group for the immunological characterization of leukemias (EGIL) classifies 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 ($mCD3^+$, $CD1a^-$) (Bene et al., 1995), from the most immature to the mature stage. In addition, the more recently proposed TCR-based classification system, divides T-ALL in four stages: immature stage (cytoplasmic (c) $TCR\beta^-$, surface (s) $CD3^-$), pre-αβ stage ($cTCR\beta^+$, $sCD3^-$, $pT\alpha^+$), TCRαβ ($sCD3^+$, $TCR\alpha\beta^+$), and TCRγδ ($sCD3^+$, $TCR\gamma\delta^+$) (Asnafi et al., 2003). 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 or signatures, which are associated with the underlying expression of particular oncogenes (Ferrando et al., 2002; Homminga et al., 2011; Soulier et al., 2005).

Similarly to other types of cancer, T-ALL genesis and progression are driven by a combination of intrinsic genetic lesions and extrinsic events that are dependent on the interaction with the stroma. The cooperating effects of these alterations culminate in differentiation arrest, uncontrolled cell growth, and clonal expansion of T-cell precursors.

1.2.1. Cell-intrinsic mechanisms

Although T-ALL presents often with a normal diploid karyotype, numerical and structural chromosomal abnormalities may also occur. Indeed, many studies have identified several structural chromosomal abnormalities that contribute to leukemogenesis in addition to other genetic alterations. These genetic abnormalities are variably present and were classified in type A and type B (Meijerink, 2010; Van Vlierberghe et al., 2008). Type A abnormalities usually involve chromosomal translocations, large genomic deletions or amplifications that are essentially mutually exclusive and define T-ALL subgroups (Table 1.1). Chromosomal

translocations involving TCR gene rearrangements and gene fusions are a common consequence of physiologic TCR rearrangements that occur in normal T cell development. Frequently, these lead to ectopic expression of oncogenic transcription factors in developing thymocytes and differentiation arrest (Ferrando and Look, 2003; Ferrando et al., 2002; Reinherz et al., 1980). Therefore, type A abnormalities are generally considered driving chromosomal abnormalities or initiating events. Although the oncogenes involved are usually activated by chromosomal rearrangements, they can also be overexpressed by other means, such as activating deletions or other mutations, epigenetic mechanisms, or upstream regulatory molecules (Ferrando and Look, 2003; Ferrando et al., 2002; de Leval et al., 2009; Van Vlierberghe et al., 2008). T-ALL oncogenic transcription factors include basic helix-loop-helix (bHLH) family members such as TAL1/SCL, TAL2, LYL1; LIM-only domain (LMO) genes like LMO1 and LMO2; homeobox family members including TLX1/HOX11, TLX3/HOX11L2, HOXA, and NKX2-1; and also MYB proto-oncogene and MEF2C (Homminga et al., 2011; Van Vlierberghe et al., 2008). Frequently, chromosomal translocations or inversions result in gene fusions that are expressed as constitutively activated chimeric oncoproteins (e.g., SIL-TAL1, PICALM-MLLT10, and MLL-MLLT1) (Van Vlierberghe et al., 2008).

Table 1.1. Common type A genetic abnormalities detected in T-ALL.

Genetic abnormality	Genes involved	Subgroup	Effect
Translocations involving TCR and Gene Fusions	*TAL1*, *LMO2*, *LYL1*	TAL/LMO	Differentiation impairment
	TLX1, *TLX3*	TLX1/3	
	HOXA cluster	HOXA	
	MYB, *NKX2-1*/*NKX2-2*	Proliferative	
	MEF2C	Immature	
	SIL-TAL1	TAL/LMO	
	PICALM-MLLT10	HOXA	
	MLL-MLLT1	HOXA	
	SET-NUP214	HOXA	

Type B abnormalities include gene-specific small deletions, translocations, duplications and point mutations in proto-oncogenes or tumor suppressor genes (Table 1.2). These are found in different combinations throughout all major T-ALL subgroups and seem

to synergize with type A abnormalities during T-cell leukemogenesis. Several genes encoding members of signaling pathways are affected by activating point mutations and include *RAS*, *JAK1*, *JAK3*, *FLT3*, *IL7R* (Zhang et al., 2012), *PI3K*, and *AKT* (Gutierrez et al., 2009). Similarly, and besides being involved in low frequency T-ALL translocations, the *NOTCH1* proto-oncogene can also carry activating mutations, as reported for more than 50% of T-ALL patients (Palomero et al., 2007; Weng et al., 2004). The Notch signaling pathway is also activated in T-ALL due to inactivating point mutations in its negative regulator *FBXW7* (Thompson et al., 2007). Other inactivating mutations or deletions occur in the Ras inhibitor *NF1* (Balgobind et al., 2008) and in the PI3K negative regulator *PTEN* (Gutierrez et al., 2009), leading to activation of Ras and PI3K/AKT/mTOR signaling pathways, respectively. Gene fusions like *ETV6*/*TEL-JAK2* or *BCR-ABL1* are translated as constitutively active kinases and lead to aberrant signaling activation (Carron et al., 2000; De Keersmaecker et al., 2005). In addition, deletions in *CDKN2A* cell cycle regulator lead to disruption of p53-induced apoptosis pathway (Hebert et al., 1994). Cell cycle defects are also originated due to inactivating deletions in *RB1*, which encodes the tumor suppressor retinoblastoma (Rb) protein, and to translocations in *CCND2* that lead to the activation of cyclin-D2, and consequent inactivation of Rb (Van Vlierberghe and Ferrando, 2012). Tumor suppressor transcription factors have also been shown to be inactivated in T-ALL including WT1, LEF1, GATA3, RUNX1 or BCL11B (Gutierrez et al., 2010, 2011; Tosello et al., 2009; Zhang et al., 2012). Recent studies have also shown high frequency of somatic mutations in epigenetic regulators. Inactivating mutations in *PHF6* and *EZH2* leading to chromatin remodeling alterations were reported in addition to alterations in genes involved in DNA methylation/demethylation such as *TET1* (Peirs et al., 2015). Finally, mutations affecting ribosomal proteins, which are essential components of the translational machinery, were also recently revealed (De Keersmaecker et al., 2013).

When reviewing the current list of oncogenes and tumor suppressor genes involved in T-ALL development, it becomes clear that these are linked to thymocyte differentiation and self-renewal or general cellular functions like proliferation and survival, cell cycle control, and DNA repair (De Keersmaecker et al., 2005).

Table 1.2. Common type B genetic abnormalities detected in T-ALL.

Genetic abnormality	Genes involved	Function	Main effects
Activating Mutations	*NOTCH1*	Transcription activation mediated by ICN	Self-renewal
	LCK	Signal transduction from the TCR complex	Proliferation
	FLT3	Cytokine receptor	Proliferation and survival
	JAK1 / *JAK3*	Signal transduction from cytokine receptors	Proliferation and survival
	RAS	Signal transduction	Proliferation and survival
	IL7R	Common γ-chain cytokine receptor	Proliferation and survival
	AKT	Signal transduction	Proliferation and survival
	PI3K	Signal transduction	Proliferation and survival
Inactivating Mutations and Deletions	*FBXW7*	Cyclin E, Myc and NOTCH1 targeting	Self-renewal
	NF1	Ras inhibition	Proliferation and survival
	PTEN	Signal transduction (PI3K inhibition)	Proliferation and survival
	WT1	Tumor supressor transcription factor	Survival
	LEF1	Tumor supressor transcription factor	Proliferation and survival
	PHF6	Epigenetic regulator	Chromatin remodeling
	EZH2	Epigenetic regulator	Chromatin remodeling
	CDKN2A	Cell cycle regulator	Cell cycle (CC) activation
	RB1	Cell cycle regulator	CC activation
Translocations	*CCND2*	Cell cycle regulator	CC activation
	MYC	Cell growth transcription factor	Proliferation and survival
	ABL1 fusions	Signal transduction	Proliferation and survival
	TEL-JAK2	Signal transduction	Proliferation and survival

1.2.2. Microenvironmental factors

Although the roles of genetic lesions and epigenetic alterations as steps toward T-ALL development have been largely appreciated, the various aspects on the impact of the microenvironment have been mostly overlooked. It is now recognized that cancers develop in complex microenvironments composed of a dynamic and interactive mixture of different cell types and cytokines plus growth factors, which may sustain cancer-cell growth, invasion, and metastization (Tlsty and Coussens, 2006). In lymphoid cancers, the microenvironment often consists of variable numbers of malignant cells intermixed in a matrix of the so-called stromal cells (e.g., endothelial and epithelial cells, fibroblasts, DCs, macrophages), and also often non-malignant lymphocyte infiltration (Herreros et al., 2008). In fact, tumor cells and the surrounding stroma evolve together through continuous communication through paracrine and/or juxtacrine signaling, which culminates in the creation of permissive or selective microenvironments that support malignant cells to survive, grow, and resist to immune recognition and elimination. An altered microenvironment may disrupt the homeostasis and favor the early steps of transformation, or even promote protection against therapeutic intervention (Raaijmakers, 2010; Zhang et al., 2012). In this context, the malignant cells may respond passively to physiological signals or even become unresponsive to deleterious signals provided by their local microenvironment. Alternatively, cancer cells may more actively modulate stromal cells to change their gene expression profile and thus create more appropriate microenvironments for cancer cell growth and survival (Hanahan and Weinberg, 2000). Importantly, there is reliable evidence that neoplastic cells may interact with the surrounding environment in a bidirectional manner resulting in the acquisition of a competitive advantage by the malignant cells during oncogenesis (Colmone et al., 2008; Scupoli et al., 2003, 2007).

Notably, the thymus, an organ specialized in T cell development, contains a dynamic microenvironment with an high concentration of growth factors, cytokines and stromal cells necessary for thymopoiesis, making it also a potential permissive location for leukemogenesis and tumor progression. Normal thymocytes arise in the thymus where they interact with thymic stromal cells (TSCs) and thus promote T-cell development process and to maintenance of the stromal cell pool and its three-dimensional organization (Boehm et al., 2003). Normal thymocytes divide, differentiate and egress the thymus whereas leukemic T cells undergo a differentiation block and continue to grow possibly under the support of TSCs until some of them eventually acquire the capacity of microenvironment-independent

growth (Hiai et al., 1981). The absolute requirement for the presence of the thymus in murine leukemogenesis is illustrated by results showing that thymectomy impairs T-cell leukemia development induced by γ-irradiation (Kaplan, 1950), murine leukemia viruses (McEndy et al., 1944) or Ikaros protein deficiency (Dumortier et al., 2006). Moreover, a recent study has shown that lack of competition between bone marrow-derived progenitors and T-cell precursors developing in the thymus causes T-cell leukemia that shares many properties with human T-ALL (Martins et al., 2014). Cell competition was thus considered a tumor suppressor mechanism because it acts to eliminate older precursors in the thymus that may display upregulated or mutated genes responsible for stemness and thus become genomically unstable during the ageing process, favoring transformation. Also, the age-related gradual loss of thymic structure and function (termed involution), possibly in combination with other factors, was shown to impact on T-ALL development. In a study, each T-ALL subtype was shown to correlate closely with the stage of thymocyte maturation arrest regardless of age, but the incidence of the different subtypes differed markedly (Asnafi et al., 2004), probably reflecting age-related alterations in the thymocytes at risk of oncogenic transformation, alterations in stroma composition, or variable latency. In addition, a recent genome-wide sequencing study reported a correlation between age and number of somatic mutations in T-ALL, showing that particular genes are preferentially affected in adults versus children (De Keersmaecker et al., 2013).

In addition to their role in normal T-cell development, the establishment of adhesive contacts and the release of cytokines have crucial roles in regulating growth and survival of leukemic cells within the microenvironments where T-ALL develops (Balkwill, 2004). For instance, stromal cells expressing ICAM-1 were reported to favor the survival of T-ALL cells expressing lymphocyte function-associated antigen 1 (LFA-1) integrin (Winter et al., 2001). Likewise, chemokines produced by the bone marrow, thymic stromal cells, and other organs are important signaling molecules involved in T-ALL survival, proliferation, but mostly infiltration. For example, T-ALL cells with activated Notch signaling were shown to express CCR7, which promoted leukemic cell migration to CCL19-expressing CNS and infiltration (Buonamici et al., 2009; Ma et al., 2014). Moreover, the chemokine receptors CXCR4 and CCR9 expressed by leukemic cells were shown to mediate extramedullary organ infiltration in response to their ligands (Crazzolara et al., 2001; Qiuping et al., 2004). In addition, CCL25 to CCR9 signaling was reported to mediate T-ALL resistance to apoptosis and proliferation (Qiuping et al., 2004). In another study, CCR9 expression was shown to be induced by

NOTCH1 in T-ALL cells and to regulate proliferation and chemotaxis towards its ligand (Mirandola et al., 2012).

Additional reports have provided experimental evidence implicating stromal cell-induced activation of signaling in T cells during T-ALL leukemogenesis. Thymic and BM epithelial cells have been shown to promote T-ALL cell survival and proliferation *in vitro* by producing interleukin (IL)-7 (Scupoli et al., 2003, 2007). IL-7 was also reported to be involved in T-ALL progression and dissemination *in vivo* (Silva et al., 2011). Another cytokine, IL-18 produced by BM-derived stromal cells in inflammatory microenvironments, was also shown to support T-ALL progression *in vivo* probably due to enhanced proliferation (Uzan et al., 2014).

Cytokines and growth factors provided by stromal cells exert a protective effect not only on the bulk of the leukemic cell population, but also on leukemia initiating cells (LICs). LICs constitute a rare population of leukemic cells that are enriched in its ability to initiate leukemia in serial transplantation in immunodeficient mice (Cox et al., 2007). In T-ALL, Notch signaling activation induced by stromal cells is required for LIC activity mainly through the maintenance of self-renewal and engraftment abilities of these cells (Armstrong et al., 2009). Moreover, NOTCH1-induced high-level insulin-like growth factor (IGF)-1 receptor (IGF1R) expression was proved to be required for LIC activity in T-ALL. Moreover, inhibition of IGF1R signaling in response to IGF1/2 in the microenvironment halts T-ALL cell growth, survival, and interferes with disease establishment and progression (Medyouf et al., 2011).

In addition, sustained calcineurin activity in leukemic cells has been detected in mouse models of T-ALL induced by TEL-JAK2 or ICN1. *In vitro* culture of leukemic cells in the absence of stromal cells showed that calcineurin activity was rapidly lost, suggesting that specific signals from the tumor microenvironment may be essential for its maintenance (Medyouf et al., 2007). A recent study has also shown that calcineurin activation in T-ALL cells was important for survival, proliferation and motility in *ex vivo* studies. *In vivo*, it was essential to promote LIC activity but the stromal-derived factors responsible for its activation and consequent effects remain unknown (Gachet et al., 2013).

Notch signaling induction in leukemic cells was also reported to occur by microenvironmental elements. For instance, the escape of human T-ALL cells from dormancy to acquire a tumorigenic phenotype was associated with DLL4 expression by endothelial cells in the tumor microenvironment and increased NOTCH3 signaling in human T-ALL cells. Moreover, neutralization of DLL4 greatly reduced endothelial-cell mediated

activation of NOTCH3 signaling in T-ALL cells and blocked tumorigenesis (Indraccolo et al., 2009). Indeed, in addition to NOTCH1 point mutations found in T-ALL samples, overexpression of the NOTCH3 protein has been reported in virtually all cases of T-ALL, irrespective of any gross abnormalities in the *NOTCH3* locus (Bellavia et al., 2002). In addition to its expression in endothelial cells (Mailhos et al., 2001), DLL4 is also expressed by TECs, where it is indispensable for T-cell development (Hozumi et al., 2008). Interestingly, NOTCH1 activation in T-ALL cells also enhances the receptivity of T-ALL cells for signals provided by the microenvironment by inducing the expression of receptors for extracellular ligands, such as the IL-7 receptor (i.e., IL-7R) (González-García et al., 2009), the CCL19/CCL21 and CCL25 chemokine receptors (i.e., CCR7 and CCR9, respectively) (Buonamici et al., 2009; Mirandola et al., 2012), and IGF1R (Medyouf et al., 2011).

Recently, a role for the microenvironment in the activation of another signaling pathway commonly involved in T-ALL was unveiled. Ras enhanced signaling has been implicated in the pathogenesis of approximately 50% of T-ALL cases (von Lintig et al., 2000) but activating mutations in key components such as KRAS and NRAS are rare (Ahuja et al., 1990) and mostly found in ETP-ALL (Zhang et al., 2012). By studying Ras signaling activation in the absence of *RAS* somatic mutations, increased expression of the Ras activator guanine nucleotide exchange factor (RasGRP1) was found to cooperate with cytokine receptor signaling to activate Ras and contribute to T-ALL proliferation and leukemogenesis (Hartzell et al., 2013).

Finally, 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. 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 (dos Santos et al., 2008). As T-ALL in this mouse model originates in the thymus (Carron et al., 2000; dos Santos et al., 2007), RelB-dependent non-hematopoietic thymic stromal cells are most likely involved in T-ALL leukemogenesis (Dos Santos et al., 2010).

Altogether, T-cell development is strictly regulated by cytokines and growth factors produced by the microenvironments where they develop. Several of these factors may also support malignant T cells and thus be involved in the pathogenesis of T-ALL. Signaling pathways activated by these factors impact on strictly controlled processes such as

proliferation, cell cycle control, and survival of T-lymphocytes undergoing thymopoiesis. Moreover, molecular cues provided by stromal cells were also proven to be essential for T-ALL cell dissemination and LIC activity. Nevertheless, microenvironmental dependence of T-ALL may rely on the context of cell-intrinsic alterations found in T-ALL cells. If occurring aberrations alter the activity of members of signaling pathways normally activated by molecular cues provided by stromal cells (e.g., *IL7R* mutations), the pro-oncogenic role of these cells may be attenuated or even become irrelevant. In other situations, mutant proteins may render T-ALL cells more responsive to extracellular signals, as exemplified by *PTEN* mutations allowing more sustained Akt activation. Nevertheless, the identification of mutations in genes encoding components of signaling pathways commonly activated by stromal cell-derived signals further confirms their key role in T-ALL development.

1.2.3. Contribution of cell-autonomous and noncell-autonomous factors to T-ALL development

From the previously mentioned studies, it is clear that T-ALL develops by a multistep process whereby thymocytes accumulate genetic and epigenetic alterations. Although these cell-intrinsic mechanisms are major contributors to the molecular pathogenesis of T-ALL, cooperation from extrinsic factors produced by microenviromental cells constitutes a requisite for the development of overt T-ALL.

The way all these factors cooperate and contribute to leukemogenesis may be complex, but one hypothesis considers a hierarchical model in which leukemic stem cells (LSCs) are generated from T-cell progenitors before fully malignant T-ALL cells are generated. LSCs correspond to cancer stem cells that were detected in hematological malignancies including T-ALL and have the ability to initiate the disease when transplanted to recipient mice (Chiu et al., 2010), meaning that these have LIC activity. According to this model, a subset of T-cell progenitors suffer the initiating genetic event, which consists usually in genetic lesions that lead to the ectopic expression of oncogenic transcription factors (Type A abnormalities; Table 1.1). These cells are designated pre-LSCs because they acquire self-renewal abilities but they need to acquire further genetic mutations (Type B abnormalities; Table 1.2) to clonally evolve and generate LSCs. Interestingly, some of these secondary events are activating mutations in cytokine-activated signaling pathways that promote clonal expansion of LSCs independently of the thymic niche (e.g., *NOTCH1*, *IL7R*, *MYC*, *KRAS*, and *PTEN*) to generate overt T-ALL and dissemination (Gerby et al., 2014;

Tremblay and Curtis, 2014). Moreover, some studies support the notion that disease relapse is originated by a population of pre-LSCs that resist to therapeutic interventions (Mullighan et al., 2008; Tzoneva et al., 2013).

Table 1.3. Microenvironmental factors involved in T-ALL development.

Factors involved		Effect on T-ALL cells	References
Stromal cells	**T-ALL**		
ICAM-1	LFA-1	Survival	(Winter et al., 2001)
CXCL12	CXCR4	Chemotaxis, extramedullary organ infiltration	(Crazzolara et al., 2001)
CCL25	CCR9	Chemotaxis, infiltration, resistance to apoptosis, proliferation	(Qiuping et al., 2004) (Mirandola et al., 2012)
IL-7	IL-7R	Survival, proliferation, T-ALL progression	(Scupoli et al., 2003, 2007) Silva et al., 2011
IL-18	IL-18R	Proliferation, T-ALL progression	(Uzan et al., 2014)
DLL4	NOTCH3	Angiogenesis, tumor growth *in vivo* and escape from dormancy; T-ALL progression	(Indraccolo et al., 2009) (Minuzzo et al., 2015)
IGF1	IGF1R	Survival, proliferation, LIC activity	(Medyouf et al., 2011)
unknown	Calcineurin	Survival, proliferation, motility, LIC activity	(Medyouf et al., 2007) (Gachet et al., 2013)
Il-2, Il-7, Il-9	Cytokine receptors	Proliferation, T-ALL leukemogenesis	(Hartzell et al., 2013)

1.2.4. T-ALL therapeutics and potential new targets

T-ALL was once profoundly associated with dismal prognosis, but the introduction of intensive combination chemotherapy protocols has led to remarkable improvements in survival. Some patients are also treated with radiotherapy and, in case of high-risk or relapsed disease, a bone marrow transplant is indicated. Thus, over 80% of children with T-ALL achieve long lasting complete remissions (Pui et al., 2008). Still, an unresolved issue in the

treatment of T-ALL is its propensity to relapse and chemoresistance development (Pui and Evans, 2006). In contrast to the favorable response to therapy in patients at diagnosis, the prognosis of T-ALL patients with relapsed leukemia remains poor (Aifantis et al., 2008). Moreover, these patients may develop long-term adverse effects derived from therapeutics that include neurocognitive defects, growth deficiency, and increased risk of developing second cancers, including acute myeloid leukemia (AML) and CNS tumors (Robison, 2011). 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.

When envisioning a therapeutic strategy for T-ALL, several players in disease development may constitute targets for rational therapy. First, the common oncogenic transcription factor(s) aberrantly expressed in T-ALL would be appealing targets. Even though their activation is not sufficient to induce leukemogenesis in the absence of secondary alterations, in some cases targeting the initiating event is enough to eliminate leukemic cells since the secondary events are unlikely to replace its function (so-called oncogenic addiction). For instance, the Lmo2 complex was successfully targeted in T-ALL by inhibition of interactions with other molecules proven essential for its oncogenic function (Appert et al., 2009). Second, signaling pathways aberrantly activated due to mutations or upstream activating mechanisms constitute key events in the pathogenesis of T-ALL and an excellent opportunity for molecular targeting. In fact, different combinations of factors cause T-ALL but it usually results in the deregulation of common signaling pathways for which several inhibitors have been already developed (Zhao, 2010). The potential targets are integrative members of signaling pathways such as the phosphatidylinositol 3-kinase/Akt/mammalian target of rapamycin (PI3K/Akt/mTOR) pathway (Bertacchini et al., 2015), Notch pathway (Weng et al., 2004), nuclear factor-kappaB (NF-κB) pathway (Dos Santos et al., 2010; Zhao, 2010), and Ras pathway (Gibbs et al., 1994; von Lintig et al., 2000). Recently, new studies supported the value of targeting the Calcineurin/Nuclear Factor of Activated T Cells (NFAT) pathway (Gachet et al., 2013), Wnt signaling pathway (Ng et al., 2014), and janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway (Maude et al., 2015). Third, the evidence that inappropriate regulation of epigenetic factors is perturbed by for instance mutations in key players responsible for DNA methylation or post-translational histone modifications (Peirs et al., 2015) or imbalances in micro RNA (miRNA) expression (Mavrakis et al., 2011) during malignant transformation, created an interest in targeting epigenetic mechanisms. Finally, some of the signaling pathways aberrantly activated in T-

ALL are induced by microenvironmental factors rather than cell-intrinsic mechanisms. In this case, the receptors responsible for receiving 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 pose potentially a reduced risk of resistance and tumor recurrence. However, specifically disrupting the tumor microenvironment is challenging since it has diverse capacities to induce both beneficial and adverse consequences for tumorigenesis.

In fact, tailored therapies consisting of a combination of drugs directed to a specific molecular target plus chemotherapy, or several drugs targeting different members of signaling pathways (e.g., combined inhibition of PI3K/AKT and NOTCH1) (Bertacchini et al., 2015) should be adapted to take into account the disease intrinsic heterogeneity and the extensive crosstalk between signaling pathways in this context (Zhao, 2010). Therefore, understanding the underlying disease mechanisms is of great value to be translated into more rational and effective therapeutic strategies. To this end, the T-ALL models that were developed have been of extreme importance not only to unveil T-ALL pathologic mechanisms, but also to test new experimental therapeutic approaches (Appert et al., 2009).

1.2.5. Models to study T-ALL

Since the first report of the establishment of a T-ALL cell line in 1965 (Foley et al., 1965), several cell lines have become available for immunological and molecular studies either as models for T-cell leukemias/lymphomas or even as representatives of its respective normal counterpart in the thymus. These cell lines provide unique *in vitro* model systems for studying cellular and molecular events involved in the proliferation and differentiation of normal and leukemic T cells. Importantly, these cell lines were often established from primary T-ALL cells collected from patients (Morikawa et al., 1978; Pegoraro et al., 1985; Schneider et al., 1977; Smith et al., 1988) and also reflect the wide heterogeneity regarding immunophenotypic and genotypic characteristics (Burger et al., 1999). Therefore, when using T-ALL cell lines as experimental models for malignant or normal T cells, it is necessary to consider the particular differentiation stage and gene expression profile of each individual line. Importantly, T-ALL human cell lines and primary samples are also currently studied *in vivo* following transplantation to immunodeficient mice as xenograft models (Maude et al., 2015; Poglio et al., 2015; Silva et al., 2011).

These xenograft models and other animal models provide an invaluable tool for understanding the mechanism of leukemogenesis and testing new therapies. Although alternative models like transgenic zebrafish (*Danio rerio*) overexpressing Notch (Chen et al., 2007) or c-Myc (Langenau et al., 2003) under the control of *Rag2* promoter have been developed (Stoletov and Klemke, 2008), the mouse (*Mus musculus*) is still the most widely used experimental organism to model human T-ALL. Mice have one enormous advantage over other experimental organisms: they are genomically, physiologically and anatomically the closest to humans of any animal for which powerful genetic approaches and tools are feasible.

One mouse model of T-ALL widely used was developed by transduction of bone marrow cells with activated intracellular NOTCH1 (ICN1) and transplantation to lethally irradiated mice (Aster et al., 2000; Pear et al., 1996). Bone marrow reconstituted mice develop aggressive leukemia, showing NOTCH1 oncogenicity when constitutively activated. Moreover, transgenic mice can be created by introducing engineered genes carrying molecular alterations similar to those recognized in cases of human T-ALL into the mouse genome and expressing it in the appropriate cellular compartment. Transgenic mice overexpressing known oncogenes, such as LMO2 (Drynan et al., 2001), TAL1 (O'Neil et al., 2001), or LYL1 (Zhong et al., 2007), develop T-cell leukemia that exhibits pathologic characteristics similar to human T-ALL. In some cases, leukemia presents with long latencies, not only due to particular timing and levels of transgene expression, but also due to requirements for additional genetic mutations to occur or other events that allow T-cell leukemogenesis to develop independently of the thymic cell niche, thus mimicking the multistage nature of human T-ALL.

The TEL-JAK2 fusion gene, identified in a t(9;12)(p24;p13)-positive T-ALL patient, was also used to create a transgenic mouse model. A transgene bearing the TEL-JAK2 complementary DNA was placed under the transcriptional control of the EμSRα enhancer/promoter to express the fusion protein in lymphoid cells (Carron et al., 2000). The expression of TEL-JAK2 resultes in constitutively activated Janus tyrosine kinase 2 (JAK2) (Lacronique et al., 1997) and TEL-JAK2 transgenic (TJ2-Tg) mice were found to develop T-cell leukemia with high penetrance, which was fatal in 8 to 22 weeks of age (Carron et al., 2000). TEL-JAK2-induced leukemia was histological and phenotypically similar to human T-ALL, the leukemic cells being mainly a mixed population of CD24-positive $CD4^+CD8^+$ DP thymocytes, an immunophenotype normally only seen in the thymus, and immature $CD8^+$ SP thymocytes (Carron et al., 2000; dos Santos et al., 2007). In TJ2-Tg mice, the expression of

the TEL-JAK2 fusion protein functions as the initial genetic "hit" in T-ALL initiation. Therefore, additional secondary events accumulate during the disease latency, which may be variable and acquired in a different order between different mice. This notion is supported by the phenotypic heterogeneity observed in diseased mice, the oligoclonality observed in leukemic cells collected from these mice, and the extended time for disease development, all in accordance with the multistep nature of T-ALL (Carron et al., 2000; Ihle and Gilliland, 2007).

The TEL-JAK2 oncoprotein appears to activate substrates similar to those activated by the normal JAK2 tyrosine kinase. Among them, the STAT5 transcription factor is essential to its transforming activity in cellular and animal models (Lacronique et al., 2000; Schwaller et al., 2000). Moreover, TEL-JAK2 can activate other effectors that are not normally involved in JAK2 signaling but are critical to its oncogenic properties. Supporting it is the fact that IKKα-dependent NF-κB activation was shown to contribute to cell survival mediated by the TEL-JAK2 fusion protein in the murine lymphoid Ba/F3 cell line (Malinge et al., 2006; Santos et al., 2001).

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 (Adélaïde et al., 2006), the JAK/STAT signaling pathway is frequently activated (Benekli et al., 2003; Vainchenker and Constantinescu, 2013; Weber-Nordt et al., 1996) by several mechanisms including *IL7R*, *JAK1*, *STAT5B*, and *PTPN2* mutations. Moreover, transgenic expression of STAT5 mutant induces murine T-ALL (Kelly et al., 2003). Notably, JAK inhibitors have shown therapeutic potential in xenograft models (Maude et al., 2015; Waibel et al., 2013).

1.3. THE LTα1β2/LIGHT TO LTβR SIGNALING AXIS

1.3.1. The lymphotoxin-β receptor

The human Lymphotoxin-β Receptor (LTβR) genetic locus (*TNFRSF3* or *LTBR*) is located on chromosome (Chr) 12 (mouse Chr 6), adjacently to the TNFR1 gene (*TNFRSF1A*) and CD27 genes (Crowe et al., 1994; Force et al., 1995). The *LTBR* locus contains 10 exons spanning 16.2 kb. Its promoter region resembles that of a housekeeping gene (Force et al.,

1995). Nevertheless, it has been shown to be regulated by compounds such as dexamethasone (Muller et al., 2001). Alternative LTβR transcript variants encoding different isoforms have been reported such as a transcript encoding an NH_2-terminally truncated form of the LTβR identified in a pancreatic ductal carcinoma cell line (Fujiwara et al., 2005). The LTβR full-length transcript is approximately 2.2 kb in size and 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 by cytoplasmic domain (CD) (Figure 1.3). Like other TNF superfamily receptors, LTβR displays four TNF cysteine-rich domains (CRD) in the ECD domain, which confer the specificity and the affinity for its cognate ligands (Crowe et al., 1994). Unlike other TNFR superfamily members, LTβR does not contain a death domain in the cytoplasmic tail but contains rather a proline-rich membrane proximal region (Crowe et al., 1994) and two binding sites for members of the TNF Receptor-Associated Factors (TRAF) family of zinc RING finger proteins (Ganeff et al., 2011). Indeed, TRAF-2 (Kuai et al., 2003), -3 (VanArsdale et al., 1997), -4 (Krajewska et al., 1998) and -5 (Nakano et al., 1996) have been reported to associate with the LTβR. Moreover, within this domain, distinct regions mediate self-interaction and trafficking of the receptor, and the activation of downstream signaling pathways like NF-κB and others leading to cell death (Figure 1.3) (Force et al., 2000).

The murine LTβR shows high homology with its human counterpart and binds the homologous ligands with similar specificity. Furthermore, the murine and human LTβR-encoding genes are located in a region of conserved synteny in close linkage with the TNFR1-encoding gene (Force et al., 1995).

As expected from having a housekeeping gene-like promoter, LTβR has been shown to be constitutively expressed on a wide variety of cells of lymphoid and visceral tissues such as epithelial and endothelial cells, follicular dendritic cells (FDCs), fibroblasts, and myeloid cells (e.g., monocytes, dendritic cells, and mast cells), but strikingly not on lymphocytes (Force et al., 1995; Murphy et al., 1998; Seach et al., 2008; Stopfer et al., 2004). Since the only two known ligands for LTβR, $LT\alpha_1\beta_2$ and LIGHT, are expressed in lymphocytes (Browning et al., 1997; Mauri et al., 1998; Ware et al., 1992), this pattern of expression suggests that signals mediating LTβR activation are paracrine in nature.

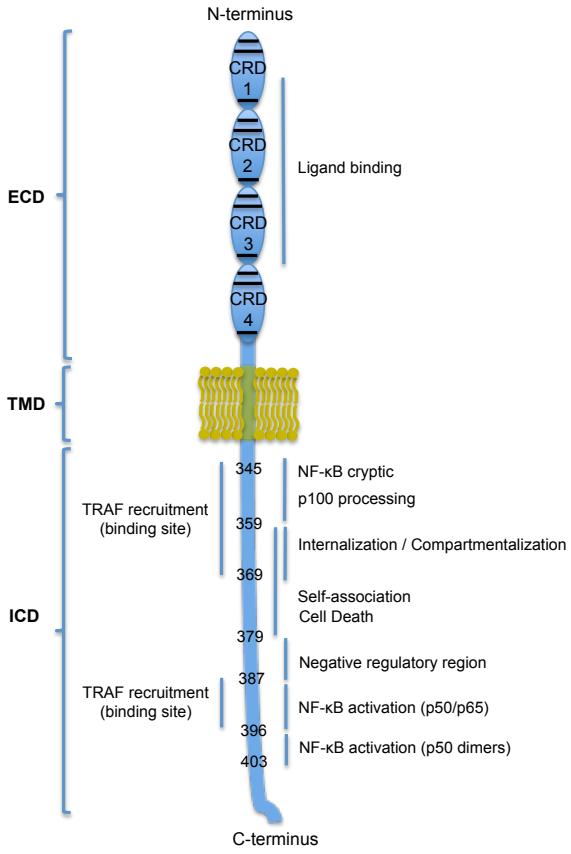


Figure 1.3. Schematic representation of human LTβR structure. LTβR main domains, and mapping of discrete regions determined by deletion mutant studies and functional outcomes. ECD, extracellular domain; TMD, transmembrane domain; ICD, intracellular domain; CRD, cysteine-rich domains

1.3.2. The LTβR ligands: $LT\alpha_1\beta_2$ and LIGHT

The genes encoding lymphotoxin (LT) α, the TNF superfamily, member 1 (*TNFSF1* or *LTA*) and LTβ, the TNF superfamily, member 3 (*TNFSF3* or *LTB*), reside in a tightly linked locus within the MHC class III region on Chr 6 in humans (Chr 17 in mice), flanking the gene encoding TNFα (*TNFSF2* or *TNFA*), while the LTβ-encoding gene is oriented in the opposite direction of TNFα- and LTα-encoding genes (Browning et al., 1993; Lawton et al., 1995; Nedospasov et al., 1986; Nedwin et al., 1985).

The *LTA* locus spans approximately 2 kb and contains 4 exons (Nedwin et al., 1985). The full-length mRNA (1.4 kb) encodes a 205-amino acid glycosylated (N- and O-linked) protein, LTα or TNFβ (20-25 kDa), in which exon 1 encodes the 5' untranslated region (UTR), exon 2 encodes the remainder of the 5' UTR and the majority of the signal peptide, and exons 3 and 4 encode the mature protein. In addition, exon 4 contains a long 3' UTR (Nedwin et al., 1985). In immune cells, the *LTA* promoter region has been shown to contain

NFAT, STAT, AP-1, NF-κB and Sp1 transcription factor binding sites (Shebzukhov and Kuprash, 2011). The respective murine counterpart is highly homologous, including the promoter region (Paul and Ruddle, 1988). In addition, an alternate core promoter responsible for transcriptional regulation was identified in the region between the transcription and translation initiation sites (downstream segment) in T and B human cell lines that contains an additional Sp1 binding site and an initiator element (Inr). Additionally, the same group reported a T-cell specific repressor element that may be responsible for transcription inhibition in unstimulated T cells (Yokley et al., 2013). Interestingly, several transcript variants were identified in human T lymphocytes (Yokley et al., 2013) and peripheral blood mononuclear cells (PBMC) (Smirnova et al., 2008), which presented different levels of expression before and after induction upon specific stimulation conditions. Although their functional significance is not fully understood, the *LTA* transcripts and their relative abundance are likely influenced by the cell type and the nature of the stimuli. These transcripts have been shown to be inducible by T cell mitogens (Zinetti et al., 1998), phorbol ester and ionomycin (Voon et al., 2001), TCR activation through anti-CD3/28 treatment or TCR cross-linking (Millet and Ruddle, 1994), cytokines (e.g., IL-4, IL-2) (Lu et al., 1998; Worm et al., 1998), viral proteins (Lee et al., 2005; Paul et al., 1990) and by the $LT\alpha_3$ soluble protein form (Messer et al., 1990).

Similarly to *LTA*, the human *LTB* locus spans approximately 2 kb and contains 4 exons (Browning et al., 1993). The full-length mRNA is 0.9 kb long and encodes LTβ, a 244-amino acid glycosylated transmembrane protein (33 kDa) (Browning et al., 1993). The exon 4, which encodes the extracellular domain, is highly conserved between the mice and human LTβ-encoding genes. Additionally, the murine and human genes are organized similarly and have an equivalent location in the MHC locus, but they are differently spliced (Pokholok et al., 1995): the murine mRNA retains the region corresponding to intron 2 in the human *LTB* locus, thus converting exons 2 and 3 into a single large exon that results in the creation of a long stalk between the extracellular domain and the transmembrane domain of the LTβ protein (Lawton et al., 1995; Pokholok et al., 1995). Two transcript variants of human LTβ mRNA were identified: the functional one consisting of 4 exons, and an alternative one, in which the exon 2 is eliminated resulting in a LTβ truncated form that lacks the majority of the extracellular domain of the native protein, impairing its normal assembly with LTα (Warzocha et al., 1997). Regardless this observation, the physiological significance of the LTβ truncated form is still unknown. In the upstream sequence of the putative transcription initiation site of *LTB*, which is highly conserved between mice and humans, the presence of

some binding sites for known transcription factors such as NF-κB, Ets, MyoD and Sp1/Egr-1 (Kuprash et al., 1996; Pokholok et al., 1995; Shebzukhov and Kuprash, 2011) have been reported. *LTB* has been shown to be constitutively expressed (Kuprash et al., 2002; Millet and Ruddle, 1994; Voon et al., 2004) but still inducible to higher levels by several stimuli such as T cell mitogens (Zinetti et al., 1998), phorbol ester and ionomycin (Voon et al., 2001), TCR activation (anti-CD3/28 or TCR cross-linking) (Millet and Ruddle, 1994), cytokines (e.g., IL-7, TNFα, $LT\alpha_3$) (Honda et al., 2001; Voon et al., 2001, 2004) and viral proteins, especially in hepatocytes (Haybaeck et al., 2009; Simonin et al., 2013).

LTα- and LTβ-encoding genes display a restricted and similar pattern of expression, being expressed in hematopoietic cells such as activated T, B and NK lymphocytes (Browning et al., 1997; Ware et al., 1992), DCs (De Trez et al., 2008) and lymphoid-tissue inducer (LTi) cells (Cupedo et al., 2004). In contrast to the LTα mature protein, which lacks a transmembrane domain, LTβ is a type II transmembrane glycoprotein (N-linked) consisting on a short N-terminal cytoplasmic domain, a transmembrane domain and a C-terminal extracellular domain (Browning et al., 1993). Therefore, when expressed in the absence of LTβ, LTα forms soluble $LT\alpha_3$ homotrimers stabilized primarily by interactions between hydrophobic and aromatic side chains (Eck et al., 1992; Force et al., 1995; Williams-Abbott et al., 1997). When LTα is expressed together with LTβ, these proteins oligomerize generating cell-surface $LT\alpha_1\beta_2$ heterotrimers (Androlewicz et al., 1992; Browning et al., 1995; Force et al., 1995). The LTα subunit contributes primarily to the conformation of the heterotrimer (Williams-Abbott et al., 1997), while the LTβ subunit provides the membrane anchor for the $LT\alpha_1\beta_2$ heterotrimer and the specificity for LTβR binding (Crowe et al., 1994). Following cell activation, *LTA* induction is faster than *LTB* induction (Browning et al., 1993), so the basal levels of *LTB* mRNA may be important to transport LTα to the cell surface as the $LT\alpha_1\beta_2$ heterotrimer (instead of $LT\alpha_2\beta_1$ or even soluble LTα), and LTα production is probably the rate-limiting step this process (Browning et al., 1993; Kuprash et al., 2002; Millet and Ruddle, 1994). Cell-surface $LT\alpha_1\beta_2$ heterotrimers are upregulated through lymphocyte activation, but also by cytokine and chemokine induction including IL-2 on human peripheral blood T cells (Ware et al., 1992), and IL-4 and IL-7 cytokines in addition to the CCL19 and CCL21 chemokines in murine splenic T cells (Luther et al., 2002). Abnormal expression of $LT\alpha_1\beta_2$ in infected hepatocytes (Haybaeck et al., 2009; Simonin et al., 2013) and in the cervical epithelium (Kim et al., 2011) by viral proteins was also reported and shown to be involved in cancer development.

The other known LTβR ligand is encoded by the human TNF superfamily, member 14 (*TNFSF14*) or *LIGHT* gene and spans 5.1 kb. It is located within an MHC-like region on Chr 19 (Chr 17 in the mouse), recognized as an MHC paralog region, adjacent to other TNF superfamily member genes such as the CD27 ligand (*TNFSF7*) and the CD137 ligand (*TNFSF9*) (Granger et al., 2001; Misawa et al., 2000). *LIGHT* expression is inducible and its promoter presents the motifs for AP-1, NF-κB and Oct-1 binding, as other TCR signaling-inducible genes (Granger et al., 2001). The LIGHT full-length transcript is composed of 4 exons (2.8 kb) and is translated in a 29 kDa N-glycosylated type II transmembrane protein (Granger et al., 2001). The first exon encodes the cytoplasmic tail, the transmembrane domain and the beginning of the extracellular stalk region, exons 2 and 3 encode the remaining stalk region and part of the trimerization domain, and finally, exon 4 encodes the remainder of the trimerization domain plus the receptor-binding domain (Granger et al., 2001). LIGHT monomers form homotrimers at the cell surface of activated lymphocytes (Mauri et al., 1998), which can be shed upon proteolytic cleavage (Granger et al., 2001). An alternative, less abundant spliced isoform that contains an internal deletion and encodes a nonglycosylated cytosolic protein lacking the transmembrane domain was also identified, but its function remains unknown (Granger et al., 2001). Similarly to lymphotoxins, the LIGHT-encoding gene displays a restricted expression pattern being mainly expressed on activated peripheral blood T lymphocytes (Mauri et al., 1998), monocytes, granulocytes and immature dendritic cells (Tamada et al., 2000; De Trez et al., 2008; Zhai et al., 1998). Conversely, in mucosal tissues, $CD4^+$ T cells and NK cells were shown to constitutively express cell-surface LIGHT (Cohavy et al., 2005). Its gene was shown to be expressed also in thymic stromal cells such as DCs, fibroblasts, endothelial and epithelial cells (Seach et al., 2008).

LTβR signaling and its output is complex due to overlapping patterns of ligand-receptor binding involving LTα/LTβ-composed ligands and LIGHT (Figure 1.4). LTβ and/or LTα form 3 distinct ligands: soluble $LT\alpha_3$ and two lymphotoxin membrane-bound heterotrimers with different stoichiometry, $LT\alpha_1\beta_2$ (major form) and $LT\alpha_2\beta_1$, which is likely an extemporaneous minor form, expressed *in vitro*, representing less than 10% of LTαβ heterotrimers (Browning et al., 1995). $LT\alpha_3$ binds TNFR1, TNFR2 (Bossen et al., 2006; Chaplin and Fu, 1998), and herpes virus entry mediator (HVEM) (Bossen et al., 2006; Mauri et al., 1998). Since its discovery, LTβR is still the only known receptor for $LT\alpha_1\beta_2$ (Browning et al., 1995), while $LT\alpha_2\beta_1$ may bind LTβR with low affinity (Browning et al., 1995; Crowe et al., 1994; Williams-Abbott et al., 1997), and bind both TNFR1 and TNFR2 (Mauri et al., 1998). LIGHT forms only homotrimers, which can bind and activate both LTβR and HVEM

(Mauri et al., 1998), a receptor expressed by T, B and NK lymphocytes, DCs and monocytes (Kwon et al., 1997), and the soluble decoy receptor 3 (DcR3), which acts as a negative regulator (Yu et al., 1999).

Although published *in vitro* studies report several putative interactions between ligands and receptors, their physiological relevance *in vivo* still remains questionable. While the role of $LT\alpha_1\beta_2$ and LIGHT to LTBR signaling and LIGHT to HVEM have been increasingly characterized, the role of $LT\alpha_3$-mediated TNFR activation and the functional significance of the $LT\alpha_2\beta_1$ minor form *in vivo* are not completely understood.

Even though both $LT\alpha_1\beta_2$ heterotrimers and LIGHT homotrimers are often anchored to the cell membrane, in certain contexts they can be shed from the cell surface. For instance, $LT\alpha_1\beta_2$ can be shed from activated human T cells, upon proteolysis mediated by matrix metalloproteinase (MMP)-8 and TNFα converting enzyme (TACE), to induce the expression of pro-inflammatory genes on synovial fibroblasts from rheumatoid arthritis patients (Young et al., 2010). LIGHT can also be actively shed from the cell surface of $CD4^+$ T lymphocytes by MMPs in rheumatoid arthritis (Pierer et al., 2007) but also in the context of immune cell regulation (Murphy and Murphy, 2010). Although the shed soluble form of LIGHT also binds and activates HVEM, the membrane-bound homotrimer shows enhanced effects (Cheung et al., 2009; Granger et al., 2001; Morel et al., 2000). Interestingly, the membrane-bound form of LIGHT expressed in T lymphocytes has been shown to co-stimulate these cells upon ligation of the TCR, when bound to an agonistic antibody or to its receptor DcR3, a phenomenon denominated reverse signaling (Ware, 2005). Nevertheless, the biological significance of these different forms is still not fully understood especially regarding LTβR activation. In certain contexts LIGHT shedding may induce distal functional effects on LTβR activation or may serve as a mechanism of inactivation.

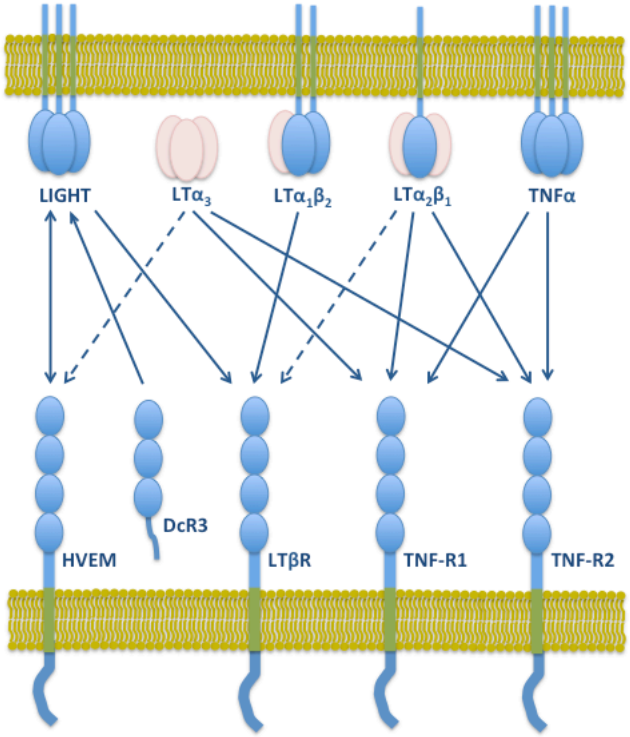


Figure 1.4. LTβR as part of a network of shared ligand-receptor interactions within the TNF superfamily. $LT\alpha_1\beta_2$ and LIGHT bind LTβR. In addition, LIGHT binds also HVEM and DcR3 (in human cells). $LT\alpha_3$ binds HVEM with low affinity in addition to TNFR1 and TNFR2, which are the two known receptors for TNFα. A minor form of the LTαβ heterotrimer, $LT\alpha_2\beta_1$, binds LTβR and also TNFR1 and TNFR2. (Dashed lines indicate weak interactions; arrowheads indicate known signaling directions).

1.3.3. LTβR activation, signal transduction and target genes

The TNFR superfamily members are typically activated by ligand-induced trimerization or even higher order clustering through binding to monomer-monomer interfaces (Ware, 2005). As expected, the central initiating event for LTβR signaling is receptor aggregation but, differently from other TNF receptors, LTβR binds only two sites in the $LT\alpha_1\beta_2$ heterotrimer, the LTα-LTβ (higher affinity) and LTβ-LTβ' (lower affinity) interfaces (Sudhamsu et al., 2013). Similarly, LIGHT has been shown to present only two high-affinity binding sites for LTβR (Eldredge et al., 2006). The binding of $LT\alpha_1\beta_2$ or LIGHT to LTβR brings 2 receptor molecules in close proximity (Eldredge et al., 2006; Sudhamsu et al., 2013) and the LTβR self-interaction region in the cytoplasmic domain promotes receptor aggregation and consequent conformational changes (Force et al., 2000). Since like other TNFSF receptors, LTβR has no intrinsic kinase or other enzymatic activity encoded by its cytosolic domain, the previous events lead to the sequential recruitment of cytosolic adaptor proteins to the cytoplasmic region of LTβR forming the so-called LTβR-induced signaling complexes. The

main adaptors are TRAF proteins as suggested by the ability of LTβR to bind TRAF2, 3, 4, and 5. These proteins may activate or repress signaling initiation leading to gene transcription through different signaling pathways such as the classical and alternative NF-κB, c-Jun N-terminal kinase (JNK), or even signaling pathways leading to cell death (Norris and Ware, 2007). LTβR-dependent downstream signaling can also be initiated independently of its cognate ligands either by anti-LTβR agonistic antibodies that mimic receptor aggregation (Mackay et al., 1996; VanArsdale et al., 1997), or by overexpression of the receptor in cell lines leading to self-association (Dhawan et al., 2008; Wu et al., 1999). These observations support the existence of regulatory mechanisms that limit the expression and/or aggregation of the receptor. Also, although the naturally occurring ligands are membrane-bound, the recombinant soluble forms of $LT\alpha_1\beta_2$ and LIGHT are also capable of binding and activating LTβR (Chen et al., 2000; Ganeff et al., 2011; Kim et al., 2005). Other TNFSF ligands are subject to proteolysis, allowing them to act in a soluble form (e.g., TNFα and RANKL) (Grell et al., 1995; Lum et al., 1999) or to inhibit signaling (e.g., FasL) (Voss et al., 2008). The fate of $LT\alpha_1\beta_2$ and LIGHT following LTβR binding, and whether these two ligands can induce reverse signaling in this context, remains unveiled (Juhász et al., 2013).

Despite LTβR activation has been reported to induce gene expression through ASK-MKK-JNK-dependent AP-1 activation (Chang et al., 2002; Kim et al., 2005) and LTβR interacts with the AP2 adaptor complex/clathrin to mediate unknown NF-κB-independent functions (Ganeff et al., 2011), its role on cell death and NF-κB activation has been a major topic of research. Despite the fact that LTβR lacks a cell death domain in its cytosolic domain, it has been shown to induce cell death in certain cellular contexts. In some cancer cell lines (e.g., HT-29, WiDr, Hep3BT2 and MCF-7), recombinant forms of its ligands, $LT\alpha_1\beta_2$ and LIGHT, and LTβR agonistic antibodies induce cell death with slow kinetics (36-72h) in the presence of IFN-γ (Browning et al., 1996; Chen et al., 2000) or through LTβR overexpression and self-association independently of ligands and IFN-γ (Wu et al., 1999). Furthermore, LTβR activation was reported to arrest tumor growth in xenograft models (Browning et al., 1996; Lukashev et al., 2006). However, the molecular mechanism is currently not fully understood. Some studies reported that recruitment of mainly TRAF3, but also TRAF2 and TRAF5 (Force et al., 1997; Kim et al., 2005; Kuai et al., 2003; VanArsdale et al., 1997), to LTβR and the consequent production of reactive oxygen species (ROS) (Chen et al., 2000, 2003) induce cell death with mixed characteristics of apoptosis and necrosis (Wilson and Browning, 2002). A caspase-independent mechanism involving the activation of the apoptosis signal-regulating kinase 1 (ASK1) has been suggested (Chen et al., 2003). On

the other hand, LTβR was shown to induce caspase activation and mitochondrial-dependent apoptosis (Hu et al., 2013; Kuai et al., 2003). In addition to its inherent complexity, the mechanism of cell death induced by LTβR has not been fully understood because it may vary depending on cell type and the nature of the LTβR-activating stimulus. Furthermore, the activation of the receptor on one cell using soluble recombinant ligands or LTβR agonists may not fully mimic the effects of membrane-bound LIGHT or $LT\alpha_1\beta_2$ expressed on another cell.

It is generally accepted that LTβR signaling bifurcates at the level of receptor-bound TRAF proteins with TRAF3 leading to cell death and NF-κB inhibition, since some TRAF3 mutants block cell death but not NF-κB activation (VanArsdale et al., 1997). Two distinct signaling pathways that lead to the activation of NF-κB transcription factors have been described, termed classical (or canonical) and alternative (or noncanonical). These are often activated by different stimuli in a cell type-specific manner, require different IκB kinase (IKK) complexes, and induce different NF-κB complexes that translocate to the nucleus and target distinct genes (Hayden and Ghosh, 2014). Unlike the prototypical TNF receptors that activate exclusively the classical NF-κB pathway (e.g., TNFR1 and TNFR2), LTβR binding by its ligands leads to both classical and alternative NF-κB pathway activation (Dejardin et al., 2002; Ganeff et al., 2011). The activation of one or the other NF-κB signaling pathway is spatially and temporally regulated by LTβR trafficking (Ganeff et al., 2011) and varying levels of receptor cross-linking may be required for distinct conformational changes and the activation of different signal transduction pathways. Furthermore, the classical and alternative NF-κB signaling pathways control distinct patterns of gene expression (Dejardin et al., 2002) and may be differentially involved in the functions attributed to LTβR signaling.

To activate the classical NF-κB signaling pathway, LTβR engagement leads to TRAF2 recruitment to its cytoplasmic domain and IκBα phosphorylation and degradation by the proteasome (Bista et al., 2010) mediated by IKKβ and IKKγ. These events lead to the activation of p50-RelA heterodimers (Dejardin et al., 2002; Müller and Siebenlist, 2003). TRAF3 has been shown to inhibit TRAF2 recruitment when upregulated by an unapparent mechanism (Bista et al., 2010). When LIGHT or $LT\alpha_1\beta_2$ accumulates at the surface of inducer cells, higher-order clusters of LTβR may form on the target cell, that seemingly trigger dynamin-2-dependent endocytosis of the receptor (Ganeff et al., 2011). During this process, the LTβR tail was shown to remain exposed towards the cytosol and to compete with NIK for the binding of its inhibitory complex composed by TRAF3/TRAF2/cIAP1/2. As a consequence, the constitutive proteasomal degradation of NIK is alleviated, leading to NIK

accumulation and activation of IKKα. These events lead to p100 processing to p52 and the translocation of p52/RelB dimers to the nucleus (Ganeff et al., 2011; Sanjo et al., 2010). In this case, TRAF3 inhibits NF-κB signaling by being part of a complex that mediates NIK targeting to proteasome degradation and, thus inhibits the processing of p100 to p52 (Bista et al., 2010). The activation of the alternative NF-κB pathway through NIK and IKKα, constitutes an essential component downstream the LTβR for lymphoid organogenesis, whereas for thymic organogenesis it is activated in cooperation with other receptor, namely the receptor activator of NF-κB (RANK) (Matsushima et al., 2001; Mouri et al., 2011).

In terms of kinetics, ligand binding to LTβR can induce a rapid and transient activation of the classical NF-κB pathway, followed by a delayed but sustained activation of the alternative pathway (Dejardin et al., 2002; Müller and Siebenlist, 2003). The delayed activation of the alternative pathway may be at least partially due to the necessity of *Nfkb2* gene transcription (encoding p100), which is mediated by the classical pathway (Dejardin et al., 2002; Müller and Siebenlist, 2003). Alternatively, LTβR activation may induce the alternative pathway alone to eventually produce NF-κB dimers containing RelA and RelB (Basak et al., 2007). Through the activation of p50/RelA heterodimers, LTβR signaling promotes for instance the upregulation of proinflammatory molecules including the CCL4/macrophage inflammatory protein (MIP)-1β, MIP-2 and vascular-cell adhesion molecule 1 (VCAM-1) in mouse embryonic fibroblasts (MEFs) (Dejardin et al., 2002), and CXCL1 and CXCL2, intercellular adhesion molecule 1 (ICAM-1), VCAM-1 and E-selectin in endothelial cells (Madge et al., 2008). Conversely, LTβR-mediated activation of p52/RelB heterodimers results in the production of lymphoid chemokines such as the EBl1-ligand chemokine (ELC/CCL19), secondary lymphoid tissue chemokine (SLC/CCL21), stromal cell-derived factor-1α (SDF-1α/CXCL12), B lymphocyte chemoattractant (BLC/CXCL13) and the cytokine B cell activation factor (BAFF), being all involved in lymphoid organogenesis and homeostasis (Dejardin et al., 2002; Seach et al., 2008). Gene Ontology analysis performed by Lovas et al. (2008), confirmed that LTβR-NF-κB target genes were predominantly involved in the regulation of immune responses. 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 (Lovas et al., 2008). The previously described findings on LTβR-mediated signal transduction and related effects are summarized in Figure 1.5.

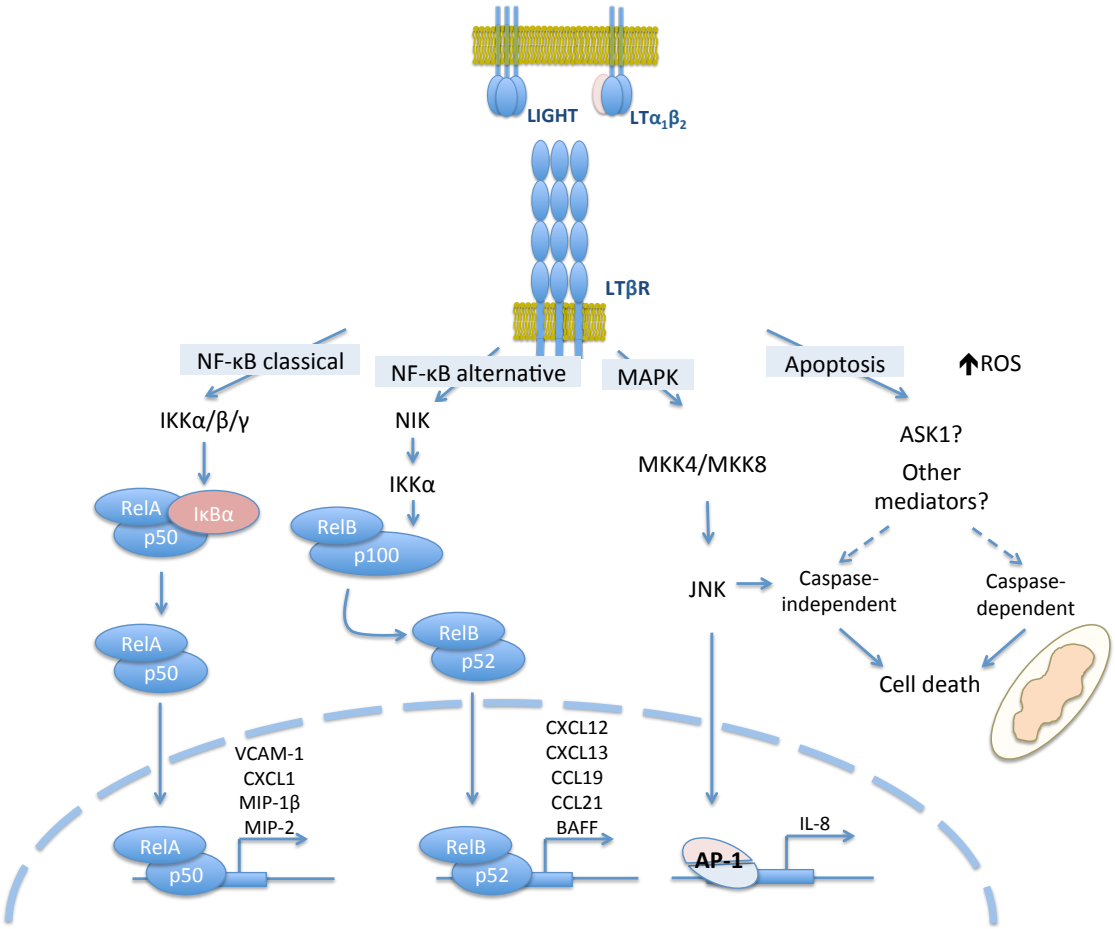


Figure 1.5. Summary of LTβR-mediated signal transduction for target-gene expression and cell death. Activation of LTβR signaling axis by LIGHT or $LT\alpha_1\beta_2$ induces gene expression and cell death. NF-κB classical pathway induction involves activation of IKK complex, IKK-mediated IκBα phosphorylation, and subsequent degradation, resulting in nuclear translocation of RelA/p50 heterodimers, which induce the expression of pro-inflammatory cytokines, chemokines, and adhesion molecules. 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.

1.3.4. Biological functions of the LTβR signaling pathway

Like other members of the TNF superfamily of ligands and receptors, $LT\alpha_1\beta_2$/LIGHT to LTβR signaling is critically involved in lymphoid organogenesis and the maintenance of secondary lymphoid structures, in addition to its roles related to the regulation of immune responses, including inflammation, tissue homeostasis, and innate immunity. These functions are in keeping with the profile of gene expression induced by $LT\alpha_1\beta_2$/LIGHT through LTβR signaling.

Lymphoid organogenesis is largely associated with signaling by the $LT\alpha_1\beta_2$ heterotrimer through LTβR as shown in studies where the signaling was blocked (Ettinger et al., 1996; Rennert et al., 1996), and reverse genetic modeling studies in which LTβR, LTα, LTβ and LIGHT targeted gene knockouts were generated (Fütterer et al., 1998; Koni et al., 1997; Scheu et al., 2002; De Togni et al., 1994). LTβR knockout mice lack most secondary lymphoid organs, including peripheral and mesenteric lymph nodes (LNs), Peyer´s patches and gut-associated lymphoid tissues (GALT) (Fütterer et al., 1998). LTα knockout mice lack peripheral LNs and Peyer´s patches although some mesenteric lymph node-like structures were observed in few mice (Banks et al., 1995; De Togni et al., 1994). Unexpectedly, most LTβ knockout mice conserved mesenteric LNs and cervical lymph node-like structures (Alimzhanov et al., 1997; Koni et al., 1997), a phenotype that could be improved by LIGHT-deletion, meaning that LIGHT can compensate for LTβ absence in mesenteric lymph node development (Scheu et al., 2002). In addition, all LTα-, LTβ- and LTβR-deficient mice, but not LIGHT-deficient mice presented splenic structural defects. Discrepancies in the effects of ligand/receptor gene inactivation led to the assumption that an alternative, yet unknown ligand for LTβR or nonspecific interactions may account for these differences.

In the adult, LTβR signaling was reported to be critically involved in the adaptive immune response against pathogens due to its intervention in processes such as DCs homeostasis and expansion (Kabashima et al., 2005; Wang et al., 2005), and lymphocyte maturation and survival (Banks et al., 2005; Elewaut et al., 2000; Silva-Santos et al., 2005; Wang and Fu, 2003). Furthermore, its activation is continuously required for the maintenance of the integrity and organization of microenvironments from secondary lymphoid organs (Ettinger et al., 1996; Fütterer et al., 1998; Rennert et al., 1996). For example, LTβR is important for the development and structural maintenance of fibroblastic reticular cells (FRCs) in LNs and spleen (Chai et al., 2013; Zhao et al., 2014). In the spleen its activation was also shown to be essential for FDC differentiation (Tumanov et al., 2002). Accordingly,

LTβR-deficient mice present disrupted FDC and germinal center formation and, consequently deficient B cell affinity maturation (Fütterer et al., 1998). LTβR signaling is also important for the trafficking of lymphoid and other hematopoietic cells, namely in the recruitment, migration and organization inside the organs, and emigration to other tissues (Ansel et al., 2000; Boehm et al., 2003; Luther et al., 2002; Seach et al., 2008). Moreover, it is involved in the regulation of acute inflammatory reactions and in the development of inflammation-associated neolymphoid structures (Cupedo et al., 2004; Kratz et al., 1996). In this process, LTβR-dependent stromal cell differentiation into reticular networks and induction of chemokines, cytokines and adhesion molecules play a critical role. Furthermore, it favors the recruitment of hematopoietic cells to lymphoid compartments by instructing the development and function of high endothelial venules (HEVs) (Browning et al., 2005).

Importantly, LTβR signaling has been shown to be a key player for thymic medullary epithelial cell differentiation (Mouri et al., 2011) and the maintenance of the thymic structure (Boehm et al., 2003), considered essential for central tolerance induction. In this context, T cell development and selection, and the maintenance of the thymic microenvironments require reciprocal interactions between thymocytes and stromal cells and the LTβR signaling is a critical mediator of this thymic crosstalk (Boehm et al., 2003). LTβR is expressed on virtually all stromal cells of the thymus (Seach et al., 2008). Conversely, the LTβR ligands are expressed in CD4 SP and CD8 SP thymocytes (Boehm et al., 2003). This expression pattern and the fact that LTβR stimulates the expression of adhesion molecules and chemokines, suggests that this receptor may be involved in the creation of microenvironments suitable for productive T cell interactions. The thymi of LTβR-deficient mice exhibit an aberrant microarchitecture characterized by a blurred demarcation between cortex and medulla and reduction of essentially Aire$^-$ mTEC subset (Boehm et al., 2003; Lkhagvasuren et al., 2013; Milićević et al., 2008; Venanzi et al., 2007). Further studies showed that SP thymocytes could activate LTβR leading to TRAF3 derepression and NIK-induced alternative NF-κB signaling pathway activation, which is indispensable for mTEC development (Burkly et al., 1995; Jenkinson et al., 2013; Kajiura et al., 2004). The LTβR-deficient mice also exhibit signs of autoimmunity characterized by the development of auto-antibodies to multiple organs (Boehm et al., 2003) and the presence of lymphocytic infiltrates in the perivascular areas of various organs (Fütterer et al., 1998), as previously reported also for LTα-, LTβ and LIGHT-deficient mice (Alimzhanov et al., 1997; Banks et al., 1995; Scheu et al., 2002). These autoimmune and inflammatory effects are believed to be a secondary effect of thymic structural defects in *Ltbr* knockout mice, resulting from the

impaired development of mTECs (Boehm et al., 2003), reduced expression of TEC-derived CCL19 and CCL21 chemokines and consequent defects in thymocyte migration from the cortex to the medulla (Lkhagvasuren et al., 2013; Seach et al., 2008; Zhu et al., 2007), but also reduced expression of some *Aire*-independent tissue-restricted antigens (TRAs) (e.g., casein β, casein κ and C-reactive protein) (Seach et al., 2008).

Altogether, the information extracted from experiments with LTβR knockout mice corroborates the conclusion that, in the thymus of adult mice, lymphocytes bearing $LT\alpha_1\beta_2$ and inducing LTβR signaling in mTECs, in association with signals mediated by RANK and CD40 (Akiyama et al., 2008; Hikosaka et al., 2008; White et al., 2008), have an important role in maintaining the integrity, organization and function of thymic microenvironments and in the control of autoimmunity. Furthermore, since LTβR and RANK have a cooperative action on immature mTECs from fetal thymus, these receptors share also a role in thymic organogenesis. In this context, it was reported that LTβR activation induces RANK expression, the activation of which by RANKL-expressing lymphoid tissue inducer cells is vital for mTEC development (Mouri et al., 2011). A possible similar mechanism involving the adult thymus was not clearly demonstrated to date.

In addition to these studies based on genetic inactivation, other reports revealed that excessive LTβR signaling has a dramatic impact in the thymus. LTα and LTβ overexpression in T lymphocytes induced thymic atrophy mediated by a synergistic effect of TNFR1 and LTβR signaling in radioresistant stromal cells (Heikenwalder et al., 2008; Liepinsh et al., 2009). In this context, thymic involution was most likely caused by stromal cell death, especially involving cortical TECs, by alterations in the microarchitecture of the thymus and by aberrant T-cell development. Consequently it resulted in compromised immune competence. These reports suggest that LTβR and TNFR1 signaling may contribute to thymic atrophy upon chronic infection or inflammation (Liepinsh et al., 2009) and during aging (Heikenwalder et al., 2008), and highlight the importance of the strict control of $LT\alpha_3$ and $LT\alpha_1\beta_2$ expression in the thymus. Likewise, T-cell restricted overexpression of LIGHT, resulted in a similar phenotype regarding the reduced size and altered structure of the thymus (Wang et al., 2001). Furthermore, it promoted the apoptosis of DP thymocytes. As LIGHT blockade was shown to result in the rescue of thymocytes from apoptosis, a role for LIGHT to LTβR signaling in T-cell negative selection and central tolerance induction was disclosed (Wang and Fu, 2003; Wang et al., 2001). In contrast, LIGHT overexpression was shown to activate mature T cells in the periphery, leading to inflammation and tissue destruction, mainly in the intestinal mucosa (Shaikh et al., 2001). These peripheral effects were not

unexpected since LIGHT is a costimulatory molecule for T-cell activation, proliferation and IFN-γ secretion. However, these functions are likely mediated by LIGHT to HVEM signaling (Scheu et al., 2002). In fact, LIGHT studies are complicated to interpret by the fact that it binds both LTβR and HVEM and, in specific contexts, LTβR binding may be attenuated by HVEM (Bechill and Muller, 2014). Furthermore, LIGHT to LTβR and HVEM binding can be competitively limited by the DcR3 decoy receptor.

Considering the pleiotropic functions of LTβR signaling it is thus not unexpected that, when deregulated, it leads to autoimmune and inflammatory diseases (Tumanov et al., 2007) like rheumatoid arthritis (Bekiaris et al., 2013; Young et al., 2010), Sjögren´s syndrome (Gatumu et al., 2009), autoimmune pancreatitis (Seleznik et al., 2014), hepatitis (Haybaeck et al., 2009) or colitis (Stopfer et al., 2004). Although LTβR and its ligands are recognized as key players in immunity, currently it is known that they are involved in many other biological processes such as liver regeneration (Anders et al., 2005; Tumanov et al., 2009), hepatitis (Chen et al., 1997; Lowes et al., 2003), hepatic lipid metabolism (Lo et al., 2007), and adipocyte differentiation (Bénézech et al., 2012). Importantly, LTβR signaling has also been reported to be involved, not only in cell death and tumor growth inhibition, but also in cancer development and progression (Drutskaya et al., 2010; Wolf et al., 2010), what explains the paradoxical roles identified in this context. The context-dependent roles for LTβR signaling in cancer are reviewed next.

1.4. LTβR SIGNALING IN CANCER DEVELOPMENT

LTα, also known as TNFβ, was the first of the two lymphotoxins to be discovered and it was named this way because it was a cytokine similar to TNFα that induced cytotoxicity when added to cells in culture (Ruddle, 2014). Indeed, several studies reported that when LTβR was directly activated in some carcinoma and sarcoma cells it promoted growth inhibitory and apoptotic effects (Browning et al., 1996; Dhawan et al., 2008; Hu et al., 2013; Lukashev et al., 2006). In addition, LTβR was shown to mediate anti-tumor effects also by other mechanisms such as tumor sensitization to chemotherapeutic agents and radiation (Hu et al., 2013; Lukashev et al., 2006), and stimulation of host-mediated anti-tumor immune responses.

In the latter case, the anti-tumoral effects were achieved either by inducing the expression of pro-inflammatory cytokines and chemokines that function as chemoattractants for activated lymphocytes (Lukashev et al., 2006; Winter et al., 2007), or by inducing the differentiation of high endothelial venules that mediate lymphocyte trafficking to normal organs and tumors (Browning et al., 1995; Martinet et al., 2013).

Paradoxically, a wide body of evidence has implicated LTβR in pro-carcinogenic mechanisms. In a variety of contexts, LTβR was shown to have direct cell-intrinsic oncogenic properties in cancer cells due to overexpression and self-oligomerization without the requirement for ligand-induced activation (Dhawan et al., 2008; Force et al., 2000; Fujiwara et al., 2005; Or et al., 2010). Conversely, when induced by ligand expressing cells, it can promote tumor angiogenesis (Daller et al., 2011; Hehlgans et al., 2002), chronic inflammation (Ammirante et al., 2010; Haybaeck et al., 2009; Wolf et al., 2014), and modulate cancer microenvironments into pro-growth and survival niches (Heinig et al., 2014; Lau et al., 2014; Rehm et al., 2011). Indirect effects in the thymus or in the local microenvironment may also lead to the suppression of anti-tumor immune responses (Kim et al., 2011; Zhou et al., 2009). LTβR involvement in cancer is not unexpected since, in addition to the fact that several cancer cells from different origins express LTβR (Dhawan et al., 2008; Hu et al., 2013; Lukashev et al., 2006), in some cases its expression has been shown to be upregulated throughout cancer progression and metastization (Dhawan et al., 2008; Hu et al., 2013; Or et al., 2010). Furthermore, LTβR activation and NF-κB induction are closely linked and NF-κB constitutive activation has been increasingly recognized as a hallmark of several types of cancer. LTβR-mediated NF-κB signaling activation in cancer cells may result from either exposure to pro-inflammatory stimuli in the tumor microenvironment or from genetic alterations in components of this pathway.

Usually, immune cells either normal or malignant, and cancer cells infected by virus, such as human papillomavirus 16E6 (HPV16) (Simonin et al., 2013) and hepatitis C virus (HCV) (Kim et al., 2011), express the $LT\alpha_1\beta_2$ and/or LIGHT ligands. On the other hand, LTβR is mainly expressed by stromal cells such as FDCs and fibroblasts (Heinig et al., 2014; Lau et al., 2014; Rehm et al., 2011), and cancer cells (Ammirante et al., 2010; Daller et al., 2011), excluding B, T and NK cells (Table 1.4).

1.4.1. LTβR cell-intrinsic pro-carcinogenic roles

As mentioned before, LTβR may act as an oncogene promoting the survival and/or proliferation of cancer cells. One of the first studies reporting LTβR transforming ability was done in the context of pancreatic ductal carcinoma (PDC). Construction of a retroviral cDNA expression library from total RNA isolated from the PDC cell line MiaPaCa-2 led to detection of an NH2-terminally truncated form of LTβR with transforming activity *in vitro* and *in vivo*. Interestingly, full-length LTβR protein was also shown to display transforming activity (Fujiwara et al., 2005). Supporting the notion that LTβR was pro-oncogenic, LTβR was found to be overexpressed and consistently activated in 7.3% cases of nasopharyngeal carcinoma (NPC) due to a 12p13.3 amplification (Or et al., 2010). In a nasopharyngeal epithelial cell line, LTβR overexpression was shown to contribute to increased NF-κB activity and cancer cell proliferation. Importantly, LTβR knockdown inhibited *in vivo* tumor growth in an NPC xenograft model (Or et al., 2010). Further demonstrating the importance of NF-κB activation in NPC development, genetic alterations affecting NF-κB signaling were identified in cases without *LTBR* gene amplification (Chung et al., 2013).

Further highlighting the role of LTβR as an activator of NF-κB in cancer promotion, a search for genetic abnormalities in multiple myeloma revealed mutations in multiple members of classical and alternative NF-κB pathways including *LTBR* amplifications in one cell line and one primary patient sample. This mutation was shown to result in LTβR overexpression and constitutive activation of the noncanonical NF-κB pathway (Keats et al., 2007).

LTβR was also reported to be oncogenic in melanoma. Dhawan and coworkers have shown that LTβR expression is upregulated in human melanoma cells and melanocytic lesions, when compared to normal melanocytes. As a result, the NF-κB pathway was often activated leading to cell proliferation and increased invasiveness (Dhawan et al., 2008). Activation of LTβR *ex vivo* appeared to be ligand-independent, as ligand expression was not detected in cultured melanoma cells and inhibition of LTβR ligand binding had no effect in NF-κB activation or cell growth (Dhawan et al., 2008). Nonetheless, in a previous study it was shown that LIGHT is frequently expressed in human melanoma metastasis and melanoma-derived microvesicles, and it was suggested that LIGHT interaction with LTβR in stromal cells may induce the T-cell attractant CCL21 chemokine and play a role in regulating T-cell infiltration of neoplastic tissue (Mortarini et al., 2005).

1.4.2. LTβR pro-carcinogenic roles mediated by interactions with the tumor microenvironment

It is generally accepted that the interaction of immune cells with tumor cells can either restrain or promote tumor progression. Thus, depending on the chemokine milieu in the tumor microenvironment, tumor-infiltrating immune cells can mediate a response against tumor-specific antigens to repress tumor growth or rather help tumors to subvert the adaptive immune responses. Likewise, as a signaling axis involved in immune cell communication, LTβR can also contribute to tumor microenvironmental alterations that may promote inflammation and angiogenesis.

Tumor and stromal cells respond to injury, infection, and tissue damage by producing cytokines and chemokines that chemoattract immune cells (Chovatiya and Medzhitov, 2014). As a result, immune cells migrate to the tumor microenvironment where they secrete inflammatory, pro-angiogenic and in some cases pro-tumorigenic growth and survival factors, therefore having pro-carcinogenic actions that facilitate tumor growth, invasiveness, and metastization (Coussens and Werb, 2002).

1.4.2.1. LTβR-induced angiogenesis

The importance of angiogenesis for the growth of solid tumors is well recognized. As tumor growth and metastasis require persistent new blood vessel formation, under selective pressure, a developing tumor transits from an avascular phase to an angiogenic phase, the so-called angiogenic switch (Hanahan and Folkman, 1996). This switch is controlled by a balance between pro- and anti-angiogenic factors that may be secreted by the tumor cells themselves or by cells in the tumor microenvironment, in particular immune cells and other stromal cells. It is known that the expression of pro- and anti-angiogenic factors by cancer cells is controlled directly by oncogenes, tumor suppressor genes and transcription factors, but also indirectly by microenvironmental factors. Yet, the roles and the interplay among the various inflammatory cytokines and chemokines during the angiogenic switch are still not completely understood.

Shedding light on a role for LTβR in angiogenesis, Hehlgans and colleagues have shown that LTβR signaling ablation inhibits angiogenesis and tumor growth (Hehlgans et al., 2002). In this report and further studies using the methylcholanthrene-induced murine fibrosarcoma model (BFS-1), it was show that LTβR activation by $LT\alpha_1\beta_2$-expressing T and B lymphocytes and also LIGHT, induced the expression of the angiogenic mediator

CXCL2/MIP-2 through NF-κB activation and, consequently, increased angiogenesis and solid tumor growth *in vivo*. The described pro-tumorigenic effect of LTβR activation was shown to be due to modulation of the tumor microenvironment and not cell-intrinsic mechanisms because *ex vivo* treatment of fibrosarcoma cells with an agonistic anti-LTβR monoclonal antibody did not increase proliferation or survival (Daller et al., 2011; Hehlgans et al., 2002). Recently, the same investigators have identified another player in this process. They found that host-derived tumor-infiltrating $CD45^+$ hematopoietic cells expressed the mouse β-defensin 14 (mBD14), which was shown to mediate $CD19^+$ and $B220^+$ lymphocyte chemoattraction through CCR6 stimulation. These lymphocytes expressed $LT\alpha_1\beta_2$ and possibly LIGHT that, by interaction with LTβR in fibrosarcoma cells induced CXCL2 expression and initiated a proangiogenic pathway leading to increased tumor vascularization and growth (Röhrl et al., 2012).

1.4.2.2. LTβR-induced chronic inflammation

In the above-mentioned studies on the role of LTβR in fibrosarcoma development, tumor-infiltrating immune cells were responsible for the initiation of an acute inflammatory response. This, in turn created a permissive microenvironment for tumor progression through angiogenesis induction (Daller et al., 2011; Hehlgans et al., 2002; Röhrl et al., 2012). Differently from this situation, inflammation often contributes to cancer development when it is not clearly resolved and becomes persistent. Indeed, it is well established that chronic inflammation in the tumor microenvironment constitutes a hallmark of cancer (Colotta et al., 2009; Mantovani et al., 2008). Furthermore, NF-κB signaling has been pinpointed as a key molecular link between chronic inflammation and cancer (DiDonato et al., 2012). As an upstream activator of NF-κB, LTβR has also been found to be involved in immune cell infiltration, chronic inflammation, and carcinogenesis in models of hepatocellular carcinoma (HCC) and prostate cancer (Ammirante et al., 2010; Haybaeck et al., 2009; Wolf et al., 2014).

Haybaeck and coworkers have described the involvement of LTβR signaling in the development of chronic virus-induced hepatitis and HCC. First, LTβR and its ligands LTα, LTβ and LIGHT, but also proinflammatory chemokines like CCL2, CCL3, and CXCL10, were foun to be upregulated in human primary samples of hepatitis B virus (HBV)- or HCV-induced chronic hepatitis and HCC. Furthermore, a causal link between HCV infection and *LTBR*, *LTA*, *LTB*, *LIGHT* and inflammatory chemokine expression was shown *in vitro* using

the human hepatoma cell line Huh-7.5. In transgenic mice expressing high levels of LTα and LTβ in the liver, LTβR signaling induced chronic hepatitis characterized by inflammation with lymphocytic infiltrates of T and B cells and hepatocyte apoptosis. Both B and T lymphocytes, which expressed LTβR ligands (including $LT\alpha_1\beta_2$), and canonical NF-κB signaling pathway induction following LTβR activation in hepatocytes, were shown to be required for LTβR-induced chronic hepatitis and HCC development. In this mouse model, rather than having direct oncogenic properties, LTβR signaling generated an inflammatory hepatic microenvironment driving oncogenesis (Haybaeck et al., 2009). Supporting the previous study, Simonin and coworkers have shown that LTβ expression is induced by the HCV core protein NS5B in Huh7 human hepatoma cells. Using a transgenic mouse model of inflammation-induced liver tumorigenesis with targeted expression of the full length HCV open reading frame in hepatocytes, it was shown that LTβ expression induction in hepatocytes was followed by NF-κB activation, chemokine neosynthesis, and intra-tumoral recruitment of macrophages and T and B lymphocytes (Simonin et al., 2013). Using another model in which mice are exposed to long-term choline-deficient high-fat diet, Wolf and colleagues identified $CD8^+$ T and NKT cells recruited to the liver as key players in the development of steatosis and its progression to hepatocellular carcinoma (Wolf et al., 2014). They found that these immune cells interacted with hepatocytes leading to their activation and to the release of soluble factors including LIGHT and lymphotoxin, which induced liver tumorigenesis through LTβR-induced classical NF-κB signaling activation in hepatocytes (Wolf et al., 2014).

Cancer therapy-induced cell death can also elicit an inflammatory response that may contribute to therapeutic failure rather than to success. This is the case of castration-resistant metastatic prostate carcinoma, the emergence of which constitutes a major clinical complication and is responsible for most prostate cancer mortality. Using subcutaneous allografts of the mouse androgen-dependent prostate cancer (CaP) cell line myc-CaP in castrated FVB mice, Ammirante and colleagues unveiled a mechanism underlying the emergence of castration-resistant prostate cancer. They have found that following androgen ablation therapy, the death of androgen-deprived cancer cells induced an inflammatory response with concomitant production of CXCL13 and other inflammatory chemokines, and recruitment of leukocytes, most importantly B cells, into the regressing tumor. IKKβ activation in B cells, presumably by inflammatory cytokines, induced the expression of $LT\alpha_1\beta_2$. Through LTβR activation in prostate cancer cells, these proteins led to IKKα activation and nuclear translocation to promote androgen-independent growth and survival,

together with STAT3 activated by other non-identified factors (Ammirante et al., 2010). In this study, it was not determined the mechanism by which death of androgen-deprived CaP triggers the inflammatory response. However, it is known that the uncontrolled tumor growth, which leads to disruption of tissue architecture and lack of blood supply, may result in necrosis and the consequent release of damage associated molecular patterns (DAMPs). These molecules are produced by cells and tissues involved in host defense against microbial infections (defensins), passively released by necrotic cells or actively secreted by stimulated inflammatory cells (expressing HMGB1) (Scaffidi et al., 2002). In a recent study by He and coworkers using the TRAMP model of human prostate cancer, HMGB1 was shown to be induced during tumor progression and to be required for the activation and accumulation of T cells within the prostate tumor. The tumor-infiltrating T cells expressed $LT\alpha_1\beta_2$ and, through LTβR activation, promoted the recruitment of macrophages into the tumor presumably by inducing monocyte chemotactic protein 1 (MCP-1) upregulation. This mechanism contributed to the progression from hyperplasia to invasive prostate carcinoma (He et al., 2013). Differently from the study by Ammirante and coworkers, the inflammatory response in this case was mediated by $LT\alpha_1\beta_2$-expressing T cells rather than B cells, and LTβR signaling contributed to cancer progression from hyperplasia to malignant carcinoma (He et al., 2013). Together, these studies show that LTβR may contribute to several developmental phases of the same type of cancer and involving different cellular players.

1.4.2.3. Immune evasion mediated by LTβR

Another mechanism through which LTβR signaling was described to be involved in cancer development is by interfering with the host anti-cancer adaptive immune response. Because $LT\alpha_1\beta_2$-LTβR plays a role in self-tolerance induction (Boehm et al., 2003; Fütterer et al., 1998; Seach et al., 2008; Zhu et al., 2007), blocking this signaling axis might rescue tumor-reactive T cells from clonal deletion in the thymus and therefore prevent cancer development. To test this hypothesis, Zhou and coworkers used the TRAMP model, bearing a T-cell receptor specific for the SV40 large T antigen. They found that targeted inactivation of the LTα gene impaired efficient negative selection of tumor-reactive T cells in the thymus, resulting in a decrease in cancer incidence and in a milder malignant phenotype. Confirming the impact of LTβR signaling in prostate cancer development, short-term blocking of LTβR also rescued T cells from clonal deletion and consequently reduced the progression of primary prostate cancer and prevented metastasis (Zhou et al., 2009). Since LTβR signaling

increased the host resistance to prostate cancer, this mechanism was suggested as a non-antigen-based strategy of immune prevention for patients with high genetic risk for cancer. Similarly, cervical cancer was reported to develop due to suppression of cellular immunity, but in this case through a mechanism involving lymphotoxin signaling locally in the tumor microenvironment. In this context, Kim and colleagues have shown that the E6 oncogene from HPV16, which is responsible for oncogenic transformation of the cervical epithelium, induces LTα, LTβ and LTβR gene expression in HeLa cervical cancer cells. This resulted in NF-κB activation and consequent MHC I downregulation followed by resistance to cytotoxic T-lymphocyte-mediated lytic activity and cancer cell evasion (Kim et al., 2011).

1.4.2.4. Induction of a pro-tumorigenic niche supported by LTβR-expressing stromal cells

Recently, some studies of great relevance to this thesis, unveiled a role for LTβR signaling in lymphoid malignancies. In this context, $LT\alpha_1\beta_2$-LTβR signaling was shown to mediate paracrine tumor-stromal interactions conditioning the microenvironment to establish attractive tumor-permissive niches in secondary lymphoid organs. Rehm and colleagues identified the homeostatic chemokine receptor CCR7 as a determinant factor in dictating the location and survival of B-cell lymphoma cells within secondary lymphoid organs. In the *Eμ-Myc* transgenic mouse model of aggressive human B-cell lymphoma, they found that CCR7 regulates lymphoma cell dissemination to LNs and to the T-cell zone of the spleen, where through $LT\alpha_1\beta_2$ expression, cancer cells stimulate LTβR in glycoprotein 38-positive FRCs. This crosstalk results in the expansion of the stromal FRC network and in the release of chemoattractant homeostatic chemokines (e.g., CCR7 ligands, CCL19 and CCL21), and trophic factors (e.g., indian hedgehog) from FRCs that confer a survival advantage to lymphoma cells (Rehm et al., 2011). In another recent report, the authors used the murine *Eμ-Tcl1* model of B-cell chronic lymphocytic leukemia (B-CLL) to show that the CXCR5-CXCL13 signaling axis mediates leukemic B cell access to a stromal compartment enriched with FDCs in the spleen. In this location, leukemic B cells and FDCs engaged an LTβR-mediated reciprocal crosstalk in which $LT\alpha_1\beta_2$-expressing leukemic cells stimulate the differentiation of FDC networks and CXCL13 production. On the other hand, FDCs provide protection and proliferative stimuli to leukemic cells presumably by producing B-cell growth factors (Heinig et al., 2014). In both studies, the inhibition of LTβR-mediated interactions between malignant cells and their microenvironment impaired disease progression and

though was pointed as a possible strategy to complement traditional cytotoxic therapies (Heinig et al., 2014; Rehm et al., 2011).

In addition to hematopoietic malignancies, solid cancers can also take advantage of LTβR signaling to modulate the tumor microenvironment in their favor. In a recent report, it was shown that ovarian cancer cells maintain reciprocal interactions with stromal cells through LTβR (Lau et al., 2014). It was demonstrated that human ovarian cancer primary cells overexpress $LT\alpha_1\beta_2$ and induce chemokine expression in LTβR-expressing cancer-associated fibroblasts through NF-κB signaling. One of the chemokines induced in stromal fibroblastic cells was CXCL11, which promoted proliferation and migration of ovarian cancer cells. This specific expression pattern of LTβ and LTβR was also identified in endometrial cancer, suggesting that it may be a common phenomenon in gynecologic malignancies (Lau et al., 2014).

1.4.3. Determinants of LTβR anti- and pro-carcinogenic roles

Altogether, the studies previously described have disclosed several but perhaps not all factors influencing the pro- or anti-carcinogenic activities of LTβR signaling. Several variables such as the tumor type, the progression stage, the cancer-intrinsic genetic and epigenetic alterations, the status of activated signaling pathways, the microenvironmental factors, and the experimental model used may ultimately determine if the overall effect of LTβR activation should be pro- or anti-tumorigenic. Moreover, the mechanisms by which LTβR may lead to foster or impede tumor progression are not completely understood. Nevertheless, the classical and alternative NF-κB signaling pathways seem to be commonly activated by LTβR in any scenarios, which corroborates the dual role of NF-κB signaling in cancer observed in different contexts (Klein and Ghosh, 2011).

Another important issue to consider when studying LTβR role in carcinogenesis is the mechanism of activation. It may be constitutively activated due to overexpression and self-oligomerization, or it may be activated only in the presence of its ligands. In the latter case, heterotypic interactions with cells present in the tumor microenvironment are usually involved (Figure 1.6). Furthermore, it is important to determine which ligand may exert the observed effects, if $LT\alpha_1\beta_2$, LIGHT or both. Importantly, the way by which ligand-induced activation of LTβR is achieved (membrane-bound or soluble ligand) or experimentally mimicked (e.g., lymphoid cells expressing the ligand, recombinant soluble ligand or soluble or immobilized agonistic LTβR antibody) should be carefully considered. These factors are

highly relevant since they may lead to different cellular outcomes. For instance, it is known that the degree of receptor clustering and the varying lifetime of the oligomerized states may lead to diverse cellular responses following receptor activation (Browning et al., 1996; Mackay et al., 1996; VanArsdale et al., 1997). Also, during the course of LTβR stimulation, which may be short or prolonged, different NF-κB complexes are activated and may result in the expression of different sets of target genes (Dejardin et al., 2002; Müller and Siebenlist, 2003).

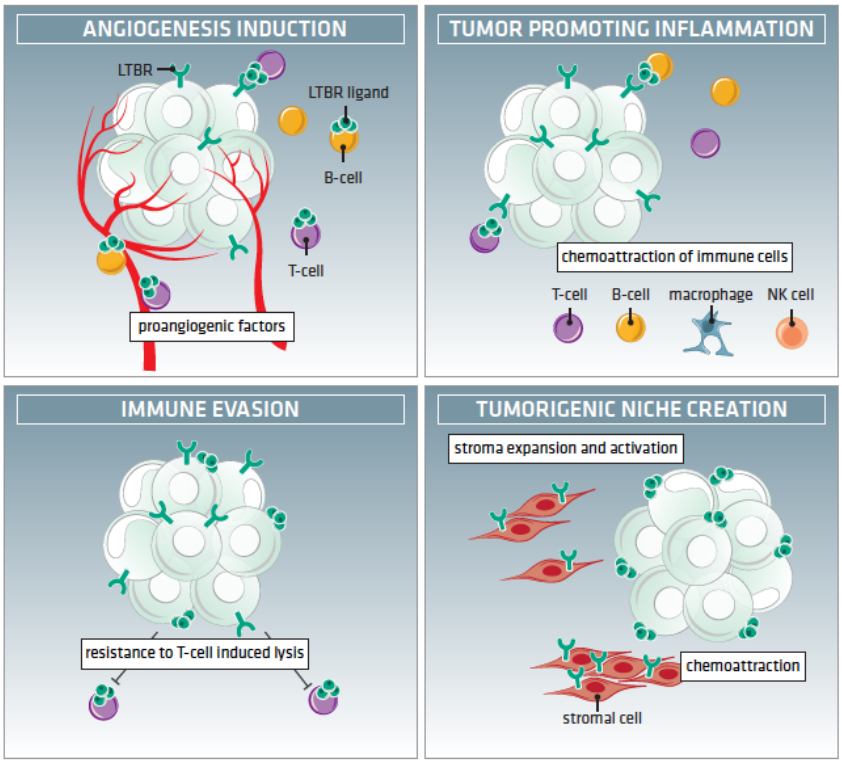


Figure 1.6. LTβR pro-tumorigenic roles in the tumor microenvironment. Activation of LTβR signaling axis favors carcinogenesis due to crosstalks between malignant cells and cells from the tumor microenvironment by four main mechanisms. When activated in tumor cells by its ligands expressed in lymphocytes, LTβR activates the expression proangiogenic factors, leading to angiogenesis induction and tumor progression. In another settings, LTβR ligand-expressing lymphocytes activate LTβR in tumor cells, which produce chemokines that attract other inflammatory cells, resulting in tumor-promoting inflammation. LTβR activation in tumor cells may also affect the recognition of tumor cells, resulting in resistance to T-cell induced lysis and immune evasion. Finally, malignant cells expressing LTβR ligands can activate the receptor in stromal cells, leading to stroma expansion and activation, followed by production of pro-survival factors and further attraction of malignant cells. All these interactions lead to the creation of a pro-tumorigenic niche.

Due to its pro-carcinogenic functions and with the exception of cancers presenting constitutive activation of LTβR, blocking of LTβR signaling between tumor cells and microenvironmental cells has been proposed as a therapy (Bjordahl et al., 2013).

Unfortunately, because of its dual functions in cancer development and progression, to develop a rational and effective cancer therapy and prevent deleterious side effects, it is imperative to learn more about the mechanisms and contexts in which contexts LTβR may exert pro-carcinogenic effects.

Table 1.4. LTβR-induced pro-carcinogenic effects in different cellular contexts.

Cancer Type	LTαβ	LIGHT	LTβR	Context	Reference
Solid Cancers					
Nasopharyngeal Carcinoma	-	-	Carcinoma	LTβR amplification causing activation of NF-κB	(Or et al., 2012)
Pancreatic Ductal Carcinoma	-	-	Carcinoma	LTβR transforming activity	(Fujiwara et al., 2005)
Melanoma	-	-	Melanoma	Autonomous growth of melanoma cells	(Dhawan et al., 2008)
Fibrosarcoma	T, B Lymphocytes	n.i.	Fibrosarcoma	Induction of angiogenesis and tumor growth	(Hehlgans et al., 2002) (Daller et al., 2010)
Liver Cancer	T and B Lymphocytes (mainly) Hepatocytes (HV-infected)	T and B Lymphocytes	Hepatocytes (mainly)	Virus-induced chronic hepatitis and HCC	(Haybaeck et al., 2009) (Simonin et al., 2013)
	$CD8^+$ and NKT Lymphocytes	$CD8^+$ and NKT Lymphocytes	Hepatocytes	Diet-induced nonalcoholic steatohepatitis and HCC	(Wolf et al., 2014)
Prostate Carcinoma	B Lymphocytes	n.d.	Carcinoma	Emergence of castration-resistant CaP	(Ammirante et al., 2010)
	T Lymphocytes	-	Prostate epithelial cells	Progression from pre-neoplasia to carcinoma	(He et al., 2013)
Cervical Carcinoma	Cervical Cancer Cells (HPV-infected)	n.d.	Cervical Cancer Cells	Immune escape	(Kim et al., 2011)
Ovarian Carcinoma	Ovarian Cancer Cells	n.d.	CAFs	Promotion of a pro-carcinogenic niche	(Lau et al., 2014)
Multiple Myeloma	-	-	MM cells	LTβR amplification causing activation of NF-κB	(Keats et al., 2007)

Table 1.4 (cont.). LTβR-induced pro-carcinogenic effects in different cellular contexts.

Hematological Cancers					
B-cell Lymphoma	Malignant B Cells	n.d.	FRCs	Promotion of a pro-carcinogenic niche	(Rehm et al., 2011)
B-CLL	Malignant B Cells	n.d.	FDCs	Promotion of a pro-carcinogenic niche	(Heinig et al., 2014)
Ovarian Carcinoma	Ovarian Cancer Cells	n.d.	CAFs	Promotion of a pro-carcinogenic niche	(Lau et al., 2014)

-, not expressed; n.i., the cells expressing it were not identified; n.d., not determined; B-CLL, B-cell chronic lymphocytic leukemia; CAFs, cancer-associated fibroblasts; CaP, prostate carcinoma; FDCs, follicular dendritic cells; FRCs, fibroblastic reticular cells; HCC, hepatocellular carcinoma; HV, hepatitis virus; HPV, human papilloma virus.

1.5. THE RANKL TO RANK SIGNALING AXIS

1.5.1. The receptor activator of NF-κB

The receptor activator of NF-κB (RANK) genetic locus (*TNFRSF11A* or *RANK*) is located in Chr 18 in humans (mouse Chr 1) and it contains 10 exons spanning approximately 61 kb (Anderson et al., 1997). Through alternative splicing, the *RANK* gene produces different isoforms truncated either in its ICD or ECD (Sirinian et al., 2013). The RANK full-length transcript is approximately 4.5 kb in size and encodes a 616 amino acid glycosylated type I transmembrane protein, consisting of an ECD with a signal secretion peptide, four CRDs, and a long cytoplasmic domain (Anderson et al., 1997). The cytoplasmic domain of RANK consists of 383 amino acids and like LTβR does not contain a death domain. It contains also several putative TRAF-binding sites that cluster in distinct regions: one located in the most C-terminal 93 amino acids, which is able to bind TRAF2/TRAF5 and TRAF6, and another membrane-proximal, which binds TRAF6 (Darnay et al., 1998; Hsu et al., 1999; Wong et al., 1998). Moreover, within the cytoplasmic domain, a region comprising amino acids 524 to 550 mediates ligand-independent oligomerization and signal transduction (Das et al., 2014; Kanazawa and Kudo, 2005). The murine RANK shows high homology with its human counterpart (Anderson et al., 1997) and binds the homologous ligand with similar specificity (see below).

RANK is expressed on mature DCs (Josien et al., 1999) and osteoclasts (Hsu et al., 1999), in mammary epithelial cells (Fata et al., 2000), thymic medullary epithelial cells (Hikosaka et al., 2008) and, at low levels, in activated T cells (Anderson et al., 1997; Josien et al., 1999) and activated B cells (Josien et al., 1999). In addition, its expression may be constitutive or, in certain contexts, inducible. Indeed, RANK expression has been shown to be inducible by TCR/CD3 activation on peripheral blood T cells (Josien et al., 1999), CD40L on DCs (Anderson et al., 1997), macrophage colony-stimulating factor (M-CSF) on osteoclast precursor cells (Arai et al., 1999), and LTβR activation on fetal thymic mTECs (Mouri et al., 2011).

1.5.2. The RANK ligand

The only known ligand for RANK has been designated receptor activator of NF-κB ligand (RANKL) or TNFSF11 but several alternative names such as TNF-related activation-induced cytokine (TRANCE), osteoclast differentiation factor (ODF) or osteoprotegerin ligand (OPGL) may also be found in the literature.

The human RANKL genomic locus (*TNFSF11* or *RANKL*) consists of 5 exons spanning approximately 45 kb on Chr 13 in humans (murine Chr 14) (Anderson et al., 1997). The full-length RANKL mRNA encodes a glycosylated type II transmembrane protein of 317 amino acids (Anderson et al., 1997; Wong et al., 1997). The murine and human RANKL proteins are highly homologous (Anderson et al., 1997). The exon 1 encodes the 5' UTR, the ICD and TMD, while exons 2 to 5 encode the ECD (Walsh et al., 2013). The ECD of membrane-bound RANKL can be cleaved by TACE at amino acids 139-140 to produce a soluble active protein (Lum et al., 1999). Nevertheless, alternatively spliced transcript variants lacking exon 1 and the TMD can occur. These encode functional soluble protein isoforms composed of only the ECD (Nagai et al., 2000; Walsh et al., 2013).

RANKL is expressed in activated peripheral T cells and predominantly in CD4 SP thymocytes, but also in CD8 SP and CD4/CD8 DN thymocytes (Anderson et al., 1997; Hikosaka et al., 2008; Josien et al., 1999; Wong et al., 1997). In T cells, RANKL expression was shown to be inducible through TCR activation using anti-CD3/CD28 or PMA plus ionomycin stimulation and is controlled by calcineurin-regulated transcription factors (Josien et al., 1999; Walsh et al., 2013; Wong et al., 1997). A regulatory region proximal to exon 1A was reported to contain consensus-binding sites for transcription factors such as

NFAT/STAT, AP-1/STAT, or STAT, which are important for transduction of activation signals in T cells (Walsh et al., 2013).

In addition to T cells and its precursors, RANKL expression was also reported in pro-B cells (Anderson et al., 1997), mature DCs (Josien et al., 1999), osteoblasts and bone marrow stromal cells (Lacey et al., 1998; Yasuda et al., 1998), and osteocytes (Nakashima et al., 2011). Various osteoactive factors including glucocorticoids, vitamin D, and TNFα have been reported to induce RANKL expression in osteoblasts (Walsh and Choi, 2014).

RANKL binds and activates RANK, its functional receptor but binds also osteoprotegerin (OPG), a secreted TNFR-related decoy receptor that sequesters RANKL, impairing RANK engagement and signaling activation (Hsu et al., 1999; Lacey et al., 1998).

1.5.3. RANK activation, signal transduction and molecular effects

Engagement of RANK with RANKL promotes its trimerization (Kanazawa and Kudo, 2005), which leads to conformational changes and recruitment of adaptor factors and docking proteins to the receptor. RANK has been shown to interact with TRAFs 1, 2, 3, 5 and 6 (Darnay et al., 1998; Wong et al., 1998). In general, interaction with TRAF family members mediates RANK-induced NF-κB and JNK pathway activation (Darnay et al., 1998; Hsu et al., 1999) in various cell types. When overexpressed, RANK self-assembles in trimers in the absence of the ligand and this is sufficient to activate NF-κB and JNK (Hsu et al., 1999). NF-κB and JNK can be also activated by recombinant soluble RANKL and RANK agonist antibodies on cells expressing RANK (Anderson et al., 1997; Hsu et al., 1999; Wong et al., 1997). Activation of these pathways promotes translocation and activation of transcripton factors including NFATc1, CREB, NF-κB, AP-1, and MITF (Walsh and Choi, 2014). Moreover, RANK-mediated gene transcription may differ depending on cell type but often involves the induction of transcription factors like c-fos and NFATc1/NFATc2 (Ishida et al., 2002; Matsuo et al., 2000).

TRAF6 is a major transducer downstream of RANK activation. Nevertheless, most studies on RANK signaling were done using osteoclast precursor cells as a model and some of the mechanisms appear to be specific to osteoclastogenesis. In this context, TRAF6 is critical for the activation of MAPKs such as p38 and JNK, as well as the classical NF-κB pathway (Darnay et al., 1999; Lomaga et al., 1999; Naito et al., 1999). Moreover, in combination with TRAF2, TRAF6 is able to activate the TAB1/2/TAK1 complex, which in combination with other upstream kinases, leads to activation of IKKβ and NF-κB, and

MAPKs and AP-1 (Mizukami et al., 2002). In addition, TRAF6, c-Src and Cbl scaffolding protein interaction with the RANK cytoplasmic tail following receptor activation, leads to PI3K and Akt activation (Arron et al., 2001; Wong et al., 1999). Interestingly, TRAF6-dependent RANK signaling was shown to be negatively regulated by IFNγ-induced ubiquitination and degradation of TRAF6 through the lysosome/autophagy system (Takayanagi et al., 2000). RANK signaling can also regulate calcium homeostasis in combination with other factors (Yang et al., 2007). Also functioning in some settings as a negative regulation mechanism of RANK function, RANK ectodomain can be shed from osteoclast precursor cells by the TACE (Hakozaki et al., 2010).

Similarly to LTβR, RANK is one of the TNFR superfamily members capable of activating both the classical and alternative NF-κB pathways. RANK-associated TRAF3 has been shown to play a key role in negatively regulating the alternative NF-κB pathway. When RANK is activated, it overcomes NIK inhibition by triggering the degradation of TRAF3 (Xiu et al., 2014). For example, in mTECs, RANK engagement induces both TRAF6/IKKβ and NIK/IKKα, which activate the classical and the alternative NF-κB pathways, respectively culminating in the expression of target genes such as *Aire* and various TRAs (e.g., C-reactive protein, insulin 2, and salivary protein 1) (Akiyama et al., 2005, 2008; Kajiura et al., 2004). Moreover, the TRAF6-induced NF-κB classical signaling pathway was shown to induce RelB expression, while NIK stabilization contributed to RelB activation and translocation to the nucleus (Akiyama et al., 2008).

Another property common to some members of TNFSF ligands and receptors is the capacity to induce reverse signaling. Besides RANK-induced signaling activation in adjacent cells, binding of RANK to RANKL-expressing cells leads to signaling pathway activation. For example, reverse signaling through RANKL induces IFNγ secretion by T cells, via a p38-dependent pathway, when these interact with RANK-expressing DCs (Wang et al., 2001).

1.5.4. Biological functions of the RANK signaling pathway

Studies employing genetically deficient mouse models have demonstrated the critical role of RANKL to RANK signaling in bone and immunity. By generating RANK knockout mice, Dougall and coworkers found that it is essential for osteoclast differentiation and bone resorption, and also lymph node organogenesis, but not for mucosal-associated lymphoid tissues, although these were reduced in the knockout mice (Dougall et al., 1999). Similarly,

RANKL genetic ablation resulted in osteopetrosis and lymph node absence, in addition to B- and T-cell defects (Kong et al., 1999).

Highlighting its essential role in bone metabolism, the RANKL-RANK-OPG system was shown to be involved in diseases like postmenopausal osteoporosis, familial expansile osteolysis and Paget's disease of bone (Silva and Branco, 2011). In addition, it was shown to play a role in the so-called cancer skeletal-related events (SREs) (e.g., pain, hypercalcemia, and fractures) that are mediated by some types of cancer that metastasize to the bone, like breast and prostate cancer (Buckle et al., 2012; Fizazi et al., 2009). RANK activation in breast and prostate tumor cells was also recognized as a player in metastization to the bone (Casimiro et al., 2013) since RANKL expression in the bone seems to chemo-attract RANK-expressing cancer cells (Jones et al., 2006). Moreover, a role for osteolysis in cancer development was supported by studies showing that osteolysis induces the release of tumor-promoting factors such as insulin-like growth factor-1 and hypoxia-inducible factor (Kuchimaru et al., 2014).

Besides their critical roles in lymph node organogenesis, RANKL/RANK signaling was also shown to be important for adaptive immunity, for example, mediating the interaction between T cells and DCs, thus favoring T-cell proliferation and DC function and survival (Anderson et al., 1997). Moreover, RANKL to RANK signaling is essential for thymic organogenesis and the establishment of central tolerance. In RANKL and RANK knockout mice the number of mTECs is reduced and the thymic medulla is underdeveloped, as a consequence of reduced crosstalk between developing thymocytes and mTECs. Since this is essential for negative selection/deletion of potentially self-reactive T cells, these mice develop autoimmune diseases (Akiyama et al., 2013; Hikosaka et al., 2008; Rossi et al., 2007). Recently, this crosstalk was shown to contribute also to the selection and survival of immunossupressive regulatory T cells (Cowan et al., 2013). Supporting a role for RANKL-RANK signaling in regulatory T-cell functions, RANKL expression on these cells was reported to ameliorate intestinal inflammation in chronic colitis (Totsuka et al., 2009).

Interestingly, several immunopathologies like rheumatoid arthritis, periodontitis and Crohn's disease are often accompanied by osteoclast activation and bone loss, a phenotype shown to be induced by T cells (Cochran, 2008; Gough et al., 1994; Moschen et al., 2005). This observation links immune function and bone metabolism, and is the basis of a new field named osteoimmunology.

Recently, RANK activation in mammary lobuloalveolar cells was implicated in physiological proliferation and development of the lactating mammary gland during

pregnancy (Fata et al., 2000). In this context, hormones and in particular progesterone, were shown to promote RANKL expression (Schramek et al., 2010). This finding prompted studies finding that the RANK-RANKL-OPG axis is implicated in breast cancer tumorigenesis and metastatic spread to bone and lungs (Palafox et al., 2012; Schramek et al., 2010; Tan et al., 2011). In one study, the critical source of RANKL that promoted RANK activation and induction of breast cancer-derived pulmonary metastases were regulatory T cells recruited to the tumor microenvironment (Tan et al., 2011). This observation implicates inflammatory factors recruited to the tumor microenvironment in RANKL-driven metastization.

Similarly, RANKL and RANK were also shown to be expressed in the brain (Kartsogiannis et al., 1999); hence it was disclosed a role for this signaling axis in inflammatory fever response via RANK-induced prostaglandin E2 (PGE2) expression by astrocytes, and physiological thermoregulation in females (Hanada et al., 2009). Furthermore, the high expression of RANKL in glioma cells was shown to mediate RANK-induced activation of the classical NF-κB pathway and expression of cytokines in microenvironmental astrocytes that promote glioblastoma invasiveness (Kim et al., 2014).

Dysregulated RANKL-to-RANK signaling was also reported in other types of solid cancer, such as osteosarcoma (Bago-Horvath et al., 2014) and oral squamous cell carcinoma (Grimm et al., 2015; Sambandam et al., 2013), and in hematologic malignancies as well. In B-cell chronic lymphocytic leukemia (B-CLL), the enhancement of leukemic cell survival was reported to rely on IL-8 induction through RANKL reverse signaling in these cells (Secchiero et al., 2006), while in acute myeloid leukemia (AML), RANK signaling is responsible for the impairment of NK cell-dependent immunesurveilance (Schmiedel et al., 2013).

Therefore, it is not surprising that the RANKL-RANK-OPG signaling axis has increasingly been recognized as a therapeutic target in several types of cancer. Indeed, this pathway is already being targeted (through the monoclonal antibody denosumab) in order to reduce SREs due to bone metastases for instance in breast cancer, prostate cancer and multiple myeloma (Buckle et al., 2012; Fizazi et al., 2009).

1.6. THESIS RATIONALE AND OBJECTIVES

Despite the improved prognosis in T-ALL therapy due to aggressive multi-agent chemotherapy, a significant subset of patients has still a poor clinical outcome as a result of massive leukemic cell dissemination and infiltration of several organs, emergence of resistance to therapy, and disease relapse. T-ALL leukemogenesis results from the combination of cell-intrinsic alterations and microenvironmental factors (Buonamici et al., 2009; Indraccolo et al., 2009; Silva et al., 2011; Van Vlierberghe and Ferrando, 2012). Moreover, the microenvironment where T-ALL develops has been implicated in protection from therapy (Gilbert and Hemann, 2010, 2011) and stimulation of LIC activity (Armstrong et al., 2009; Medyouf et al., 2011), which is believed to be linked to disease relapse. So far, only few microenvironmental factors favoring leukemogenesis have been identified as opposed to the widely explored genetic landscape of T-ALL.

In this context, RelB expression in radio-resistant, non-hematopoietic cells was reported to favor leukemia development in TJ2-Tg mice (dos Santos et al., 2008). Since T-cell leukemia originates spontaneously in the thymus of this mouse model and RelB is essential for thymic stromal cell development (Weih et al., 1995), thymic stromal cells are the potential microenvironmental cells supporting leukemogenesis. However, the upstream activators of RelB and the downstream players were not identified yet. Receptors known to activate RelB and playing a role in thymic function include members of the TNF superfamily such as LTβR, RANK and CD40 (Akiyama et al., 2012). These receptors and their cognate ligands are important mediators of thymocyte to stromal cell crosstalk modulating the thymic microenvironments to support normal T-cell development (Akiyama et al., 2012). Thus, it is plausible that T-ALL cells may also modulate the thymic microenvironment in their favor through these signaling axes.

The recently uncovered role for LTβR in cancer development in diverse contexts (Ammirante et al., 2010; Haybaeck et al., 2009), particularly in the microenvironments of lymphoid malignancies (Heinig et al., 2014; Rehm et al., 2011) led us to hypothesize that an evolving crosstalk between leukemic thymocytes expressing lymphotoxin and/or LIGHT ligands and thymic stromal cells expressing their cognate receptor takes place to favor leukemogenesis (Figure 1.7).

Importantly, a role for LTβR in T-cell leukemia was to the best of our knowledge never demonstrated and its potential involvement in T-ALL leukemogenesis could pave the way to use it as a target for therapy (Bjordahl et al., 2013). Therefore, the general aim of the present work was to investigate whether LTβR signaling may be activated and playing a role in the development of human T-ALL using the TJ2-Tg mouse model.

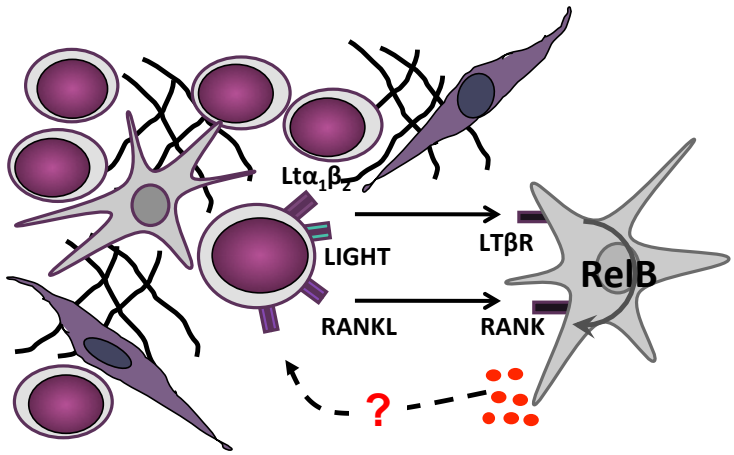


Figure 1.7. Working hypothesis. Based on previous studies, we hypothesize that during T-ALL development, an evolving crosstalk between transformed thymocytes and stromal cells through LTβR signaling axis, alone or cooperating with RANK, takes place and modulates the microenvironment into a pro-leukemogenic niche. On one hand, malignant thymocytes may promote the maturation and expantion of the stromal network; on the other hand, the stromal network may support T-ALL by producing cytokines and growth factors, previously found to contribute to leukemogenesis.

The specific aims at the outset of this work were:

(1) to verify if LTαβ/LIGHT-LTβR and RANKL-RANK signaling can occur in thymic lymphomas;

(2) to determine the mechanism by which LTβR and RANK ligands are induced in leukemic cells;

(3) to assess a possible role for LTβR an RANK in leukemogenesis;

(4) to determine if LTβR deficiency delays initial expansion of malignant cells in the thymus;

(5) to assess the tumorigenic potential of TJ2-Tg leukemic cells growing in different microenvironments (LTβR-deficient or -proficient) and their role in disease progression;

(6) to study the dynamics of lymphotoxin to LTβR signaling and determine if leukemic cells can benefit from the presence of stromal cells in terms of survival;

(7) to test the therapeutic potential of LTβR signaling blockade;

(8) finally, to determine if the mechanisms identified in TJ2-Tg leukemic T cells are conserved in human T-ALL cells.

In Chapter 2, the materials and methods strategically used to attain the above listed aims are described. The original data obtained during this research project is presented in Chapter 3. First, the results of TNF receptor and ligand expression in TJ2-Tg leukemic versus normal thymi are presented. Second, we show that LTβR and other factors modulate lymphotoxin expression at the surface of TJ2-Tg leukemic cells. Third, the survival and characterization of TEL-JAK2-induced T-cell leukemia in LTβR-deficient mice are presented. Finally, some common features between human T-ALL and TEL-JAK2-induced leukemia are analyzed. These results are then discussed in Chapter 4 focusing on their significance, concordance with previously published studies, and the possible mechanism(s) that could account for the effects observed. Also in this last chapter, some questions left unanswered are addressed, together with recommendations for future research.

CHAPTER 2

MATERIALS & METHODS

2.1. HUMAN PRIMARY SAMPLES

For the Portuguese cohort, T-ALL samples were obtained at diagnosis from peripheral blood (PB) and/or bone marrow (BM) of pediatric patients with high leukemia involvement (>85%), and enriched by density centrifugation over Ficoll-Paque (GE Healthcare). The Brazilian cohort consisted of samples from 51 pediatric patients with newly diagnosed T-ALL accrued from 2000 to 2013 at Centro Infantil Boldrini, Campinas, Brazil. Most patients (n=44) were treated according to the Brazilian GBTLI-99 Childhood ALL Treatment protocol . The use of patient samples was approved by the FCM/UNICAMP Research Ethics Committee (CAAE: 0014.0.144.146-08). Thymic samples, obtained from children undergoing cardiac surgery, were gently minced in culture medium and subsequently subjected to density centrifugation. Informed consent and Institutional Review Board approval were obtained for all sample collections in accordance with the Declaration of Helsinki.

2.2. MOUSE PROCEDURES

2.2.1. Mice

EμSRα-TEL-JAK2 transgenic (TJ2-Tg) mice, which express the TEL-JAK2 fusion protein specifically in lymphoid cells and develop T-cell leukemia (Carron et al., 2000), were provided by Dr. Jacques Ghysdael (CNRS, Orsay, France). *Ltbr* knockout mice, in which the coding exons 1 to 5 of the *Ltbr* gene were targeted for inactivation (Fütterer et al., 1998), were kindly provided by Jorge Caamaño (Institute of Biomedical Research, Birmingham, UK). *Ltbr* heterozygotic ($Ltbr^{+/-}$) and knockout ($Ltbr^{-/-}$) embryos were used to prepare mouse embryonic fibroblasts (MEFs; procedure in section 2.3.3). *Rag2* knockout mice (Shinkai et al., 1992) were obtained from the department of cryopreservation, distribution, typing, and animal archive (CDTA; CNRS, Orléans, France) and crossed to TJ2-Tg mice.

All mice were maintained in the CBME/UAlg Animal Facility (Faro, Portugal) and the experimental procedures followed recommendations for the care and use of laboratory animals from the European Commission (Directive 2010/63/UE) and Portuguese authorities (Decreto-Lei nº113/2013).

2.2.2. Mouse breeding and survival

TJ2-Tg mice were bred with *Ltbr* knockout mice on the C57BL/6 background to originate cohorts of TJ2-Tg;$Ltbr^{+/-}$ (controls) and TJ2-Tg;$Ltbr^{-/-}$ mice. Mice were monitored for the development of TEL-JAK2-induced malignancy (see 2.2.5). Only mice that succumbed to confirmed malignancy were included in survival analyses. For detection of malignant thymocytes at early stage T-ALL, TJ2-Tg mice were sacrificed at 8-weeks-of-age, and the percentage of $CD8^+CD25^+$ cells in thymocyte suspensions was determined by flow cytometry.

2.2.3. Leukemic cell transplantation

For transplantation experiments, single-cell suspensions of freshly collected primary TJ2-Tg leukemic T cells were prepared (procedure in section 2.2.5). For tumor establishment, 5×10^6 cells in a volume of 100 µl of sterile phosphate-buffered saline (PBS) solution (Lonza) were injected intravenously into the tail vein of littermate $Ltbr^{+/-}$ and $Ltbr^{-/-}$ recipient mice (7 to 15 weeks of age).

2.2.4. LTβR-Fc treatment of mice

To block LTβR signaling *in vivo*, TJ2-Tg mice received weekly intraperitoneal injections of 100 µg mLTβR-mIgG (provided by Biogen Idec, Cambridge, USA) or control ChromPure mouse IgG (Jackson Immunoresearch) for 5 consecutive weeks, starting at 5-weeks-of-age. For detection of malignant thymocytes at early stage T-ALL, TJ2-Tg mice were killed at 8-weeks-of-age, and the percentage of $CD8^+CD25^+$ cells in thymocyte suspensions was determined by flow cytometry.

2.2.5. Sacrifice, necropsy and sample collection

Unless the sacrifice time-point is stated, TJ2-Tg mice and transplantation recipient mice were monitored for leukemia development and killed by CO_2 inhalation when manifesting signs of

disease (i.e., dyspnea, lethargy, enlarged LNs or enlarged abdomen). Age-matched C56BL/6 mice were sacrificed to serve as controls, when required.

Organs collected at necropsy were kept in cold PBS until weighing and sample processing. To prepare single-cell suspensions, the organs were washed in PBS and TJ2-Tg leukemic T cells were isolated from the stroma-enriched fraction by gentle compression against a 70 μm cell strainer (BD Biosciences) with a plunger and washing with cold PBS. BM single cell suspensions were obtained by flushing cold PBS through tibiae and femora.

Mouse PB was collected from the submandibular vein by using blood lancets (Marienfeld superior). To this end, the location where the facial and submandibular veins meet at the rear end of the mandibular bone was punctured with a lancet and few drops were collected in a microtube containing 50 mM EDTA (approximarely 1 volume of EDTA (Sigma) for 10 volumes of blood). To determine white blood cell (WBC) counts in the PB, the kit Leuco-TIK® (Bioanalytic), based on Türk's solution staining of leucocyte nuclei after red blood cell lysis, was used following the manufacturer´s instructions.

2.3. CELL CULTURE

Culture of cell lines and primary TJ2-Tg leukemic T cells was performed in a laminar flow cabinet (HERAsafe Heraeus) under aseptic conditions. Cells were maintained in a incubator (HERAcell 150 Heraeus) at 37ºC under a humidified atmosphere with 5% CO_2. Monitoring of cell culture growth and cell counting on a hemocytometer (see 2.3.5) was performed on an inverted light microscope (Leica).

2.3.1. Cell lines

DND41, PF382, P12, and SUPT1 human T-ALL cell lines were provided by Hind Medyouf and Andrew P. Weng (BC Cancer Agency Research Centre, Vancouver, Canada). Human cutaneous T-cell lymphoma HUT-78 cell line was provided by Neil Perkins (ICaMB, Newcastle, UK), and Jurkat and Nalm6 cell lines were provided by João T. Barata (IMM, Lisbon). Murine MS5 bone marrow stroma (Itoh et al., 1989) and 1C6 mTEC (Mizuochi et

al., 1992) cell lines were provided by Françoise Pflumio (CEA-IRCM, Fontenay-aux-Roses, France) and Georg Hollander (University of Basel, Germany), respectively.

2.3.1.1. Maintenance and treatments

Leukemic cell lines were cultured in complete RPMI medium (Lonza) supplemented with 10% heat-inactivated fetal bovine serum (FBS; PAA), 2 mM L-glutamine (Lonza), and 100 U/ml penicillin plus 100 µg/ml streptomycin (Lonza). For activation and/or signaling pathway inhibition, leukemic cell lines were cultured at $1x10^6$ cells/ml and treated for 10 h with either DMSO (Sigma, control), 50 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and/or 10 µM BMS-345541 (IKKi; Calbiochem).

MS5 and 1C6 mTEC stromal cell lines were maintained in minimum essential medium alpha (αMEM; Gibco) or Dulbecco's modified eagle medium (DMEM; Lonza), respectively supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and 100 U/ml penicillin plus 100 µg/ml streptomycin.

2.3.1.2. Freezing and thawing

For cryopreservation, adherent cells were washed with PBS, dissociated by 0.25% Trypsin (Lonza), and resuspended in supplemented medium to halt trypsin action. Dissociated adherent cells or suspension cells were then centrifuged at 300 ×g for 5 minutes and the pellet was resuspended in freezing solution (90% calf serum (Lonza), 10% DMSO (Sigma)) at $2.5\text{-}5\times10^6$ cells per ml, and preserved in 2 ml cryotubes (Bioline). These were kept in a Mr Frosty™ freezing container (Thermo Scientific) at -80ºC overnight, and then transferred to a liquid nitrogen tank for long-term storage. Cells were thawed by rapid warming in a 37°C water bath. These were resuspended in prewarmed culture medium, centrifuged at 300 ×g for 5 min to discard the supernatant containing DMSO, and plated in fresh, prewarmed supplemented medium.

2.3.2. Primary TJ2-Tg leukemic T cells

2.3.2.1. Isolation of TJ2-Tg leukemic T cells

Single cell suspensions from freshly collected wild-type thymocytes and primary TJ2-Tg leukemic T cells for transplantation experiments, *ex vivo* treatments and co-cultures, were obtained from wild-type thymi and TJ2-Tg thymic lymphomas, respectively. These were

prepared under aseptic conditions by gentle dissociation and filtration through sterile 70 μm nylon cell strainers (BD Biosciences) and washing with PBS sterile solution (Lonza).

2.3.2.2. Maintenance and treatments

Wild-type thymocytes and primary leukemic T cells were cultured in complete RPMI medium supplemented with 10% heat-inactivated FBS (PAA), 2 mM L-glutamine (Lonza), and 100 U/ml penicillin plus 100 μg/ml streptomycin (Lonza) at 2×10^6 cells/ml. For molecular analyses, primary cells were treated with either DMSO (control), 1 μg/ml actinomycin D (ActD), 50 μg/ml cycloheximide (CHX), 10 ng/ml PMA and 250 ng/ml ionomycin (PMA+Iono) (all from Sigma-Aldrich), 10 μM Tetracyclic Pyridone 6 (InSolution Jak Inhibitor I, JAKi) or 10 μM BMS-345541 (IKKi) (both from Calbiochem), as indicated.

2.3.2.3. Freezing and thawing

Primary leukemic T cells (25×10^6) pelleted from single cell suspensions were resuspended in 1 ml of freezing solution (90% calf serum (Lonza), 10% DMSO (Sigma)) and preserved in 2 ml cryotubes (Bioline). These were kept in a Mr Frosty™ freezing container (Thermo Scientific) at -80ºC overnight, and then stored in a liquid nitrogen tank. Cells were thawed by rapid warming in a 37°C water bath. These were resuspended in ice-cold PBS, centrifuged at 300 ×g for 5 min to discard the supernatant containing DMSO, and processed for analysis.

2.3.3. Primary mouse embryonic fibroblasts (MEFs)

2.3.3.1. Isolation of primary MEFs

Ltbr$^{+/-}$ and *Ltbr*$^{-/-}$ embryos were collected from pregnant female mice at embryonic day 13.5 to 14.5. Briefly, pregnant females were sacrificed by CO_2 inhalation, the ventral surface was cleaned with 70% ethanol, and the placenta was dissected as described elsewhere (Freshney, 2006) and transferred to a clean Petri dish with PBS. In each embryo handled separately, internal organs, head and blood system cells were discarded with the free membranes and placenta. The remainder, consisting of the carcass, was then transferred to a clean Petri dish and washed five times with PBS in a laminar flow hood. Subsequently, the carcasses were transferred to sterile 10 cm cell culture dishes with 5 ml of 0.05% Trypsin/0.53 mM EDTA (Gibco) and cut finely with a razor blade. The resulting suspension was resuspended and homogenized by pipetting up and down six to seven times in order to break up the tissue, and

then placed in the incubator at 37ºC for 15 minutes. Next, 5 ml of Trypsin/EDTA was added to the plate and repeatedly pipetted up and down followed by another 10-minute incubation. Following homogenization, the cellular suspension was passed through a sterile 100 μm nylon cell strainer (BD Biosciencies) 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 (RT). The supernatant was discarded and the cell pellet resuspended in MEF culture medium (composition in section 2.3.3.2). The cellular content of each embryo was plated in one 75 cm^2 T-flask and incubated at 37 ºC with 5% CO_2. The medium was changed after six hours and one day. When a confluence of 70% was reached, MEFs were either cryopreserved or subcultured.

2.3.3.2. Maintenance

MEFs were cultured in DMEM medium (Lonza) supplemented with 10% heat-inactivated FBS (PAA), 2 mM L-glutamine (Lonza), 100 U/ml penicillin plus 100 μg/ml streptomycin (Lonza), and 0.1 mM 2-mercaptoethanol (Gibco). The medium was changed every two days and cells were split between 1:3 and 1:5 as they reached 70% confluency.

2.3.3.3. Freezing and thawing

For cryopreservation, primary MEFs were washed with PBS, dissociated by 0.05% Trypsin / 0.53 mM EDTA (Gibco), and resuspended in supplemented medium to inactivate trypsin. These were then centrifuged at 300 ×g for 5 minutes, the pellet was resuspended in freezing solution (45% DMEM, 40% FBS, 15% DMSO) at 1×10^6 cells per ml, and preserved in 2 ml cryotubes (Bioline). The cryotubes were kept in a Mr Frosty™ freezing container (Thermo Scientific) at -80ºC overnight, and then transferred to a liquid nitrogen tank for long-term storage. MEFs were thawed by rapid warming in a 37°C water bath. These were resuspended in prewarmed medium, centrifuged at 300 ×g for 5 min to discard the supernatant containing DMSO, and plated in fresh, prewarmed supplemented medium.

2.3.4. Co-cultures

TJ2-Tg leukemic T cells ($1x10^6$ cells/ml) were co-cultured in triplicate with confluent MS5 cells or *Ltbr*$^{+/-}$ and *Ltbr*$^{-/-}$ MEFs in complete RPMI medium as previously described. Co-

culture images were obtained using a Leica DM IL microscope (magnification 400x) and captured using Leica DC500 camera and Adobe Photoshop CS2 software.

For flow cytometry analysis, co-cultured leukemic cells in suspension (non-adherent) were collected. The remaining culture was washed twice with PBS and then dissociated by incubation at 37ºC with enzyme-free cell dissociation buffer (Gibco). In flow cytometry analyses, dissociated leukemic cells (adherent) were discriminated from MS5 or MEFs using the Thy1.2 T-cell marker.

2.3.5. Cell counts

To determine cell number and viability, one volume of 0.4% trypan blue solution (Sigma) was added to one volume of cell suspension and incubated for 1 min. The suspension was mixed, introduced on a hemocytometer (Loptik Labor), and cells were counted using an inverted light microscope. Viable cells (colorless) and nonviable cells (blue) were counted in five 1 mm^2- squares of one chamber and the average number of cells per square (Average count/square) was determined. The following formula was used to estimate the total number of cells in the original suspension (Total N cells):

$$\text{Total N cells} = \text{Average count/square} \times 10^4 \times \text{Dilution factor} \times \text{Total suspension volume}$$

This method was also used to determine thymocyte cellularity (total cell number in a whole thymus).

2.4. FLOW CYTOMETRY AND CELL SORTING

Single cell suspensions were stained with fluorescein isothiocyanate (FITC)-, R-phycoerythrin (PE)-, PE-cyanine 5 (PE-Cy5)-, or allophycocyanin (APC)-conjugated antibodies, and detected by a FACS Calibur flow cytometer (BD Biosciences) or sorted using a FACSAria I (BD Biosciences), as indicated. Nonviable cells were excluded from analyses by propidium iodide (PI; Sigma-Aldrich) or 7-Aminoactinomycin D (7-AAD; BioLegend) staining, and appropriate gating. The data were analyzed using CellQuest (BD Biosciences) software.

When mouse PB was analyzed by flow cytometry, the samples were treated to lyse red blood cells before initiating the staining protocol. PB samples collected in anticoagulant were transferred to 15 ml conical tubes and centrifuged at 300 ×g for 5 min at 4°C. After discarding the supernatant, the pellet was resuspended in 5 ml of red blood cell (RBC) lysis solution (composition in Table 2.5), and incubated for 5 min at RT. Then, the samples were centrifuged at 300 ×g for 5 min at 4°C, the supernatant was discarded, and the pellets were resuspended in cold PBS.

2.4.1. Immunostaining for detection of T-cell markers and RANKL

A volume of single cell suspension containing 1×10^6 cells, was added to each 5 ml round-bottom polystyrene tube (BD Falcon) plus 1 ml of ice-cold FACS solution (composition in Table 2.5). After centrifugation at 300 ×g for 5 minutes at 4ºC, the cells were stained with fluorochrome-labeled antibodies alone or in combination at the indicated dilutions (Table 2.1) in 50 µl of FACS solution, and incubated for 1 hour on ice, in the dark. Then, the cells were washed twice with 1 ml of cold FACS solution and resuspended in 1 ml of 10 mM NaN_3 in PBS solution, and analyzed. Isotype negative controls and cells stained with each antibody individually were also prepared to adjust the FACS instrument settings

2.4.2. Detection of intracellular lymphotoxin-β (LTβ)

Intracellular lymphotoxin-β was detected using a hamster anti-mouse LTβ (BBF6.BF12) monoclonal antibody (Browning et al., 1997). This antibody and the negative control, anti-KLH armenian hamster IgG Ha4/8, were provided by Jeff Browning (Boston University School of Medicine, Boston, USA). Both primary antibodies were visualized using FITC-conjugated goat anti-hamster (Armenian) IgG (BioLegend) secondary antibody. More specifically, a volume of suspension containing 0.5×10^6 cells was transferred to each 5 ml round-bottom polystyrene tube (BD Falcon) and washed with 1 ml of ice-cold PBS. For washing, cells were centrifuged at 300 ×g for 5 minutes at 4ºC and the supernatant was discarded. Then, cells were fixed with 200 µl of 0.5% paraformaldehyde (PFA; Merck) for 20 minutes at RT. To permeabilize the membranes, cells were washed 2 times with permeabilization solution (composition in Table 2.5). After discarding completely the supernatant, 1 µg of LTβ antibody (or the negative control) diluted in 100 µl of permeabilization solution was added and incubated for 1 hour on ice. Next, cells were washed two times using permeabilization solution, incubated with 0.5 µg of secondary

antibody in 50 µl of permeabilization solution, and incubated for 1 hour on ice in the dark. Finally, cells were washed 2 more times with permeabilization solution and resuspended with 500 µl of 10 mM NaN_3 in PBS solution.

2.4.3. Detection of membrane-bound LTβR ligands

Membrane-bound LTβR ligands were detected using the murine LTβR-hIgG (mLTβR-Fc) (Browning et al., 1997) and visualized using PE-conjugated AffiniPure $F(ab')_2$ fragment donkey anti-human IgG (H+L) (Jackson ImmunoResearch Laboratories) secondary antibody, which was also used alone as a negative control. The mLTβR-Fc fusion protein and human IgG were provided by Jeff Browning (Boston University School of Medicine, Boston, USA). All antibodies were prepared in 2% normal mouse serum (NMS; Jackson Immunoresearch Laboratories) in PBS to prevent non-specific staining.

To detect LTβR ligands at the cell surface alone or in combination with T-cell markers, we applied an adapted immunostaining protocol based on the one described by Ansel and coworkers (Ansel et al., 2000). In detail, a suspension containing 1×10^6 cells was transferred to each 5 ml round-bottom polystyrene tube (BD Falcon) and washed with 1 ml of ice-cold FACS solution (composition in Table 2.5). After washing, cells were centrifuged at 300 ×g for 5 minutes at 4ºC and the supernatant was discarded. To block Fc receptors prior to immunostaining, the cells were pre-incubated with Trustain fcX™ (anti-mouse CD16/32; BioLegend) at 1 µg in 100 µl of PBS for 10 minutes on ice. Cells were washed, the supernatant was discarded carefully, and 50 µl of 2% NMS containing 1 µg of mLTβR-Fc (human IgG or just 2% NMS in the negative control tubes) was added. After a 1-hour incubation on ice, the cells were washed two times with FACS solution. When indicated, cells were incubated for 1 hour on ice, in the dark with T-cell marker antibodies (Table 2.1) and then washed two more times with FACS solution. Next, cells were resuspended in 50 µl of 2.5 µg/ml donkey anti-human secondary antibody and incubated for 45 minutes on ice, in the dark. Finally, cells were washed 2 more times with FACS solution and resuspended in 1 ml of 10 mM NaN_3 in PBS solution. Where indicated, LTβ expressed at the cell surface was pre-blocked through 20 minute incubation with 1 µg of BBF6.BF12 in 200 µl of 2% NMS. Then, the cells were washed 2 times with FACS solution before the incubation with LTβR-Fc. To detect LTβ expressed at the cell surface, 1 µg of BBF6.BF12 monoclonal antibody in 50 µl of FACS solution (incubated at 4ºC for 1 hour) was used, and it was visualized using

FITC-conjugated goat anti-hamster (Armenian) IgG (0.5 µg in 50 µl of FACS solution and incubation for 1 hour at 4ºC, in the dark).

Negative controls and cells stained with each antibody individually were also prepared to adjust the FACS instrument settings and fluorochrome compensation.

Table 2.1. Antibodies used for flow cytometry analyses of T-cell markers and RANKL.

Antigen	Antibody *	Clone	Fluorochrome	Concentration (mg/ml)	Dilution
Isotype Control	Rat IgG2a, κ	RTK2758	FITC	0.5	1/200
Isotype Control	Rat IgG2a, κ	RTK2758	PE	0.2	1/200
Isotype Control	Rat IgG2a, κ	RTK2758	PE/Cy5	0.2	1/400
Isotype Control	Rat IgG2a, κ	RTK2758	APC	0.2	1/100
Murine CD3ε	Hamster IgG	145-2C11	FITC	0.5	1/200
Murine CD4	Rat IgG2b, κ	GK1.5	FITC	0.5	1/200
Murine CD4	Rat IgG2b, κ	GK1.5	PE	0.2	1/200
Murine CD8	Rat IgG2b, κ	53-6.7	PE/Cy5	0.2	1/600
Murine CD24 (HSA)	Rat IgG2b, κ	M1/69	APC	0.2	1/400
Murine CD25	Rat IgG1, λ	PC61	FITC	0.5	1/400
Murine CD44	Rat IgG2b, κ	IM7	APC	0.2	1/400
Murine CD69	Hamster IgG	M1.2F3	FITC	0.5	1/200
Murine CD90.2 (Thy1.2)	Rat IgG2b, κ	30-H12	FITC	0.5	1/500
Murine CD254 (RANKL)	Rat IgG2b, κ	IK22/5	PE	0.2	1/200

* All antibodies were purchased from BioLegend.

2.5. GENOTYPING

2.5.1. DNA extraction

Mouse tail tips, other tissues collected at necropsy or MEFs were digested, using 100 µg/ml proteinase K (Fermentas) in 500 µl of lysis buffer (composition in Table 2.5), and incubating at 55ºC overnight. The next day, the suspension was vortex mixed and incubated at 90ºC for 10 minutes to inactivate proteinase K. Then, the lysate was cooled and centrifuged at 16 100 ×g for 15 minutes. The supernatant was transferred to a new microtube, 500 µl of isopropanol (BDH Prolabo) was added, and it was mixed. To collect the precipitated DNA, the suspension was centrifuged at 16 100 ×g for 10 min. After discarding the supernatant, the DNA pellet was washed with 500 µl of 70% ethanol (BDH Prolabo) and the microtube was inverted several times to mix the contents. Then, after a centrifugation at 16 100 ×g for 5 min, the supernatant was discarded and the pellet was allowed to dry at room temperatute (RT). Finally, DNA was resuspended in TE buffer, pH 7.4 (composition in Table 2.5) and stored at 4 ºC until genotyping.

2.5.2. Polymerase Chain Reaction (PCR)

Mouse tail and MEFs genotyping was performed by PCR. To this end, 2 µl of extracted DNA was added to a mixture containing GoTaq Flexi Buffer, 15 mM $MgCl_2$, 0.2 mM PCR nucleotide mix, primers (Sigma-Aldrich, as indicated in Table 2.2), 1.25 U GoTaq DNA polymerase (Promega) and sterilized deionized water to complete a PCR volume of 25 µl. PCR amplification was performed on a C1000 Thermal Cycler (Bio-Rad), under the following conditions: initial denaturation at 95ºC for 5 minutes; 30 cycles of denaturation at 95ºC for 45 seconds; annealing at 57ºC (*Rag2*) or 63ºC (*Ltbr* and TEL-JAK2) for 45 seconds; extension at 72ºC for 1 minute; a final extension step at 72ºC for 10 minutes was also performed, ending the reaction at 12ºC. PCR products were analyzed by electrophoresis (Power Pack Basic, BioRad) in a 2% agarose (Lonza) gel prepared in TAE buffer (composition in Table 2.5) and visualized by ethidium bromide staining under UV light (Gene Flash, Syngene BioImaging).

Table 2.2. Primers used for genotyping and expected PCR results.

Target	Primer	Primer Sequence (5'- 3')	Expected Bands	Concentration PCR mix (μM)
Ltbr	LTBR4	CTG GTA TGG GGT TGA CAG CG	Wild-type 640 bp	0.35
	LTBR7	TGT CAG CCG GGG ATG TCC TG		0.35
	HSV-TK	ATT CGC CAA TGA CAA GAC GCT GG	Knockout 200 bp	0.20
TEL-JAK2	TG-TJ2(+)	GGG AAG GGA AGC CCA TCA ACC	Positive 441 bp	0.50
	TG-TJ2 (-)	CCG CAC TGT AGC ACA CTC CC		0.50
Rag2	RAG2a	ATG TCC CTG CAG ATG GTA ACA	Wild-type 246 bp	0.25
	RAG2b	GCC TTT GTA TGA GCA AGT AGC		0.25
	Neo 204	GCT ATT CGG CTA TGA CTG GG	Knockout 706 bp	0.25
	Neo 909	GAA GGC GAT AGA ACG CGA TG		0.25

2.6. mRNA EXPRESSION ANALYSIS

2.6.1. RNA extraction

The pellet collected from a cell suspension containing 10×10^6 cells or 50-100 mg of thymic tissue was resuspended in 1 ml of Trizol reagent (Invitrogen). To homogenize the cells and tissues, these were pipetted up and down several times using a 1000 μl sterile filter tip followed by consecutive passages through sterile needles with decreasing internal diameters (21G, 23G, and 26G). Then, the sample in Trizol was incubated at room temperature for 5

minutes to allow the complete dissociation of nucleoprotein complexes and it was stored at -80ºC until further use.

Total RNA was isolated using RNase-free materials and following Trizol (Invitrogen) manufacturer´s instructions. Briefly, for phase separation 200 µl of chloroform (BDH Prolabo) per ml of Trizol was added to each homogenized sample and vigorously shaken by hand for 15 seconds, followed by a 5-minute incubation at RT. The samples were centrifuged (Centrifuge 3K15, Sigma) at 12 000 ×g, for 15 minutes at 4ºC. The aqueous upper phase was transferred to a clean microtube and 500 µl of isopropanol (BDH Prolabo) was added to precipitate the RNA. The samples were gently mixed and incubated at RT for 10 minutes. Then, these were centrifuged at 12 000 ×g, for 10 minutes at 4ºC and the supernatant was discarded. To wash the RNA pellet, 1 ml of 75% ethanol (Merck Millipore) was added to the microtube and its contents were mixed. The samples were then centrifuged at 7 500 ×g for 5 minutes at 4ºC, the supernatant was discarded and the pellet was left to dry at RT for about 10 minutes. Then, the pellet was resuspended in 50 µl of DEPC-treated water (Sigma-Aldrich) by passing the solution a few times through a pipette tip, and by incubating for 10 minutes at 55ºC. Alternatively, RNA extraction from Trizol was made by using Direct-zol RNA miniprep (Zymo Research), following the manufacturer´s instructions.

The quantity and quality of the total RNA were assessed by measuring the optical density in Nanodrop 2000c spectrophotometer (Thermo Scientific) and by running it in an agarose gel or using Experion automate electrophoresis system (Bio-Rad). The samples were kept at -80ºC for long-term storage.

Possible DNA contamination from RNA samples was reduced by digestion with DNase I (Fermentas). Briefly, the corresponding volume of 1 µg total RNA was added to 1 µl of reaction buffer, 1 U DNase I and DEPC-treated water up to 10 µl. The samples were mixed and the drops collected by brief centrifugation before being incubated at 37ºC for 30 minutes. To inactivate the enzyme and terminate the reaction, 1 µl of 25 mM EDTA was added and the samples were heated at 65ºC for 10 minutes.

2.6.2. Complementary DNA (cDNA) synthesis

Total RNA was reverse-transcribed (RT) using the First Strand cDNA Synthesis Kit (Fermentas), according to the manufacturer´s instructions. Briefly, 1 µg of total RNA was treated with 0.5 µg of oligo$(dT)_{18}$ primers, 20 U of Ribolock ribonuclease inhibitor, 1 mM dNTP mix, 200 U of M-MuLV Transcriptase, 1× reaction buffer, and nuclease-free water up

to 20 µl were used. Non-template controls (NTC) containing no RNA and non-enzyme controls (NEC) were included. The samples were kept at -20ºC until used for PCR amplification or at -80ºC for long-term storage.

For quantification of *LTA* and *LTB* expression in T-ALL Brazilian cohort, total RNA (1 µg) was reverse transcribed using the ImProm II Reverse Transcriptase enzyme (Promega) and random hexamers (25 µM).

2.6.3. Semi-quantitative Reverse Transcription (RT)-PCR

In order to determine and compare the expression of a gene transcript, the cDNA resulting from the RT reaction was used as a template for PCR with gene-specific primers (Table 2.3). Unless a reference is indicated, all primers were designed using Primer 3 on the WWW for general users and for biologist programmers (Rozen & Skaletsky).

Template cDNA (2 µl diluted 1:20) was amplified in a PCR mixture containing GoTaq Flexi Buffer, 15 mM $MgCl_2$, 0.2 mM dNTP mix, 12.5 µM of each respective forward and reverse primers, 1.25 U GoTaq DNA polymerase (Promega) and sterile deionized water to complete a PCR volume of 25 µl.

The PCR reaction was performed on a C1000 Thermal Cycler (Bio Rad), under the following conditions: initial denaturation at 94ºC for 5 minutes; 26 to 30 cycles of denaturation at 94ºC for 45 seconds; annealing at 60ºC for 45 seconds; extension at 72ºC for 1 minute; and a final extension step at 72ºC for 7 minutes. PCR products were analyzed by electrophoresis in a 2% agarose gel and visualized by ethidium bromide staining under UV light, as described for genotyping.

2.6.4. Quantitative RT-PCR (RT-qPCR)

Quantitative PCR was performed on a C1000 Thermal Cycler coupled to a CFX 96 Real-time PCR detection system (Bio-Rad), using SsoFast EvaGreen Supermix (Bio-Rad) and murine or human gene-specific primers (Tables 2.3 and 2.4), according to the manufacturer´s instructions. Briefly, in a 96-well low-profile PCR micro-plate (Thermo Scientific), 18 µl of master mix (containing 10 µl of SsoFast EvaGreen Supermix, 300 nM of each primer and sterile RNase/DNase-free water) were added to 2 µl of each cDNA sample previously diluted 1:20. The wells were sealed with adhesive PCR film (Thermo Scientific) and the PCR was performed under the following conditions: initial denaturation and polymerase activation at 95ºC for 30 seconds, 40 cycles of denaturation at 95ºC for 5 seconds, followed by annealing

and extension at 60ºC for 20 seconds. To validate the reaction specificity, a melting curve was generated for each sample by submitting it to temperatures from 65ºC to 95ºC in 0.5ºC increments (2-5 seconds/step). All samples were tested in triplicate and the results expressed as the mean ± range. Negative controls, more specifically no template controls, were always included in the analyses.

RT-qPCR results were analyzed using BioRad CFX Manager software, version 3.0, and treated using the comparative C_T method ($2^{-\Delta\Delta C(T)}$ method), and the mean fold change in expression of the target gene was calculated after normalization to expression values obtained for the following reference genes: *Gapdh*, *Hprt1* (TJ2-Tg leukemic cells), *GAPDH* (human T-ALL cell lines), or 18S (human thymocytes and primary T-ALL samples).

To analyze *LTA* and *LTB* expression in the Brazilian T-ALL patient cohort, RT-qPCR was performed using the TaqMan® method. PCR reactions were carried out in 15 µl containing 5 µl of 1:25 diluted cDNA, 7.5 µl Maxima Probe/ROX qPCR Master Mix (Fermentas), 300 nM of each primer and 200 nM of TaqMan probe (primers and probes in Table 2.4). Amplifications were performed in a 7500 Fast Real-Time PCR System (Life Technologies, Carlsbad, USA). Expression values of *LTA* and *LTB* were calculated by the $2^{-\Delta\Delta C(T)}$ method, using *ABL1* as endogenous normalizer and Jurkat cells as calibrator. Efficiencies for both the normalizer and target genes were 100% ±10%.

2.6.5. Oligonucleotide array analysis

Oligonucleotide array analyses were performed by our collaborators, Dr. J.A. Yunes and his team, in Department of Pediatrics, University of Campinas, Brazil. Briefly, total RNA was extracted from 51 pediatric T-ALL samples using the Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare). Total RNA from 2 human thymocyte samples was extracted with Trizol (Life Technologies). RNA samples were processed with the WT Expression Kit (Ambion) and GeneChip WT Terminal Labeling and Controls Kit (Affymetrix), and hybridized on Human Gene 1.0 ST Arrays (Affymetrix). Array files are publicly available through the Gene Expression Omnibus (GEO) database under the accessions GSE50999 and GSE66638. Expression values were obtained with the iterPLIER+16 algorithm in the Affymetrix Expression Console, and transformed to Z-score in a $\log_2$ scale. Molecular subtypes were classified by Hierarchical Clustering Analysis (Pearson correlation and pair wise-average linkage; http://genepattern.broadinstitute.org/) according to the expression of genes previously associated with each T-ALL subtype (Homminga et al., 2011). The Affymetrix

Complex Match table was used to identify probe sets in the original Human Genome U133 Plus 2.0 Array (Homminga et al., 2011) most related to probe sets available in the Human Gene 1.0 ST Array. Two out of the 51 T-ALL samples could not be classified and thus were excluded from analysis.

Table 2.3. Primer sequences used for RT-PCR analysis (murine).

Gene	Primer	Primer sequences (5'- 3')	Ref.
Gapdh (NM_001289726.1/NM_008084.3)	Forward	GGT GAA GGT CGG TGT GAA CG	(Seach et al., 2008)
	Reverse	ACC ATG TAG TTG AGG TCA ATG AAG G	
Hprt (NM_013556.2)	Forward	GCT GGT GAA AAG GAC CTC T	(dos Santos et al., 2007)
	Reverse	CAC AGG ACT AGA ACA CCT GC	
Tnfsf14 (NM_019418.3)	Forward	GCA CAT CTT ACA GGA GCC AAC GCC	This study
	Reverse	CGG GTA GCG GGA TGT GCG CT	
Lta (NM_010735.2)	Forward	GCT GCT CAC CTT GTT GGG TA	This study
	Reverse	GTG GAC AGC TGG TCT CCC TT	
Ltb (NM_008518.2)	Forward	TAC ACC AGA TCC AGG GGT TC	(Haybaeck et al., 2009)
	Reverse	ACT CAT CCA AGC GCC TAT GA	
Ltbr (NM_010736.3)	Forward	CCA GAT GTG AGA TCC AGG GC	(Seach et al., 2008)
	Reverse	GAC CAG CGA CAG CAG GAT G	
***Tnfsf11* (*Rankl*)** (NM_011613.3)	Forward	CGC CTC CCG CTC CAT GTT CC	This study
	Reverse	GTT CCT TCT GCA CGG CCC CC	
Tnfrsf11a (*Rank*) (NM_009399.3)	Forward	TGC TTG GTG CAT AAA GTC TG	This study
	Reverse	AGG TTT GCA TTT GTC TGT GG	
Nfkb2 (NM_001177369.1/370.1/ NM_019408.3)	Forward	TGG AAC AGC CCA AAC AGC	(Cuesta et al., 2010)
	Reverse	CAC CTG GCA AAC CTC CAT	
Socs2 (NM_007706.4/NM_001168655.1/ 656.1/657.1)	Forward	GTT GCC GGA GGA ACA GTC CC	(Zellmer et al., 2009)
	Reverse	TCG GTC CAG CTG ACG TCT TAA C	

Table 2.4. Primer sequences used for RT-PCR analysis (human).

Gene	Primer	Primer sequences (5'- 3')	Ref.
GAPDH (NM_002046.4/ NM_001256799.1)	Forward	ACA TCA TCC CTG CCT CTA CTG	(Villablanca et al., 2008)
	Reverse	ACC ACC TGG TGC TCA GTG TA	
18S rRNA	Forward	GGA GAG GGA GCC TGA GAA ACG	(Cardoso et al., 2011)
	Reverse	CGC GGC TGC TGG CAC CAG ACTT	
LTA (NM_001159740.2/ NM_000595.3)	Forward	CCC ACC AGT GGC ATC TAC TT	This study
	Reverse	CAG CCC TGG ATA CAC CAT CT	
LTB (NM_009588.1)	Forward	GAG GAC TGG TAA CGG AGA CG	(Haybaeck et al., 2009)
	Reverse	GGG CTG AGA TCT GTT TCT GG	
LTA (Taqman) (NM_001159740.2/ NM_000595.3)	Forward	CCT CAA ACC TGC TGC TCA CCT	This study
	Reverse	AAC CAT CCT GGA GGA AGG CA	
	Probe	TTG GAG ACC CCA GCA AGC AGA ACT CA	
LTB (Taqman) (NM_009588.1/ NM_002341.1)	Forward	CAG AGG AGG AGC CAG AAA CAG A	This study
	Reverse	TAG CCC CTG CCC CTT CAG	
	Probe	GAG CGC CTA TGA GGT GGG CAG CTG	
ABL1 (Taqman) (NM_007313.2/ NM_005157.5)	Forward	TGG AGA TAA CAC TCT AAG CAT AAC TAA AGG T	This study
	Reverse	GAT GTA GTT GCT TGG GAC CCA	
	Probe	CCA TTT TTG GTT TGG GCT TCA CAC CAT T	

2.7. PROTEIN EXPRESSION ANALYSIS

2.7.1. Protein extraction

Adherent cells were washed with PBS, trypsinized, and resuspended in the indicated culture medium. These cells and cells growing in suspension were collected by centrifugation, washed with ice-cold PBS and centrifuged again to discard the supernatant. The cell pellet was homogenized in 100 µl of RIPA buffer (composition in Table 2.5) with freshly added

protease inhibitor cocktail (Complete™, Roche), and incubated on ice for 20 minutes. Cell debris were removed by a 16 100 ×g centrifugation at 4°C for 15 minutes. The supernatant containing total protein extract was collected for further use.

2.7.2. Measurement of protein concentration

Protein concentrations were determined using Micro BCA™ Protein Assay Kit (Thermo Scientific) according to the manufacturer's instructions.

2.7.3. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The extract containing 30 μg of total protein was mixed with loading buffer (composition in Table 2.5) and boiled at 95°C for 5 minutes. The samples were cooled on ice and collected by brief centrifugation. Protein samples were separated in an 8% SDS-polyacrylamide mini-gel (composition in Table 2.5), in a vertical chamber (Bio-Rad) filled with running buffer (composition in Table 2.5). When the bromophenol blue front of migration exited the gel, the latter was recovered and used for Western blotting analysis.

2.7.4. Western Blotting

The proteins separated in the SDS-PAGE mini-gel were transferred a nitrocellulose membrane (Amersham™Protran™, GE Healthcare) in a wet electrotransfer procedure using Tris-glycine transfer buffer (composition in Table 2.5). The membrane was recovered and, to block non-specific proteins, it was incubated in blocking solution (PBS containing 0.1% Tween 20 (Merck) and 5% non-fat milk powder (Merck)) for 1 h at RT, with gentle agitation. Then, the membrane was incubated at 4°C overnight with gentle agitation with a solution containing the indicated primary antibodies in blocking solution. The next day, the membrane was washed three times for 5 minutes in PBS-T (0.1% Tween 20 in PBS solution), and the membrane was further incubated with the appropriate secondary antibodies conjugated with horseradish peroxidase (HRP) for 2 hours at RT with gentle agitation. The primary antibodies used were anti-LTβR goat polyclonal IgG (W-15, Santa Cruz) at 0.4 μg/ml and anti-actin mouse monoclonal IgG (clone C4, MP Biomedicals) diluted at 1 to 10000. The secondary antibodies used were peroxidase-conjugated Affinipure mouse anti-goat IgG (H+L) and peroxidase-conjugated Affinipure goat anti-mouse IgG (H+L) (Jackson ImmunoResearch Laboratories) at 0.16 μg/ml. After three more washes in PBS-T, the proteins were detected by incubating the membrane with enhanced chemiluminescence solution (ECL; Pierce® ECL

Western Blotting Substrate, Thermo Scientific) and exposing to FUJI Medical X-Ray films (FUJIFILM). X-Ray films were processed using G153 developer (AGFA) and G354 fixer (AGFA) in AGFA CP1000 film processor (AGFA Healthcare).

2.8. HISTOLOGY AND HISTOCHEMISTRY METHODS

2.8.1. Histology

Mouse organs collected during necropsy were fixed in FAA solution (composition in Table 2.5) for 2 hours. Then, these were washed with PBS solution and kept at 4ºC in PBS, protected from light. Fixed specimens were processed for paraffin embedding by dehydration through an ethanol gradient (50% for 1 hour, 70% for 3 hours, 80% for 1 hour, and 96% for 1 hour), followed by further incubation with 100% ethanol (two consecutive 5-hour incubations), 50% ethanol/50% xylene solution (2 hour incubation), 100% xylenes (two consecutive 2 hour incubations), and melted paraffin (MP 52-54ºC, Merck; two consecutive 4 hour incubations), using an automatic tissue processor (Tissue Processor Tissue-Tek® II, Sakura). Using metal base molds, the specimens were then embedded in melted paraffin and allowed to harden using a tissue embedding console system (Tissue-Tek® thermal, dispensing and cryoconsole: Sakura). Five to seven μm thick sections were cut from the paraffin blocks using Microm HM 340 E rotary microtome (Thermo Scientific). Sections were flattened on a 37ºC-heated water bath, collected onto microscope slides (VWR) pre-treated with aminopropyltriethoxysilane (APTES, Sigma), and allowed to dry.

Before proceeding for hematoxylin and eosin staining, the sections were deparaffinized and rehydrated (2 consecutive incubations in xylenes for 10 minutes, 100% ethanol for 5 minutes, 90% ethanol for 5 minutes, 70% ethanol for 5 minutes, 40% ethanol for 5 minutes, and 2 consecutive incubations with bi-distilled water for 2.5 minutes). Then, the sections were stained by immersing the slides in Accustain® Hematoxylin solution (GILL N. 3 – 6 g/l, Sigma-Aldrich) diluted 1:5 for 5 minutes, followed by a washing step in tap water for 10 minutes, a 2 minute incubation with 0.5% Eosin solution (Fluka Analytical), and one last washing step. Next, the sections were dehydrated and diaphonized by incubation with ethanol and xylene (40% ethanol for 5 minutes, 70% ethanol for 5 minutes, 100% ethanol for 5 minutes, and 2 consecutive incubations in xylenes for 5 minutes). Finally, the

sections were mounted between the slide and a coverslip using Entellan® mounting medium (Merck Millipore). After drying overnight, slides were visualized under white-light in an Axio Imager Z2 fluorescence microscope (Carl Zeiss).

2.8.2. Immunohistochemistry (IHC)

Mouse thymi collected during necropsy were cleaned and frozen directly in Tissue-Tek® O.C.T.™ Compound (Sakura) by rapidly cooling on dry ice. Five-µm thick frozen sections were cut using Research Cryostat Leica CM 3050 S, and collected in Superfrost® Plus adhesion slides (Thermo Scientific). The sections were fixed in acetone previously cooled at -20ºC for 10 minutes, at RT with gentle agitation, rehydrated with PBS in 3 quick passages, and washed three times for 5 minutes with PBS-T solution (0.1% Tween-20 in PBS solution) under gentle agitation. Then, these were incubated in blocking solution (20% FBS (PAA), 10% bovine serum albumin (BSA, Sigma-Aldrich) and 0.1% Tween 20 in PBS solution) for 1 hour, followed by an overnight incubation at 4ºC in a humidified chamber with primary antibodies diluted in blocking solution: 0.16 µg/ml rat anti-mouse CD31 (clone MEC 13.3; BD Biosciences) and 1 µg/ml rabbit anti-mouse keratin 5 (Krt5) polyclonal (AF 138; Covance) antibodies, or 10 µg/ml rabbit anti-mouse Exodus-2 (Ccl21; Peprotech) and anti-mouse Krt8 rat IgG diluted at 1:100 (TROMA 1; provided by Rolf Kemler, Max Planck Institute, Freiburg, Germany). After 3 washes in PBS-T solution, sections were incubated for 1 hour at RT in a humidified chamber with 1.5 µg/ml DyLight 488-conjugated AffiniPure Goat anti-Rat IgG (H+L) and DyLight 594-conjugated AffiniPure Goat anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories) secondary antibodies followed by 3 more washes in PBS-T solution. The slides were mounted in Mowiol (4-88, Sigma) with DAPI (Biotium) at 1.5 µg/ml by covering with coverslips, and allowed to dry overnight at RT in the dark. The next day, stained sections were visualized using an Axio Imager Z2 fluorescence microscope (Carl Zeiss).

2.9. STATISTICS

Statistical analyses were performed using GraphPad Prism 5 software. Statistical tests were used as indicated in figure legends. Kaplan-Meier survival curves were plotted and the *P*-value was calculated using the log-rank Mantel Cox test. A *P*-value below 0.05 was considered statistically significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Only statistically significant results were indicated in figures.

2.10. MICROARRAY DATA SETS AND DATA ANALYSIS

Analyses of publically available microarray data sets were performed by our collaborator, Dr. Ravi K. Kalathur. Briefly, microarray data sets from publicly available Gene Expression Omnibus database were obtained, including gene expression data from the Microarray Innovations In Leukemia (MILE) study (GSE 13159) to evaluate the expression of *LTA* and *LTB* in subtypes of leukemia compared to non-leukemia and healthy bone marrow (Haferlach et al., 2010). To compare the expression profile of TJ2-Tg mouse T-ALL with that of different stages of mouse thymocyte differentiation, thymocyte gene expression data was obtained from the Immunological Genome Project (Heng et al., 2008) and expression data from TJ2-Tg leukemic cells (Waibel et al., 2013) (GSE15907 and GSE51243, respectively). All datasets were pre-processed using RMA (Robust Multi-array Average) as implemented in the R/Bioconductor environment (Gentleman et al., 2004). The $\log_2$ signal intensities of the annotated genes were obtained and the co-efficient variation for each gene was computed. The top 1% (n=163) of genes was thus obtained, which showed least co-efficient variation. Further, hierarchical clustering of samples was performed using the complete linkage analysis method implemented in the TM4 software on the above mentioned mouse datasets. In addition to the clustering analysis, functional enrichment analysis of the 163 gene dataset was performed using Gene Set Enrichment Analysis (GSEA) to identify enriched biological processes, molecular functions and pathways (Subramanian et al., 2005). The statistical significance of enrichment was estimated and all the functional categories and pathways whose FDR was ≤ 0.05 were considered.

Table 2.5. Commonly used solutions and gels.

PB sample treatment	**RBC Lysis Solution pH 7.2**	160 mM NH_4Cl 170 mM Tris-HCl, pH 7.4 Mix 1 volume of 170mM Tris-HCl with 9 volumes of 160 mM NH_4Cl	Sigma-Aldrich Sigma-Aldrich
Flow Cytometry	**FACS Solution**	PBS 3% FBS 10 mM NaN_3	Sigma-Aldrich PAA Sigma-Aldrich
	Permeabilization Solution	PBS 0.5% BSA 0.1% Saponin	Sigma-Aldrich Sigma-Aldrich BDH Prolabo
Histology	**FAA Solution**	10% Formaldehyde 75% Ethanol 5% Glacial acetic acid	Sigma-Aldrich BDH Prolabo BDH Prolabo
DNA Extraction	**Lysis Buffer**	200 mM NaCl 100 mM Tris-HCl, pH 8.3 5 mM EDTA 1% Triton X-100	BDH Prolabo Sigma-Aldrich Sigma-Aldrich Sigma-Aldrich
	TE Buffer, pH 7.4	10 mM Tris-HCl, pH 7.4 1 mM EDTA, pH 8	Sigma-Aldrich Sigma-Aldrich
Agarose Electrophoresis	**TAE Buffer, pH 8.3**	40 mM Tris base 20 mM glacial acetic acid 1 mM EDTA	Sigma-Aldrich BDH Prolabo Sigma-Aldrich
SDS-PAGE and Western Blotting	**PBS, pH 7.4**	130 mM NaCl 2.7 mM KCl 10 mM Na_2HPO_4 1.8 mM KH_2PO_4	BDH Prolabo Merck Merck Merck
	RIPA Buffer	10 mM Tris pH 7.4 150 mM NaCl 1 mM EDTA 1% Triton X100 0.5% Sodium deoxycholate 0.1% SDS	Sigma-Aldrich Merck Sigma-Aldrich Sigma-Aldrich Sigma-Aldrich MP Biomedicals

Table 2.5 (cont.). Commonly used solutions and gels.

SDS-PAGE and Western Blotting	**4× Loading Buffer**	62.5 mM Tris pH 6.8 20% Glycerol 2% SDS 5% β-mercaptoethanol 0.5% Bromophenol blue	Sigma-Aldrich BDH Prolabo MP Biomedicals Sigma Sigma-Aldrich
	Separating Gel	4.2 ml double-distilled water 1.6 ml 40% Acrylamide/bisacrylamide 2 ml 1.5M Tris pH 8.8 80 µl 10% SDS 80 µl 10% APS 8 µl TEMED	 Fisher Scientific Sigma-Aldrich MP Biomedicals Sigma Sigma
	Stacking Gel	2.9 ml double-distilled water 0.75 ml 40% Acrylamide/bisacrylamide 1.25 ml 0.5M Tris pH 6.8 50 µl 10% SDS 50 µl 10% APS 5 µl TEMED	 Fisher Scientific Sigma-Aldrich MP Biomedicals Sigma Sigma
	Electrophoresis Buffer	25 mM Tris pH 8.3 192 mM Glycine 0.1% SDS	Sigma-Aldrich Carl Roth GmbH MP Biomedicals
	Transfer Buffer	25 mM Tris pH 8.3 192 mM Glycine 20% Methanol	Sigma-Aldrich Carl Roth GmbH BDH Prolabo

CHAPTER 3

RESULTS

This chapter is based in the following manuscript:

Fernandes, MT, Ghezzo MN, Silveira AB, Kalathur RK, Póvoa V, Ribeiro AR, Brandalise SR, Dejardin E, Alves NL, Ghysdael J, Barata JT, Yunes JA, and dos Santos NR. Lymphotoxin-β receptor in microenvironmental cells promotes the development of T-cell acute lymphoblastic leukemia with cortical/mature immunophenotype. (Submitted for publication).

Author Contributions: the majority of the experimental work was performed by Mónica T. Fernandes, who designed experiments, collected, analyzed and interpreted data, and wrote the manuscript. Laboratory work was also performed by MNG (*Ltbr* expression studies in TJ2-Tg thymic lymphomas, CCL21 IHC, and collaboration in pre-leukemic mice experiments), ABS (*LTA* and *LTB* expression studies in human samples from the Brazilian cohort), VP (*LTA* and *LTB* expression studies in human samples from the Portuguese cohort), and ARR (flow cytometry sorting of thymocytes and RNA isolation). SRB provided and performed patient clinical analyses (Brazilian cohort). RKK performed bioinformatics and statistical analyses in publicly available data sets. NRdS conceived the study idea, designed and supervised experiments, and drafted the manuscript. ED, NLA, JG, JTB and JAY were involved in critical review, revision, and approval of the final manuscript.

3.1. LTβR LIGAND EXPRESSION AND REGULATION IN TJ2-TG LEUKEMIC THYMI

3.1.1. Leukemic T cells from TEL-JAK2 transgenic mice express lymphotoxin-β receptor ligands

TEL-JAK2-induced murine leukemia is characterized by the expansion of a mixture of monoclonal or oligoclonal immature CD8 single-positive (SP), and CD4 and CD8 double-positive (DP) T cells, two phenotypes normally only seen in the thymus (Carron et al., 2000). Therefore, it is conceivable that in this animal model the disease originates in the thymus from a T cell precursor in a more precocious developing stage (dos Santos et al., 2007), and CD8 SP plus DP subpopulations represent the phenotype progression of the transformed clone (Carron et al., 2000).

To address a possible role for lymphotoxin-β receptor (LTβR) signaling in T-cell leukemogenesis using this mouse model, we assessed the expression levels of mRNAs encoding LTα, LTβ and LIGHT in primary leukemic cells collected from diseased TEL-JAK2 transgenic (TJ2-Tg) mice. *Lta*, *Ltb* and *Light* expression was more heterogeneous in TJ2-Tg leukemic cells than in wild-type thymocyte whole population (Figure 3.1A). Importantly, these genes were significantly more expressed in leukemic cells as compared to wild-type thymocytes (on average 4- to 5-fold higher expression) (Figure 3.1A). Of note, *Lta* and *Ltb* expression levels were also higher in TJ2-Tg leukemic cells than in sorted DP and CD4 SP thymocytes, the main source of LTβR ligands in the thymus (Figure 3.1B) (Boehm et al., 2003; Hikosaka et al., 2008). Conversely, *Light* was previously shown to be expressed at equally low levels across the DN, DP, CD4 SP and CD8 SP thymocyte subpopulations (Hikosaka et al., 2008). Therefore, these data show that LTβR ligand expression is likely activated during T-cell leukemogenesis.

To determine the main source of LTβR in thymic lymphomas of TJ2-Tg diseased mice, LTβR expression was assessed by western blotting in T-cell enriched fractions prepared from wild-type thymi or thymic lymphomas. Low or undetectable levels of LTβR were found in leukemic cells, although comparable, with few exceptions to those of wild-type thymocytes (Figure 3.1C). Notably, *Ltbr* expression in TJ2-Tg leukemic cells was not detectable by RT-qPCR (data not shown). In contrast, *Ltbr* expression was detected in TJ2-Tg thymic lymphomas (Figure 3.1D) regardless the decrease in the relative proportion of stromal cells when compared to wild-type thymi (data not shown). These results indicate that

in thymic lymphomas from TJ2-Tg leukemic mice, leukemic cells express LTβR ligands while LTβR is most likely derived from stromal cells, similarly to what was previously described for the normal thymus (Hikosaka et al., 2008; Seach et al., 2008).

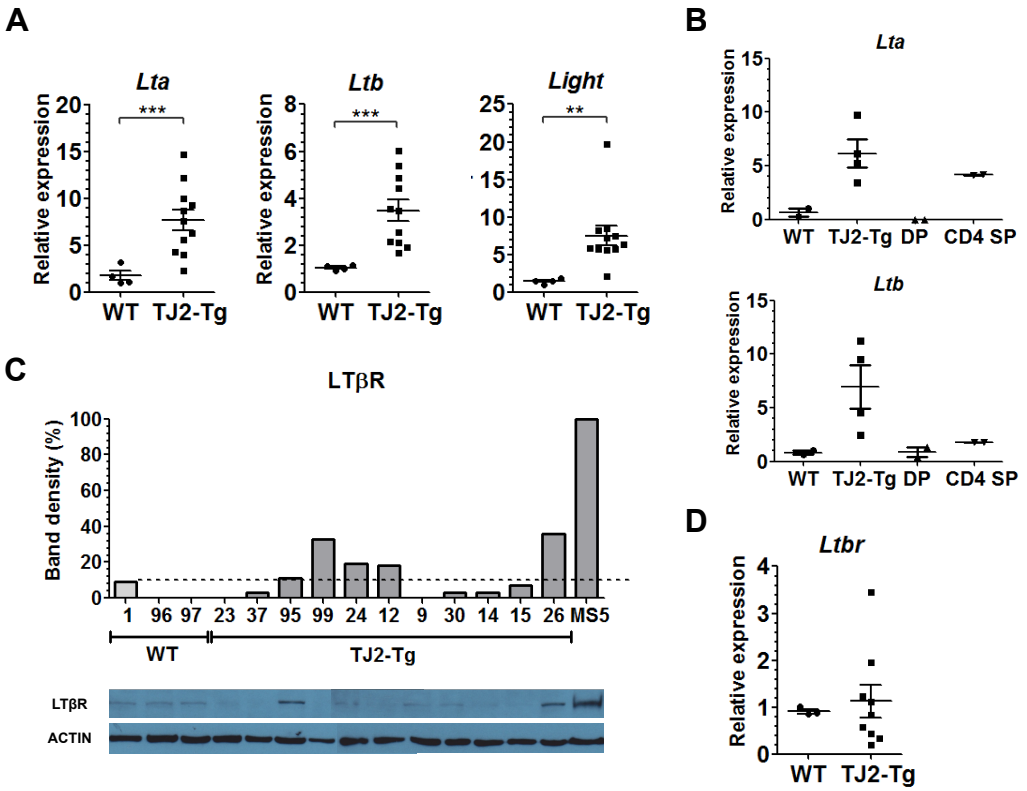


Figure 3.1. LTβR and LTβR ligand expression in TJ2-Tg mice. (**A**) RT-qPCR quantification of *Lta*, *Ltb* and *Light* in thymocyte-enriched fractions collected from wild-type thymi (WT; n=4) and leukemic cell-enriched fractions collected from TJ2-Tg thymic lymphomas (n=11). (**B**) *Lta* and *Ltb* RT-qPCR analysis in WT thymocytes (n=2), TJ2-Tg leukemic cells (n=4), and sorted CD4/CD8 double-positive (DP; n=2) and CD4 single-positive (CD4 SP; n=2) thymocytes. (**C**) LTβR protein quantification by western blotting and densitometric analysis in thymocyte-enriched fractions prepared from wild-type thymi (n=3) and leukemic cell-enriched fractions prepared from TJ2-Tg thymic lymphomas (n=11), using the Image J software. The results were normalized to the loading control (actin) and are represented as a percentage of the MS5 (positive control) band density. (**D**) *Ltbr* quantification by RT-qPCR in whole thymic lymphomas from TJ2-Tg mice (n=9) and whole wild-type thymi (n=3). All RT-qPCR results were normalized to *Gapdh* expression, and expressed as mean ± SEM. *P*-values were determined using two-tailed, unpaired Student´s *t*-test with Welch´s correction.

3.1.2. LTβR ligands are undetectable at the surface of TJ2-Tg leukemic in vivo

Previous studies have focused mostly on LTβR ligand mRNA or total cellular protein expression (Ammirante et al., 2010; Hehlgans et al., 2002; Rehm et al., 2011). However, it is

widely accepted that LTβR ligands should be presented at the surface of T cells in order to activate LTβR signaling in another cell. Therefore, to determine whether transcription of the ligands was accompanied by specific cell-surface protein expression we used LTβR-Fc, a fusion decoy protein that binds both lymphotoxin (LT or $LT\alpha_1\beta_2$) and LIGHT. LTβR-Fc binding was detected by staining with a PE-conjugated anti-human Fc antibody in flow cytometry. Despite the high expression levels of *Lta*, *Ltb* and *Light* mRNAs in TJ2-Tg leukemic cells, LTβR-Fc and anti-LTβ immunostaining revealed low to undetectable signals (Figure 3.2A). However, intracellular LTβ protein was detected on permeabilized, fixed cells (Figure 3.2C), suggesting that at least *Ltb* mRNA was indeed translated. Interestingly, upon *ex vivo* culture, both LTβ and LTβR ligands were clearly detectable on primary TJ2-Tg leukemic cell surface (Figure 3.2B,D). Importantly, anti-LTβ mAb pre-blocking showed that the LTβR-Fc fusion protein signal in *ex vivo*-cultured cells was derived from lymphotoxin only and not LIGHT expression (Figure 3.2B).

Altogether, these results suggest that LTβR ligand mRNA and protein are upregulated in leukemic cells during T-cell leukemogenesis but the surface expression of lymphotoxin is tightly regulated *in vivo*.

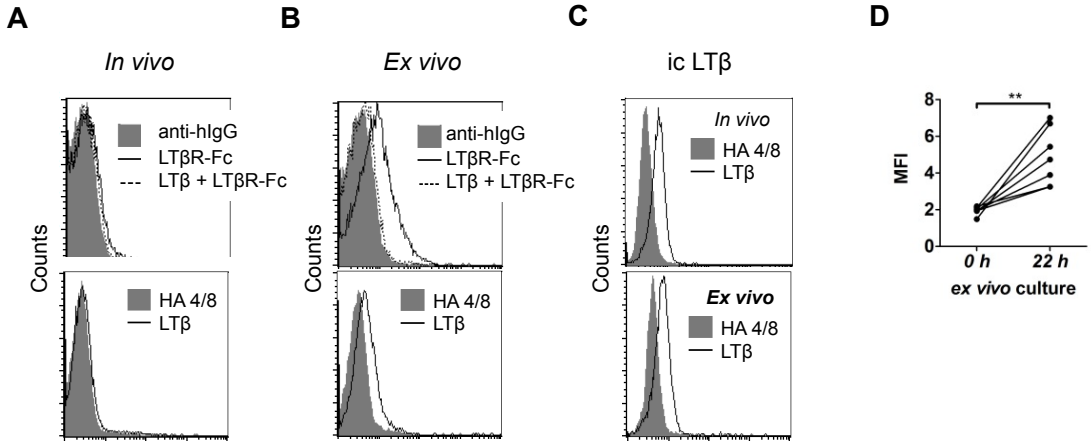


Figure 3.2. LTβR ligand expression at the surface of TJ2-Tg leukemic cells. Flow cytometry immunostaining of LTβR ligands (solid line) on representative TJ2-Tg leukemic cells with LTβR-Fc protein or anti-LTβ mAb (BBF6) on viable (**A**) freshly collected (*in vivo*), (**B**) cultured *ex vivo* for 22 h, or (**C**) fixed/permeabilized (ic) cells. The secondary antibody alone or non-specific mAb (HA4/8) were used as negative controls for LTβR-Fc and LTβ mAb, respectively (gray shading). LTβR-Fc immunostaining was also performed following anti-LTβ mAb pre-incubation (dashed line). (**D**) LTβR-Fc staining mean fluorescence intensity (MFI) for 7 different leukemic cell samples before (0 h) and after 22 h of *ex vivo* culture. *P*-value were determined using two-tailed, paired Student´s *t*-test.

3.1.3. Lymphotoxin expression in TJ2-Tg leukemic T cells is inducible by pre-TCR/TCRαβ activation

PMA (phorbol myristate acetate) has been previously reported to induce T and B cell activation together with $LT\alpha_1\beta_2$ expression (Browning et al., 1995, 1997; Ware et al., 1992). Furthermore, TJ2-Tg leukemic cells proliferate and express T-cell activation markers (e.g., CD69) when stimulated *in vitro* with PMA alone or in combination with the calcium ionophore ionomycin (dos Santos et al., 2007). Therefore, to assess if lymphotoxin expression at the surface of TJ2-Tg leukemic cells could be inducible by extracellular signals, these cells were stimulated by PMA and ionomycin, a treatment that activates protein kinase C and calcium channels and therefore mimics signaling pathway activation following TCR engagement (Nau et al., 1988). Upon overnight treatment, surface LTβR ligands were detected in wild-type thymocytes (Figure 3.3A), as previously reported (Browning et al., 1995, 1997; Ware et al., 1992), and more strongly so in TJ2-Tg leukemic cells (Figure 3.3B). Again, anti-LTβ mAb pre-blocking showed that the LTβR-Fc fusion protein signal detected in PMA/ionomycin-treated cells was derived from lymphotoxin rather than LIGHT expression (data not shown). Although we cannot exclude the possibility that LIGHT is expressed as a soluble form, these results indicate that, in addition to *ex vivo*-cultured, TJ2-Tg leukemic cells stimulated with PMA/ionomycin also express membrane-bound $LT\alpha_1\beta_2$ heterotrimers but not LIGHT homotrimers.

Since *Lta* and *Ltb* expression is induced by pre-TCR (Silva-Santos et al., 2005) and during TCR-mediated thymocyte positive selection (Irla et al., 2012), we reasoned that pre-TCR/TCR signaling could regulate lymphotoxin gene expression in these cells. To address this question, we assessed *Lta*, *Ltb*, and also *Light* expression levels in leukemic cells from TJ2-Tg;*Rag2*$^{-/-}$ mice, which arise from thymocytes blocked in the DN3 stage of T cell differentiation and lack both pre-TCR and TCRαβ gene expression (dos Santos et al., 2007). *Ltb* and *Light*, but not *Lta* expression was reduced in TJ2-Tg;*Rag2*$^{-/-}$ leukemic cells, as compared to pre-TCR/TCR-proficient TJ2-Tg leukemic cells (Figure 3.3D). Similarly to *Rag2*-proficient TJ2-Tg leukemic cells, TJ2-Tg;*Rag2*$^{-/-}$ leukemic cells did not express surface lymphotoxin, except when stimulated by PMA/ionomycin (Figure 3.3C). Although one cannot exclude that *Rag2* inactivation could induce *Ltb* and *Light* downregulation through an indirect mechanism, these data support the notion that TCR expression in leukemic cells, like in normal thymocytes (Irla et al., 2012), contributes to *Ltb* gene expression in T-ALL.

Collectivelly, these results demonstrate that surface lymphotoxin expression occurs in TJ2-Tg leukemic cells, and may be modulated by extracellular stimuli.

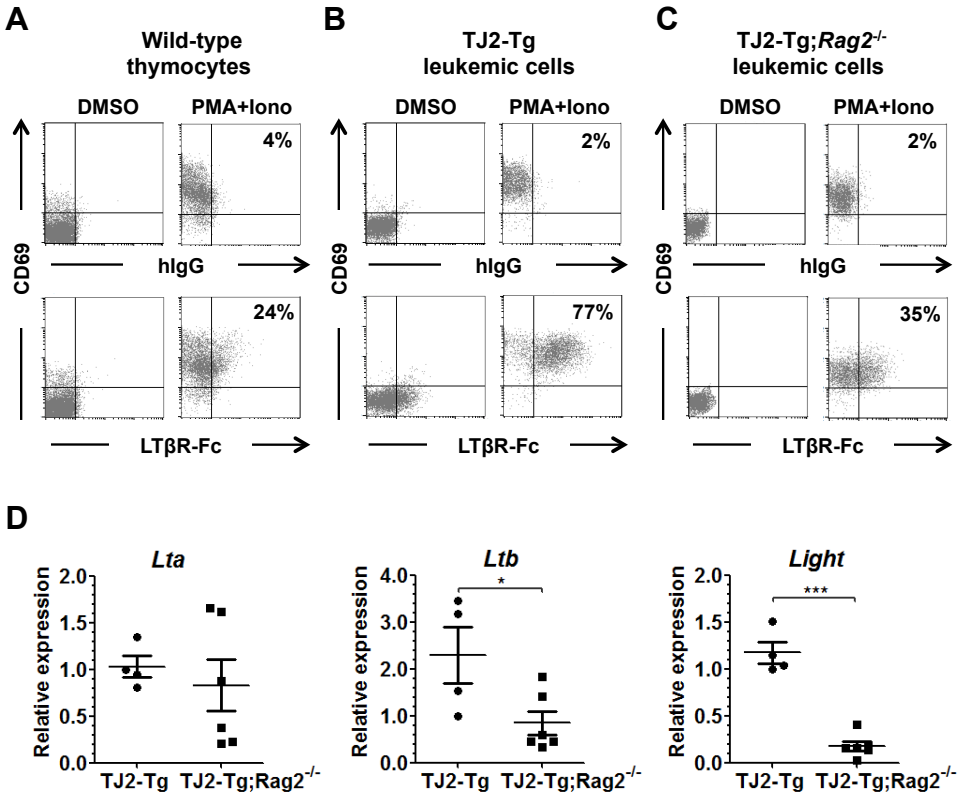


Figure 3.3. Lymphotoxin cell surface expression in thymocytes and TJ2-Tg leukemic cells is induced through pre-TCR/TCRαβ activation. Surface LTβR-Fc and CD69 immunostaining of (**A**) wild-type thymocytes, (**B**) TJ2-Tg, and (**C**) TJ2-Tg;*Rag2*$^{-/-}$ leukemic cells upon *ex vivo* treatment for 22 h with either PMA and ionomycin (PMA+Iono) or vehicle (DMSO). The percentage of cells is indicated in the respective quadrant. One representative of three independent experiments is shown. (**D**) RT-qPCR quantification of *Lta*, *Ltb*, and *Light* mRNA levels (normalized to *Gapdh*) in leukemic *Rag2*-deficient TJ2-Tg leukemic cells (TJ2-Tg;*Rag2*$^{-/-}$; n=6) and control leukemic cells (TJ2-Tg; n=4). Results are expressed as the mean ± SEM. *P*-values were determined using two-tailed, unpaired Student´s *t*-test.

3.1.4. Lymphotoxin expression in TJ2-Tg leukemic T cells depends on JAK/STAT and IKK/NF-κB signaling pathway activity

Signaling pathway activation may control the expression of *Lta* and *Ltb*. Since JAK2 is constitutively activated in TJ2-Tg leukemic cells, one possible candidate is the JAK/STAT pathway (Carron et al., 2000). Moreover, it was previously shown that NF-κB is activated in TJ2-Tg leukemic cells (dos Santos et al., 2008), and TCR deficiency correlated with reduced NF-kB activity in these cells (dos Santos et al., 2008). So, we reasoned that pre-TCR/TCR signaling could regulate lymphotoxin gene expression in these cells through NF-κB

activation. Therefore, to know how LTβR ligand expression is induced in TJ2-Tg leukemic cells, we inhibited JAK/STAT and IKK/NF-κB signaling pathways, which are known to induce *Lta* and *Ltb* expression in other cell types (Shebzukhov and Kuprash, 2011).

Treatment of leukemic cells with a pan-JAK inhibitor (JAKi) downregulated *Lta* (and the control *Socs2* gene), but neither *Ltb* nor *Light* mRNA expression (Figure 3.4A). In contrast, treatment of leukemic cells with an IKK kinase inhibitor (IKKi) markedly downregulated *Lta*, *Ltb*, and *Light* mRNAs (and the control *Nfkb2* gene) (Fig. 3.4B). Similarly, JAKi downregulated *Lta* expression, and IKKi downregulated both *Lta* and *Ltb* in wild-type thymocytes (data not shown). Therefore, JAK signaling may induce *Lta*, while NF-κB may underlie *Lta*, *Ltb* and *Light* expression in thymocytes in physiological conditions, but JAK/STAT and IKK/NF-κB constitutive activation following thymocyte transformation may lead to higher and persistent activation of these genes. Furthermore, the IKK inhibitor could block *Lta* and *Ltb* induction by PMA/ionomycin in TJ2-Tg leukemic cells (Figure 3.4C). Regarding *Light*, it was not downregulated in PMA/ionomycin-stimulated cells following treatment with IKKi (Figure 3.4C).

Together, these experiments define JAK and IKK kinases as key regulators of lymphotoxin gene expression in TJ2-Tg leukemic cells.

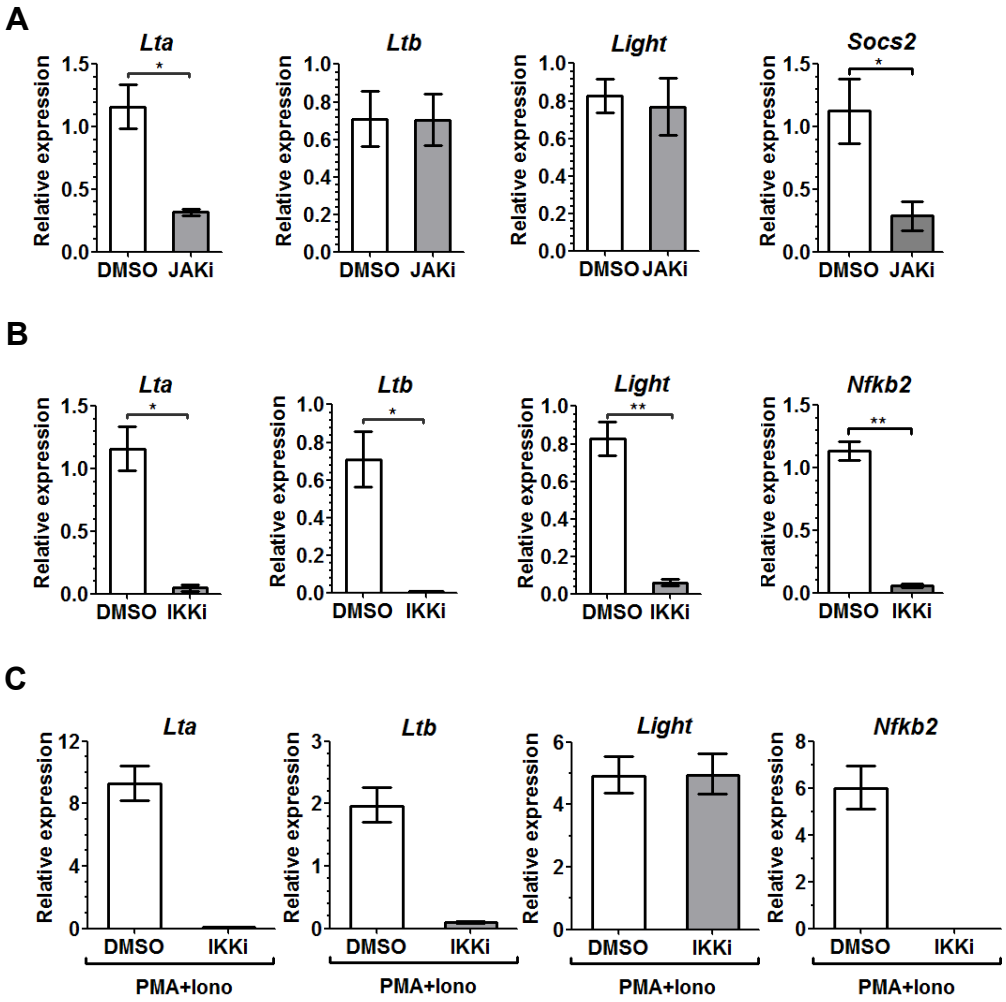


Figure 3.4. LTβR ligand gene expression in TJ2-Tg leukemic cells depends on IKK and JAK kinase activity. (**A**, **B**) *Lta*, *Ltb*, *Light*, *Socs2*, and *Nfkb2* mRNA quantification by RT-qPCR for three independent TJ2-Tg leukemic cell samples treated *ex vivo* for 10 h with either DMSO (control), a pan JAK inhibitor (JAKi) or an IKK inhibitor (IKKi). Results are expressed as mean ± SEM. *P*-values were determined using two-tailed, paired Student´s *t*-test. (**C**) *Lta*, *Ltb*, *Light*, and *Nfkb2* quantification by RT-qPCR in TJ2-Tg leukemic cells treated *ex vivo* with PMA+Iono and either IKK inhibitor (IKKi) or DMSO. Results are expressed as the mean ± range and represent one of three independent experiments. All RT-qPCR results were normalized to *Gapdh* expression.

3.2. MODULATION OF LYMPHOTOXIN AT THE SURFACE OF TJ2-TG LEUKEMIC CELLS

3.2.1. Lymphotoxin cell surface induction on TJ2-Tg leukemic T cells depends on *de novo* protein synthesis

As previously shown, LTβR ligands were hardly detectable by flow cytometry immunostaining in freshly collected TJ2-Tg leukemic T cells (Figure 3.2A), but lymphotoxin was readily detected following *ex vivo* culture (Figure 3.2B,D). To gain an insight on the mechanism responsible for $LT\alpha_1\beta_2$ upregulation, we first followed LTβR ligand expression at the surface of TJ2-Tg leukemic cells and wild-type thymocytes at particular time-points. Upon culture, LTβR ligands were upregulated in leukemic cells and DP plus CD8 SP normal thymocytes, peaking at 24 h and 12 h, respectively (Figure 3.5A). Notably, the intensity of LTβR-Fc staining in leukemic cells was more than three times higher than in its normal counterparts (Figure 3.5A). At 24h, more LT-expressing leukemic cells were also detected when compared with wild-type thymocytes (Figure 3.5C). Interestingly, the CD8 SP thymocyte subpopulation was the one that contributed mostly for LTβR ligand expression at the cell surface of wild-type thymocytes following *ex vivo* culture (Figure 3.5B,C).

Although TJ2-Tg leukemic cells were shown to express intracellular LTβ (Figure 3.2C), we could not detect intracellular LTα and LIGHT due to the lack of an antibody suitable for flow cytometry immunostaining or western-blotting. Therefore, to determine if induction of *Lta*, *Ltb* or *Light* mRNA precedes the observed increase in LTβR ligand protein expression in cultured TJ2-Tg leukemic cells, quantitative RT-PCR analysis in freshly collected and leukemic cells cultured *ex vivo* for 10 hours was performed. *Ltb* mRNA expression was consistently induced following *ex vivo* culture while *Light* was often downregulated (Figure 3.6A). The effect of *ex vivo* culture on *Lta* expression was variable (Figure 3.6A). These results indicate that the increase in $LT\alpha_1\beta_2$ surface expression can be due to *Ltb* gene induction.

The observed lymphotoxin cell surface expression following *ex vivo* culture and PMA/ionomycin stimulation could result from a combination of *de novo* protein synthesis and protein subcellular relocalization. To assess the role of *de novo* protein synthesis in surface lymphotoxin upregulation, we blocked translation and transcription by treating cells respectively with cycloheximide or actinomycin D. Both compounds blocked lymphotoxin

cell surface induction upon either *ex vivo* culture or PMA/ionomycin stimulation for 22 h (Figure 3.6B). At shorter term (6 h) *ex vivo* cultures, surface lymphotoxin was detected only after PMA/ionomycin stimulation, and in a protein synthesis-dependent manner (Figure 3.6C), indicating that PMA/ionomycin treatment induced lymphotoxin protein surface expression in leukemic cells with faster kinetics than *ex vivo* culture.

Together, these results indicate that $LT\alpha_1\beta_2$ expression at the surface of *ex vivo*-cultured and stimulated leukemic cells depends on lymphotoxin gene transcription and continuous protein synthesis.

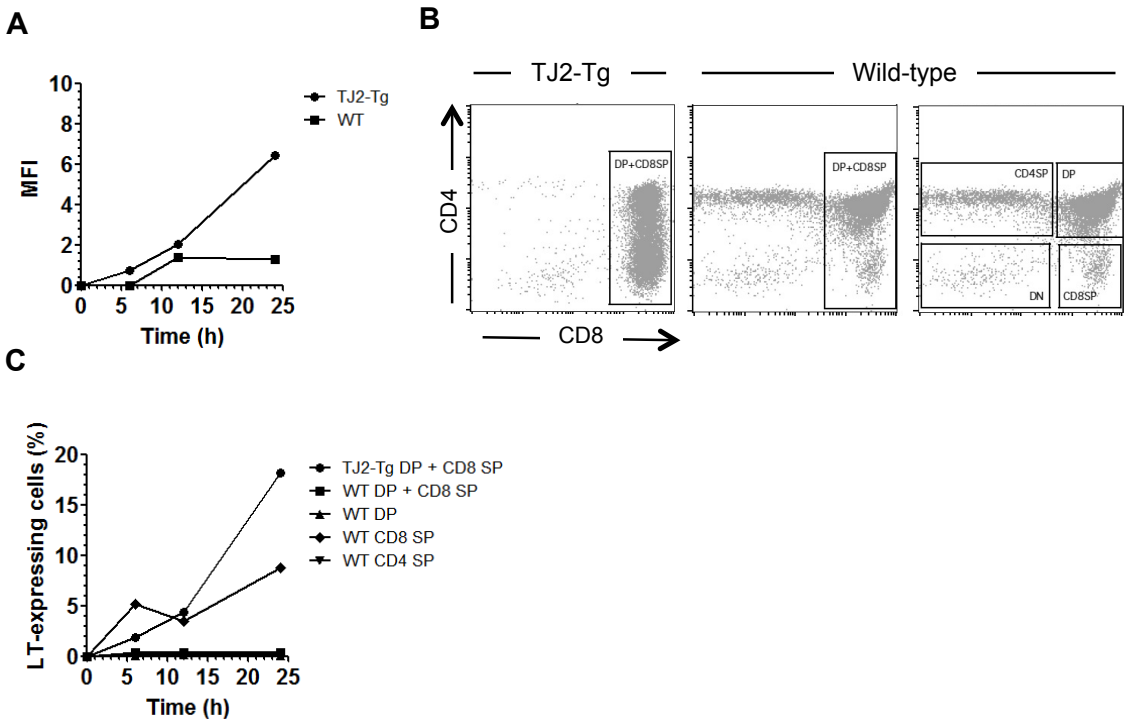


Figure 3.5. Lymphotoxin expression on TJ2-Tg leukemic cell surface is upregulated upon *ex vivo* culture. (**A**) Mean fluorescence intensity (MFI) determined by flow cytometry immunostaining with LTβR-Fc (after subtraction from the MFI of the secondary antibody alone) on TJ2-Tg leukemic cells and wild-type thymocytes (WT), *in vivo* (t = 0) or cultured *ex vivo* for the indicated periods of time. (**B**) Gating strategy used to analyze wild-type thymocyte subpopulations and TJ2-Tg leukemic cells. (**C**) Percentage of TJ2-Tg leukemic cells, WT thymocytes, and subpopulations expressing surface $LT\alpha_1\beta_2$ determined by flow cytometry with LTβR-Fc.

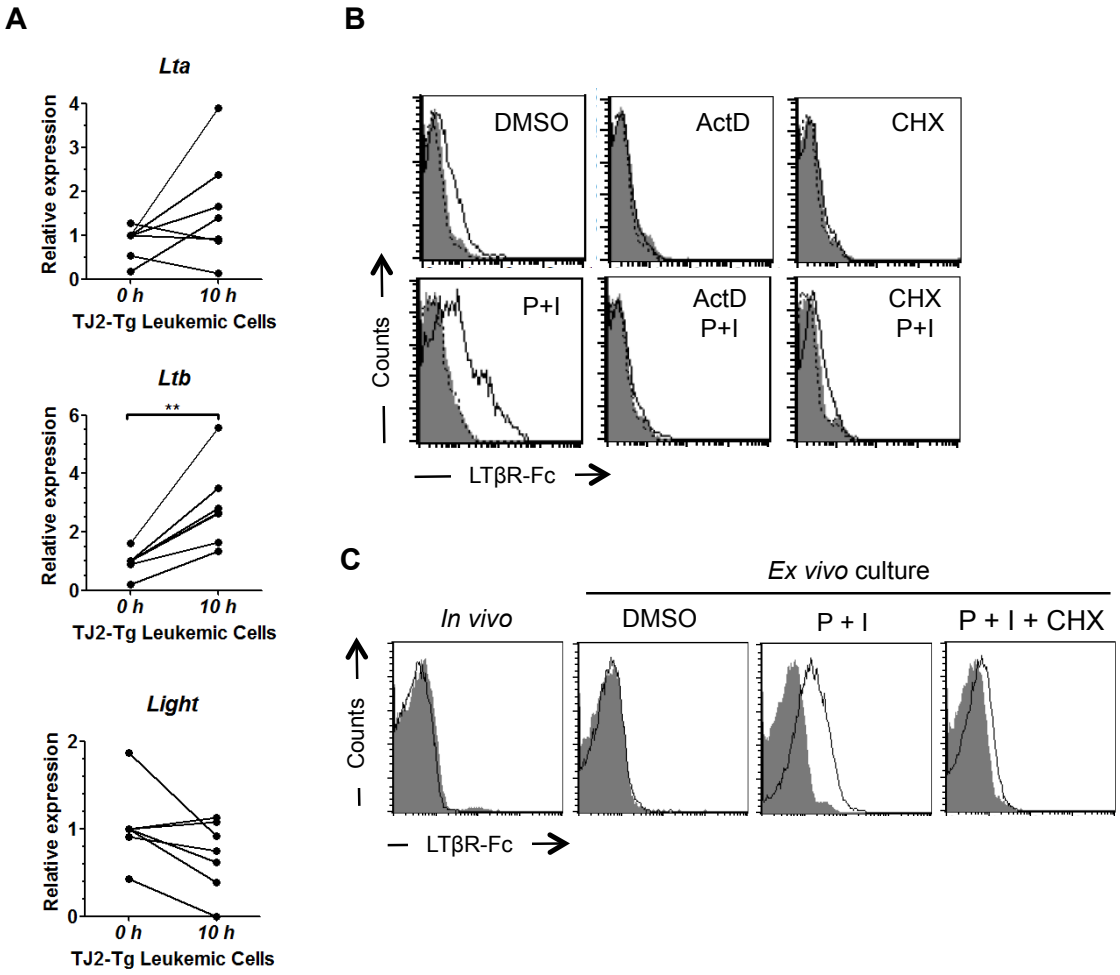


Figure 3.6. Lymphotoxin expression on TJ2-Tg leukemic cell surface depends on *de novo* protein synthesis. (**A**) RT-qPCR analysis of *Lta*, *Ltb*, and *Light* mRNA levels in TJ2-Tg leukemic cells freshly collected (0 h) or after *ex vivo* culture for 10 h (n=7). Results for each sample were normalized to *Gapdh* expression and are expressed as mean of triplicates. *P*-values were determined using two-tailed, paired Student´s *t*-test. (**B**, **C**) LTβR-Fc immunostaining of surface $LT\alpha_1\beta_2$ (solid line) on TJ2-Tg leukemic cells *in vivo* or following *ex vivo* culture for 6h or 22h, as indicated. Cells we treated either with DMSO, ActD, CHX, PMA+Iono (P+I), and P+I in combination with ActD or CHX. Cells were also pre-incubated with anti-LTβ mAb before staining with LTβR-Fc (dashed line) or incubated with a negative control (gray shading). One representative of four independent experiments is shown.

3.2.2. Microenvironmental LTβR modulates lymphotoxin expression at the surface of TJ2-Tg leukemic cells

The fact that TJ2-Tg leukemic cells expressed surface lymphotoxin upon *ex vivo* culture may indicate that factors found *in vivo* downmodulate surface LTβR ligands. Furthermore, since LTβR ligand surface expression was reported in mature thymocytes only in the absence of LTβR (Boehm et al., 2003), we decided to investigate if a regulatory loop modulating its

expression could be acting in TJ2-Tg leukemic cells *in vivo*. So, to assess the role of microenvironmental LTβR expression on membrane-bound lymphotoxin in leukemic cells, we transplanted TJ2-Tg leukemic cells into *Ltbr*$^{+/-}$ and *Ltbr*$^{-/-}$ mice. After a 3-week delay, expanded donor leukemic cells were collected from recipient mouse infiltrated organs, and the presence of LTβR ligands at the surface of leukemic cells (detected as $CD8^{+}CD25^{+}$cells) (Figure 3.7A) was analyzed. Interestingly, LTβR ligands were detected at the surface of leukemic cells freshly collected from the thymus, spleen, bone marrow and peripheral blood of *Ltbr*$^{-/-}$ but not *Ltbr*$^{+/-}$ recipient mice (Figure 3.7B-E). LTβ pre-blocking confirmed that LTβR ligand expression corresponded exclusively to lymphotoxin heterotrimers (data not shown). Because leukemic cells injected in the two groups were identical, these results demonstrate that $LT\alpha_1\beta_2$ surface expression on TJ2-Tg leukemic cells, similarly to normal mature thymocytes (Boehm et al., 2003), is downmodulated when these cells are localized in LTβR-expressing tissue microenvironments, and suggest the lack of LT detection is an indication of ongoing LTβR signaling. Moreover, this experiment was repeated by transplanting leukemic cells collected from diseased TJ2-Tg;*Ltbr*$^{-/-}$ mice and identical results were obtained (data not shown), thus confirming that the *Ltbr* genetic status in leukemic cells does not influence lymphotoxin surface expression.

To study the mechanism by which $LT\alpha_1\beta_2$ expression at the TJ2-Tg leukemic cell surface is downmodulated, a more simplistic co-culture-based model was used. The MS5 stromal cell line, which expresses LTβR (Figure 3.8A), was shown to establish functional interactions and to sustain the viability of co-cultured TJ2-Tg leukemic cells (Figure 3.8B). Therefore, to verify whether downmodulation of $LT\alpha_1\beta_2$ surface expression depends on direct cell contact, we co-cultured TJ2-Tg leukemic cells with MS5 stromal cells. As previously observed, lymphotoxin was upregulated following *ex vivo* culture in isolated TJ2-Tg leukemic cells (Figure 3.8C). Upon co-culture, leukemic cells adhering to the MS5 monolayer showed lower levels of surface $LT\alpha_1\beta_2$ than non-adhering co-cultured leukemic cells (Figure 3.8C,D). Furthermore, conditioned medium collected from co-cultured leukemic cells with MS5 cells did not induce $LT\alpha_1\beta_2$ downmodulation in leukemic cells (Figure 3.8E,F). This result further indicates that soluble factors produced in co-cultures are not responsible, at least alone, for $LT\alpha_1\beta_2$ downmodulation and that direct contact with microenvironmental cells is required for lymphotoxin downmodulation on the leukemic cell surface.

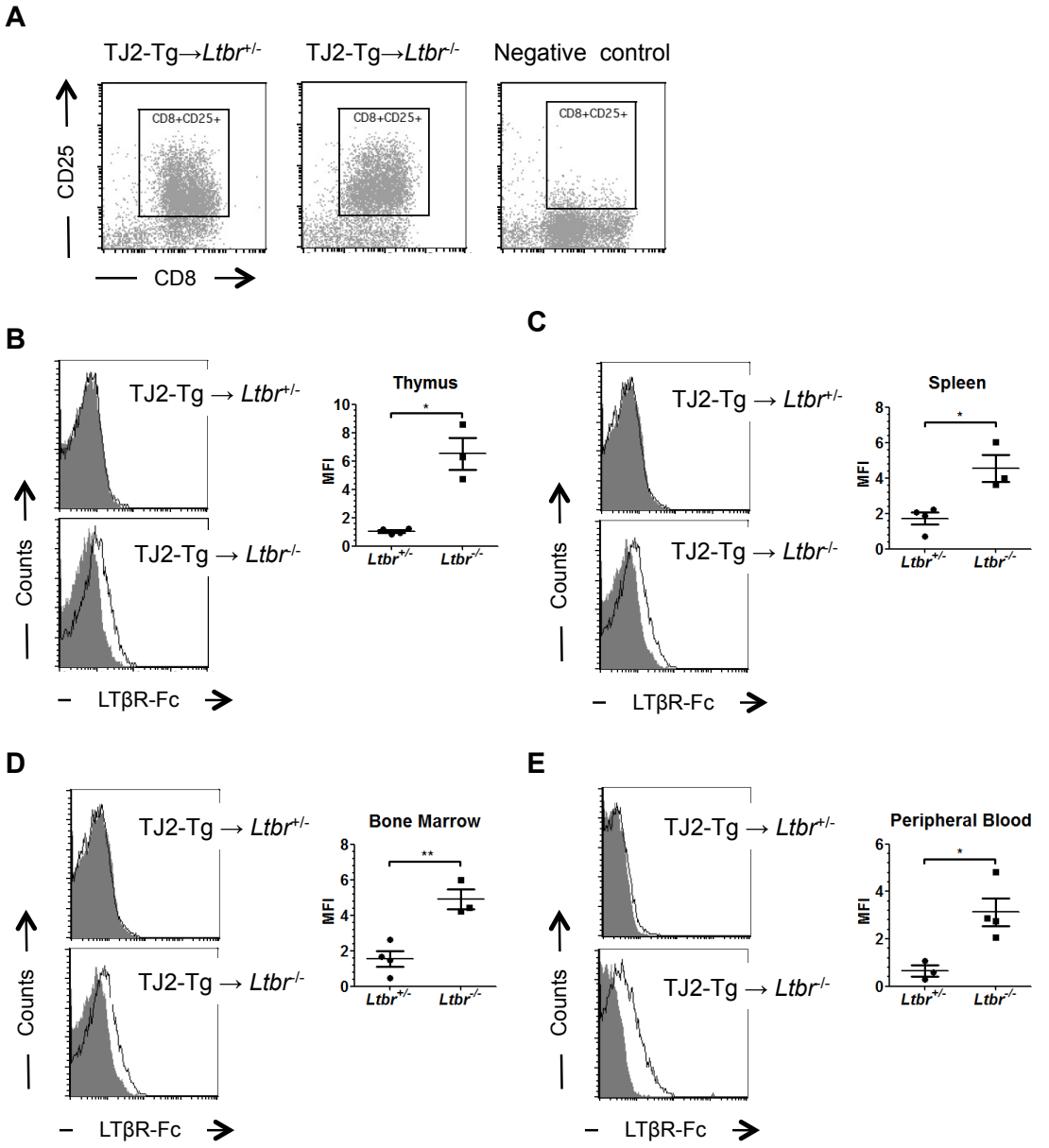


Figure 3.7. Cell surface $LT\alpha_1\beta_2$ expression on TJ2-Tg leukemic cells depends on LTβR expression in microenvironmental cells. (**A**) Cell surface immunostaining of CD8/CD25 on cells collected from the thymus of mice inoculated with TJ2-Tg leukemic cells or not (negative control) and flow cytometry gating stategy. (**B-E**) Cell surface immunostaining with LTβR-Fc (solid line) or the secondary antibody alone (negative control, gray shading) of $CD8^+CD25^+$ TJ2-Tg leukemic cells collected from the (**B**) thymus, (**C**) spleen, (**D**) bone marrow, and (**E**) peripheral blood, 3 weeks post-inoculation of *Ltbr*$^{+/-}$ and *Ltbr*$^{-/-}$ mice. LTβR-Fc mean fluorescent intensities (MFIs) subtracted from control MFIs are also indicated. One representative of three independent experiments is shown as the mean ± SEM. *P*-values were determined using two-tailed, unpaired Student´s *t*-test.

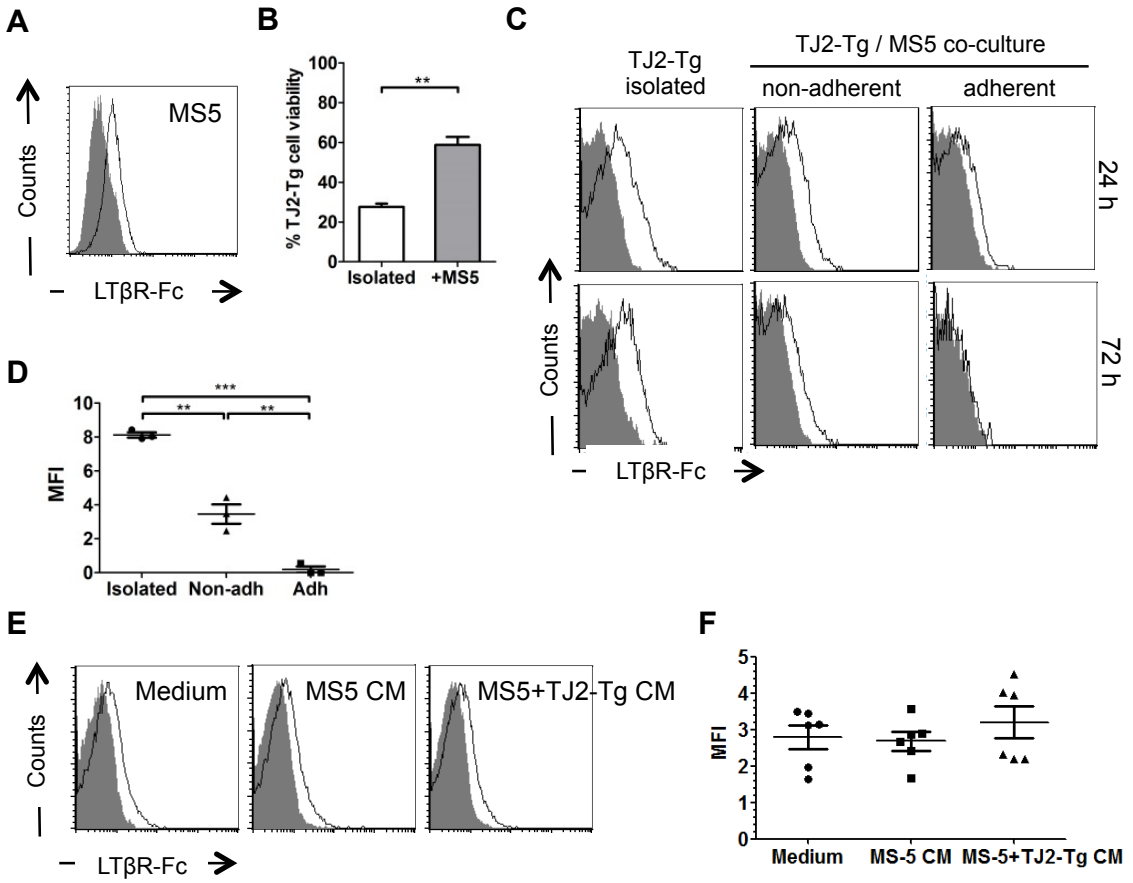


Figure 3.8. Lymphotoxin downmodulation from the surface of TJ2-Tg leukemic cells requires direct contact with LTβR-expressing MS5 stromal cells. (**A**) Flow cytometry immunostaining of MS5 stromal cells with anti-mouse LTβR antibody (black line) or the secondary antibody alone (gray shading). (**B**) TJ2-Tg leukemic cell viability upon culture for 3 days in culture medium alone (isolated) or on a layer of adherent murine MS-5 cells (+MS-5), determined by propidium iodide (PI) exclusion. Results represent three independent experiments. (**C**) Cell surface LTβR-Fc immunostaining (solid line) of TJ2-Tg leukemic cells cultured for the indicated times in culture medium alone, or on a confluent layer of MS-5 cells, detected by flow cytometry, and (**D**) corresponding LTβR-Fc MFIs of leukemic cells subtracted from the control MFIs. Leukemic cells adherent or non-adherent to MS-5 cells were analyzed by gating on Thy1.2^{+} cells. (**E**) Conditioned medium collected from TJ2-Tg leukemic and MS5 cells co-cultured for 72 h and diluted 1:2 (MS5+TJ2-Tg CM) was used to culture TJ2-Tg leukemic cells for 24 h. The $LT\alpha_1\beta_2$ heterotrimer was detected as in (C). TJ2-Tg leukemic cells cultured in RPMI complete medium (medium) or conditioned medium collected from MS5 cells alone (MS5 CM) were also analyzed as controls, and (**F**) all corresponding LTβR-Fc MFIs of leukemic cells subtracted from the control MFIs were plotted. The triplicates of two independent experiments are shown. All the results are expressed as the mean ± SEM of triplicates. *P*-values were determined using two-tailed, unpaired Student´s *t*-test.

To determine if lymphotoxin expression was modulated specifically by LTβR in stromal cells, TJ2-Tg leukemic cells were co-cultured with either *Ltbr*$^{-/-}$ or *Ltbr*$^{+/-}$ mouse embryonic fibroblasts (MEFs). After 24 h of incubation, leukemic cells localized under adherent fibroblasts (pseudoemperipolesis; Figure 3.9A) (Nishi et al., 1982). Interestingly, membrane-bound lymphotoxin was detected in leukemic cells growing in close contact (adherent) with *Ltbr*-deficient MEFs, but not in those cells adhering to *Ltbr*-proficient MEFs (Figure 3.9B), thus indicating that downmodulation of surface LT expression on leukemic cells is mediated by interaction with its cognate receptor in microenvironmental cells. These results were consistent with those obtained *in vivo*, and indicated that expression of leukemic cell surface lymphotoxin in *Ltbr*-deficient recipient mice was caused by lack of direct contact with LTβR expressed in microenvironmental cells, and not eventual indirect cellular or systemic defects.

Finally, to determine if ligation of the extracellular domain of LTβR is sufficient to induce $LT\alpha_1\beta_2$ downmodulation at the cell surface of TJ2-Tg leukemic T cells, we cultured these cells in the presence of LTβR-Fc for 24 h. By performing flow cytometry immunostaining using again LTβR-Fc, we found that LTβR-Fc added to the culture binds $LT\alpha_1\beta_2$ heterotrimers at the cell surface but does not downmodulate it (data not shown).

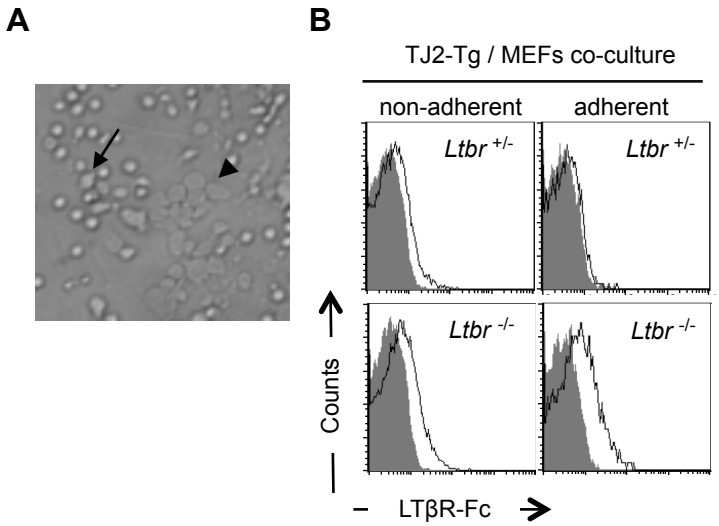


Figure 3.9. Lymphotoxin downmodulation from the surface of TJ2-Tg leukemic cells requires direct contact specifically with LTβR expressed at the surface of stromal cells. (**A**) Phase contrast microscopy picture showing TJ2-Tg leukemic cells adhering (arrowhead) and non-adhering (arrow) to MEFs, after 24 h of co-culture. (**B**) Cell surface LTβR-Fc immunostaining of TJ2-Tg leukemic cells collected from 24 h co-cultures with *Ltbr*$^{+/-}$ or *Ltbr*$^{-/-}$ MEFs. Leukemic cells adherent or non-adherent to MEFs cells were analyzed by gating on Thy1.2^{+} cells in flow cytometry.

3.3. IMPACT OF LTβR INACTIVATION ON TEL-JAK2-INDUCED LEUKEMOGENESIS

3.3.1. LTβR inactivation delays leukemia onset in TEL-JAK2 transgenic mice

To investigate if lymphotoxin expression in leukemic cells and associated LTβR activation are involved in T-cell leukemia development, we tested the impact of systemic *Ltbr* inactivation in leukemogenesis. To this end, TJ2-Tg mice were crossed with *Ltbr* knockout mice, which present no defects in CD4 and CD8 double-negative (DN) and DP thymocyte maturation (Boehm et al., 2003; Fütterer et al., 1998), the major targets for TJ2-Tg-induced malignant transformation (dos Santos et al., 2007). Demonstrating a role for LTβR in leukemogenesis, TJ2-Tg;*Ltbr*$^{-/-}$ mice developed leukemia with longer latency than TJ2-Tg;*Ltbr*$^{+/-}$ mice, median survival of 23 versus 15 weeks, respectively (Figure 3.10A). Of note, like previously found for leukemic cells transplanted to *Ltbr*$^{-/-}$ mice, those collected from *Ltbr*-deficient, but not from *Ltbr*-proficient TJ2-Tg mice expressed surface LT$\alpha_1\beta_2$ (Figure 3.10B,C), thus confirming that LT$\alpha_1\beta_2$ surface expression in both normal and malignant T cells is only detectable in the absence of the cognate receptor (Boehm et al., 2003). Since LTβR mRNA and protein was not or poorly expressed in leukemic T cells (Figure 3.1C and data not shown), our results indicate that LTβR expression in non-malignant microenvironmental cells promotes leukemogenesis.

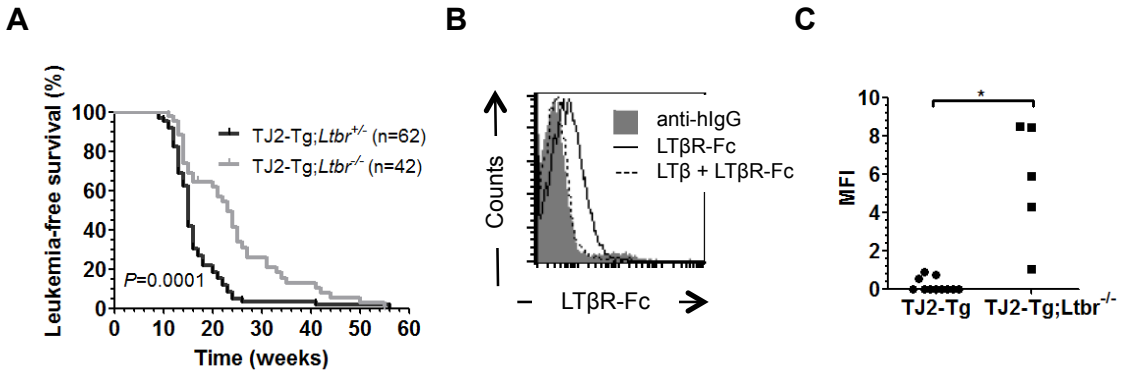


Figure 3.10. LTβR inactivation delayed TEL-JAK2-induced leukemogenesis. (**A**) Kaplan-Meier leukemia-free survival curves for cohorts of TJ2-Tg;*Ltbr*$^{+/-}$ and TJ2-Tg;*Ltbr*$^{-/-}$ mice (P = 0.0001; log-rank Mantel Cox test). Tick marks represent leukemia-free mice followed until the indicated age. (**B**) FACS immunostaining with LTβR-Fc (solid line) and the secondary antibody alone (gray shading) of one representative sample of TJ2-Tg;*Ltbr*$^{-/-}$ leukemic cells collected from the thymus. Where indicated, cells were pre-incubated with anti-LTβ mAb (dashed line). (**C**) Graph showing the LTβR-Fc mean fluorescence intensity (MFI) of TJ2-Tg (n=11) and TJ2-Tg;*Ltbr*$^{-/-}$ (n=5) leukemic cells subtracted from the MFI of cells stained with secondary antibody alone. *P*-value determined using two-tailed, unpaired Student´s *t*-test with Welch´s correction.

3.3.2. Delayed leukemia onset in TEL-JAK2 transgenic mice is not due to developmental defects in the thymus of LTβR-deficient mice

TJ2-Tg-induced T-ALL was previously shown to arise from DN/DP thymocytes (dos Santos et al., 2007). Moreover, the DP phenotype in T cells is exclusively found in the thymus under normal conditions. To confirm that the thymus is the organ where T-ALL originates, we analyzed young (8-week-old) TJ2-Tg mice presenting no signs of disease and searched for aberrant $CD8^+CD25^+$ cells in organs commonly affected by T-ALL in these mice. By doing so, we found variable proportions of leukemic cells in the thymus of most mice analyzed, while organs such as spleen, bone marrow, and the peripheral blood presented few or none (Figure 3.11). Thymocyte suspensions containing $CD8^+CD25^+$ cells generated secondary leukemias in transplanted Nude mice (data not shown), indicating that the latter cells are already transformed. Altogether, these results support the notion that leukemic cells arise in the thymus of young TJ2-Tg mice from developing thymocytes.

Since LTβR activation is involved in thymic stromal cell crosstalk with thymocytes (Boehm et al., 2003), the observed delay in leukemogenesis in the absence of LTβR could be linked to thymocyte defects. Arguing against such possibility, we found that LTβR-deficient mice of 19-22 weeks, an age when most *Ltbr*-proficient TJ2-Tg mice have developed terminal disease, presented no major defects in thymocyte cellularity (Figure 3.12A,B) and differentiation (Figure 3.12C,D). Therefore, these results indicate that the delay in TEL-JAK2-induced leukemogenesis caused by LTβR deficiency cannot be attributed to a reduction of cellular targets for transformation in the thymus.

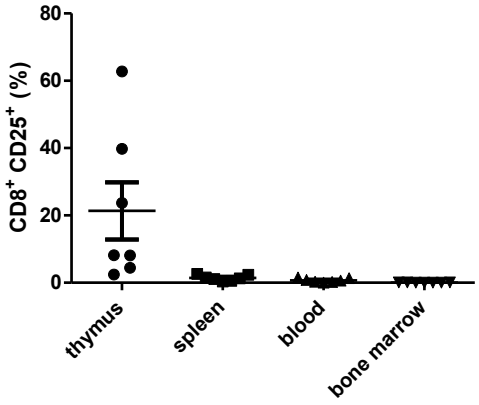


Figure 3.11. $CD8^+CD25^+$ leukemic cells originate in the thymus of TJ2-Tg young mice with no signs of disease. Leukemic cells ($CD8^+CD25^+$) were detected by flow cytometry on cell suspensions prepared from the thymus, spleen, peripheral blood, and bone marrow of 8-week-old TJ2-Tg mice.

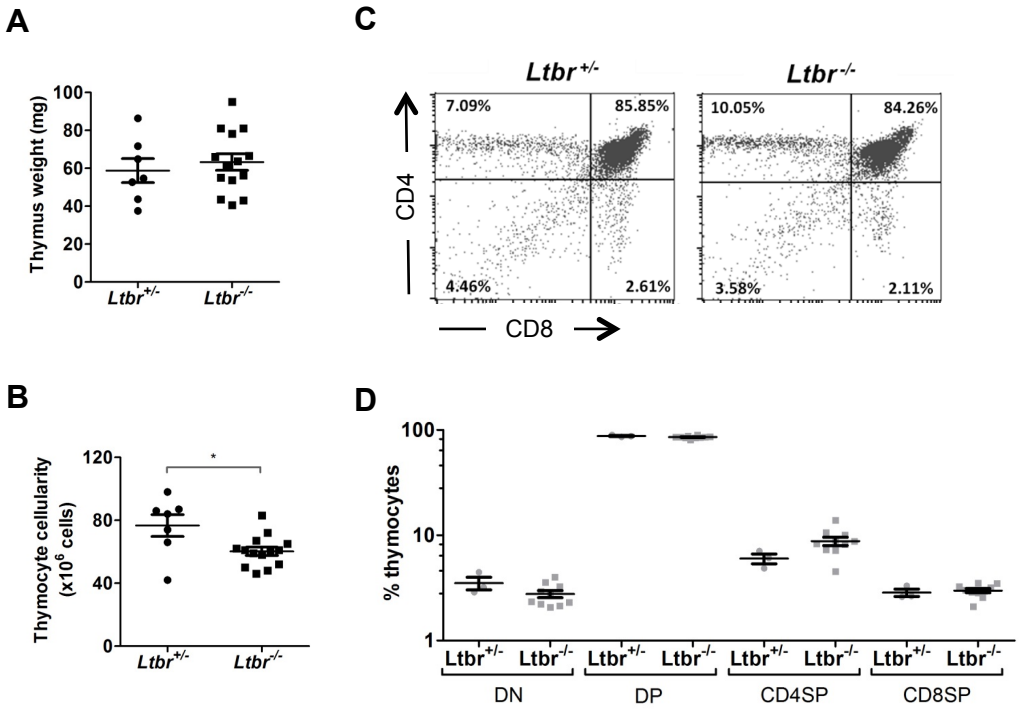


Figure 3.12. *Ltbr* deficiency does not cause major thymic alterations regarding thymocyte subpopulations in 4-5 month-old mice. (**A**) Thymic weight and (**B**) thymocyte cellularity from *Ltbr*$^{+/-}$ (n=7) and *Ltbr*$^{-/-}$ (n=14) mice aged from 19 to 22 weeks. The results are presented as the mean ± SEM. (**C**, **D**) Flow cytometry double immunostaining of cell surface CD4 and CD8 co-receptors on (**C**) representative thymocyte samples from either *Ltbr*$^{+/-}$ and *Ltbr*$^{-/-}$ mice, and (**D**) thymocytes from mice of the indicated genotype. The percentage of each thymocyte subpopulation, DN (CD4^{-}CD8^{-}), DP (CD4^{+}CD8^{+}), CD4SP (CD4^{+}CD8^{-}), and CD8SP (CD4^{-}CD8^{+}), is indicated and presented as the mean ± SEM. *P*-value determined using two-tailed, unpaired Student´s *t*-test.

3.3.3. LTβR inactivation does not affect the malignant phenotype of end-stage diseased TEL-JAK2 transgenic mice

Since *Ltbr* expression affected TEL-JAK2-induced leukemogenesis, we set out to characterize the malignant phenotype of end-stage diseased mice with or without *Ltbr* inactivation to identify possible alterations that could explain the differences in disease development observed for the two mouse cohorts. All TEL-JAK2;*Ltbr*$^{-/-}$ and TEL-JAK2;*Ltbr*$^{+/-}$ diseased mice analyzed showed typical features of disseminated T-cell leukemia, including thymic enlargement, frequently associated with pleural effusion, splenomegaly, and often non-lymphoid organ invasion (e.g., liver and lungs) (Figure 3.13A and data not shown). Macroscopic examination allowed us to divide these mice in two subgroups: some showed a predominant thymic enlargement accompanied by relatively mild splenomegaly, while others showed severe splenomegaly accompanied by relatively little

thymic enlargement. In addition, some showed high tumor burden in lymph nodes (Figure 3.13A). Notably, we found that *Ltbr* gene inactivation did not impact neither on the endpoint tumor load in lymphoid and visceral organs (Figure 3.13A), nor on the leukemic cell surface phenotype (expression of CD3, CD4, CD8, CD24 and CD25) characteristic of TJ2-Tg mice (dos Santos et al., 2007) (Figure 3.13B). The exception were lymph nodes, which were described to be absent in *Ltbr* knockout mice (Fütterer et al., 1998), and were also absent in TJ2-Tg;*Ltbr*$^{-/-}$ diseased mice (Figure 3.13A).

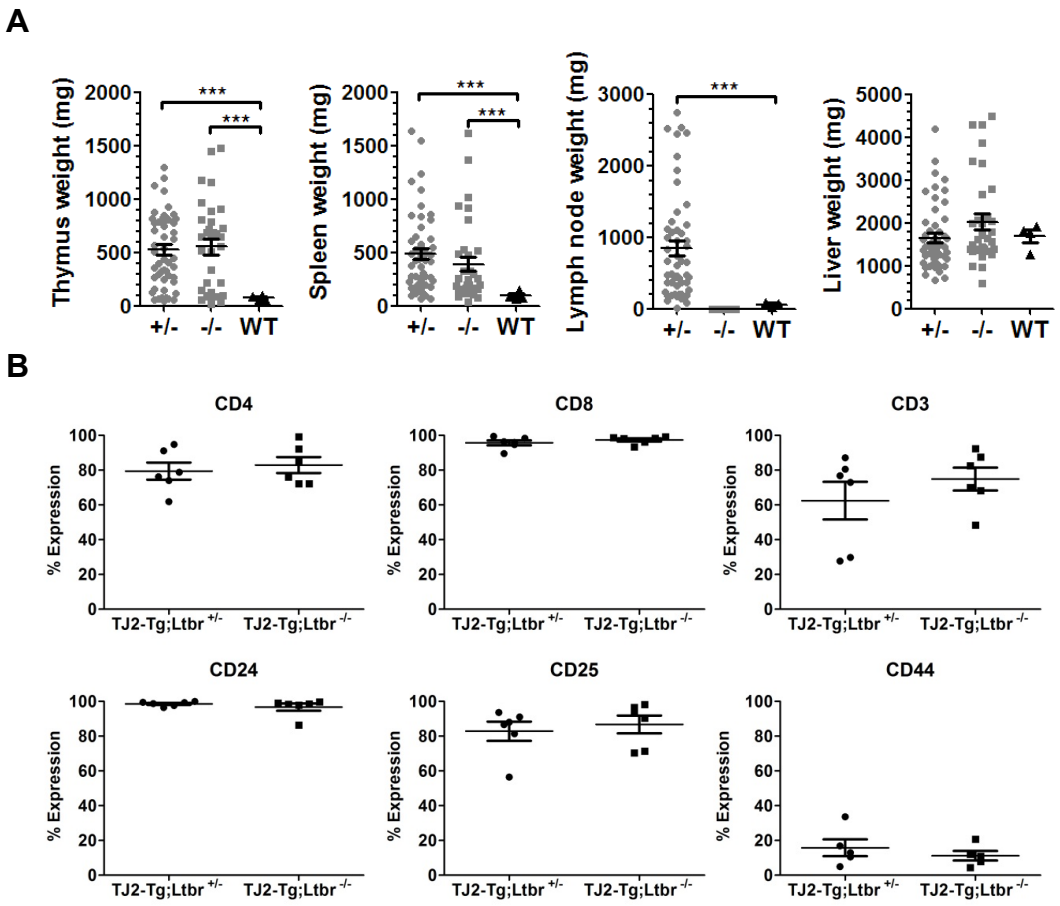


Figure 3.13. The malignant phenotype of end-stage diseased TJ2-Tg;*Ltbr*$^{-/-}$ leukemic mice is comparable to that of control TJ2-Tg;*Ltbr*$^{+/-}$ mice. (**A**) Plots representing the weights of the thymus, spleen, liver and lymph nodes (mandibular, axillary, subiliac and jejunal) collected from terminally ill TJ2-Tg;*Ltbr*$^{+/-}$ (+/-; n=51), TJ2-Tg;*Ltbr*$^{-/-}$ (-/-; n=33), and wild-type (WT; n=4) mice. (**B**) Flow cytometry cell surface immunostaining of CD4, CD8, CD3, CD24, CD25, and CD44 cell surface markers of six representative leukemic cell samples from either TJ2-Tg;*Ltbr*$^{+/-}$ or TJ2-Tg;*Ltbr*$^{-/-}$ mice. The percentage of cells expressing each marker in each sample is indicated by the mean ± SEM. *P*-values were determined using two-tailed, unpaired Student´s *t*-test with Welch´s correction.

Moreover, immunofluorescence analyses of medullary (Krt5), cortical and medullary (Krt8) and endothelial (CD31) markers showed that both diseased TEL-JAK2;*Ltbr*$^{-/-}$ and TEL-JAK2;*Ltbr*$^{+/-}$ mice presented increased vascularization and microstructural disorganization in the thymus, which did not differ significantly (data not shown).

Altogether, these analyses demonstrate that TEL-JAK2;*Ltbr*$^{-/-}$ and TEL-JAK2;*Ltbr*$^{+/-}$ mice do not show great differences regarding the malignant phenotype at end-stage disease. Therefore, the potential mechanisms fostering leukemogenesis in LTβR-proficient mice may operate at an early stage of leukemia development.

3.3.4. Lymphotoxin signaling fosters the early stages of leukemogenesis in the thymus

We have found that T-cell leukemogenesis is delayed in TJ2-Tg LTβR-deficient mice (Figure 3.10A), though LTβR signaling may play a role in leukemia initiation and/or maintenance. To investigate the timing of lymphotoxin gene upregulation during leukemogenesis, we analyzed gene expression in thymocytes from 8-week-old TJ2-Tg mice with no signs of disease and from nontransgenic littermates. Young TJ2-Tg mice were characterized by the expansion of CD8 SP and DP thymocyte subpopulations and aberrant $CD8^+CD25^+$ thymocytes (also characterized by elevated CD24 expression) at variable proportions (Figure 3.14A and data not shown), before significant thymic enlargement (Figure 3.14B,C). The appearance of these aberrant thymocytes was associated with increased overall cell size as demonstrated by FSC-H increase in flow cytometry analyses (Figure 3.14D). Moreover, the increase in malignant thymocytes was associated with higher *Lta* and *Ltb* expression, as compared to thymocytes from TJ2-Tg mice with small proportions of $CD8^+CD25^+$ cells or from nontransgenic controls (Figure 3.14E,F). Concerning *Light*, a significant increase in expression was not detected (data not shown). Therefore, these data indicate that increased lymphotoxin gene expression occurs early in TJ2-Tg-induced leukemogenesis, and support the notion that lymphotoxin signaling promotes leukemogenesis at the initial steps of disease. To test this assumption, we searched for malignant ($CD8^+CD25^+$) thymocytes in 8-week old TJ2-Tg mice of different *Ltbr* genotypes that showed no signs of disease. We found that the proportion of malignant thymocytes in the thymus was significantly reduced in *Ltbr*-deficient mice, as compared to *Ltbr*-proficient mice (Figure 3.14G). These results indicate that when LTβR is inactivated, early leukemogenesis is impaired resulting in slower progression and leukemia onset.

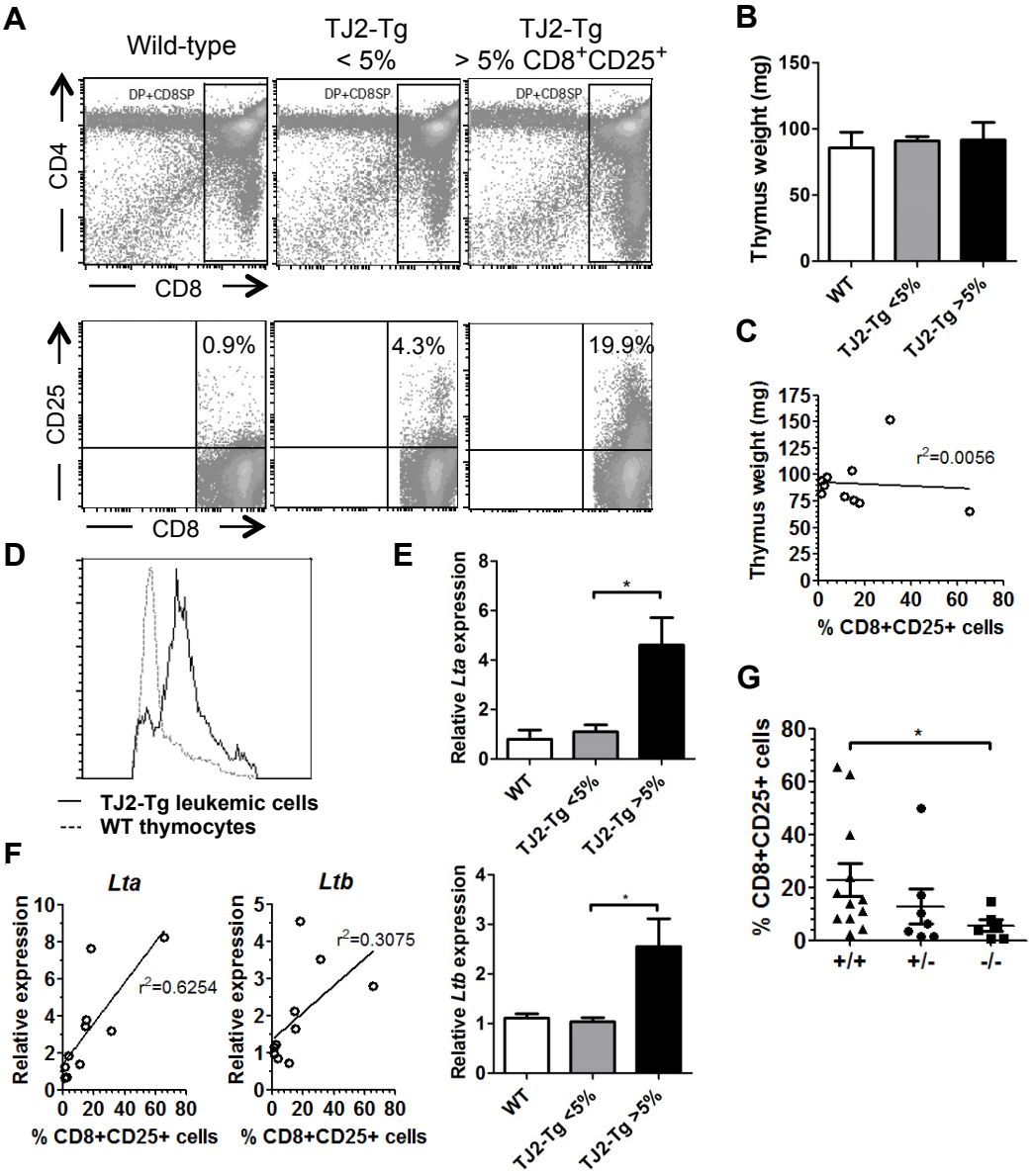


Figure 3.14. *Lta* and *Ltb* expression in TJ2-Tg thymocytes correlates with the emergence of malignant thymocytes. (**A**) CD4/CD8 (gated on viable cells) and CD8/CD25 (gated on CD4/CD8 DP plus CD8SP, viable cells) double cell surface immunostaining of representative thymocytes collected from 8-week-old wild-type (WT; n=5) mice and asymptomatic TJ2-Tg mice with less (n=4) or more than 5% $CD8^+CD25^+$ malignant thymocytes (n=6) and (**B**) the respective thymic weights. (**C**) Percentage of $CD8^+CD25^+$ thymocytes in TJ2-Tg mice (n=10) plotted with thymic weights. A possible correlation was assessed by determining the Spearman coefficient and the *P* value (*P* = 0.4047). (**D**) Thymocyte size from WT and TJ2-Tg representative mice given by forward scatter measurement. (**E**) RT-qPCR analysis of *Lta* and *Ltb* expression in thymocytes collected from 8-week-old WT (n=5) mice, asymptomatic TJ2-Tg mice with less (n=4) or more than 5% $CD8^+CD25^+$ malignant thymocytes (n=6). Results are normalized to *Gapdh* expression and presented as the mean ± SEM. *P*-value determined using two-tailed unpaired *t*-test with Welch´s correction. (**F**) *Lta* and *Ltb* mRNA expression levels plotted with the percentage of $CD8^+CD25^+$ thymocytes from 8-week-old asymptomatic TJ2-Tg mice (n=10) (mean of triplicates for each sample). The existence of possible correlations was assessed by

determining the Spearman coefficient and *P* values (*Lta*, $P = 0.0008$; *Ltb*, $P = 0.0174$). r^2 is a measure of goodness-of-fit of linear regression. (**G**) Proportion of malignant thymocytes (as identified by CD8 and CD25 positivity) in thymi from 8-week-old TJ2-Tg mice with the indicated phenotypes. Results are presented as the mean ± SEM. *P*-value determined using two-tailed, unpaired Student´s *t*-test with Welch´s correction.

3.3.5. LTβR is not required for TJ2-Tg leukemic cell engraftment and dissemination

Primary leukemic cells from TJ2-Tg mouse tumors can be transplanted to immunocompetent syngeneic recipient mice, resulting in bone marrow engraftment and invasion of lymphoid and visceral organs (Medyouf et al., 2007). To determine whether microenvironmental LTβR was also required for malignant cell leukemia engraftment and maintenance in recipient mice, TJ2-Tg leukemic cells were transplanted to $Ltbr^{+/-}$ and $Ltbr^{-/-}$ syngenic mice. When ill (after about 3-4 weeks), all recipient mice were sacrificed simultaneously for organ invasion assessment. Macroscopic and histological analyses of $Ltbr^{+/-}$ and $Ltbr^{-/-}$ recipients revealed similar dissemination to several organs including the thymus, spleen, liver, and bone marrow (Figure 3.15A,B), but not lymph nodes in $Ltbr^{-/-}$ recipients (data not shown).

To analyze the thymi from recipient mice in further detail, histological analyses were performed. Representative thymic sections stained by hematoxylin and eosin showed an altered structure and infiltration of leukemic cells in both $Ltbr^{-/-}$ and $Ltbr^{+/-}$ mice (Figure 3.15C). In addition, $Ltbr^{+/-}$ mice presented usually enlarged lymph nodes adjacent to the thymus (Figure 3.15C). Nevertheless, thymus-infiltrating leukemic cells could be identified in the vasculature and capsule of both mouse groups (data not shown). Immunofluorescence staining confirmed these observations and revealed that *Ltbr* deficiency did not impair the increased vascularization (CD31 endothelial cell staining) or altered thymic medullary structure (Krt5 epithelial cell staining) caused by leukemic cell infiltration of the thymus (Figure 3.15D). In addition, no differences were found between $Ltbr^{+/-}$ and $Ltbr^{-/-}$ infiltrated thymi regarding CCL21 chemokine expression, a well-known LTβR target (Figure 3.15E). Collectively, these results demonstrate that fully malignant leukemic cells invade equally well $Ltbr^{+/-}$ and $Ltbr^{-/-}$ recipient mouse organs. Therefore, LTβR expression and activation in stromal cells is seemingly not required for maintenance of TEL-JAK2-induced leukemia.

Taken together, the last results suggest that LTβR expression and activation in stromal cells likely plays a determinant and likely unique role in the early stages of TEL-JAK2-induced T-ALL.

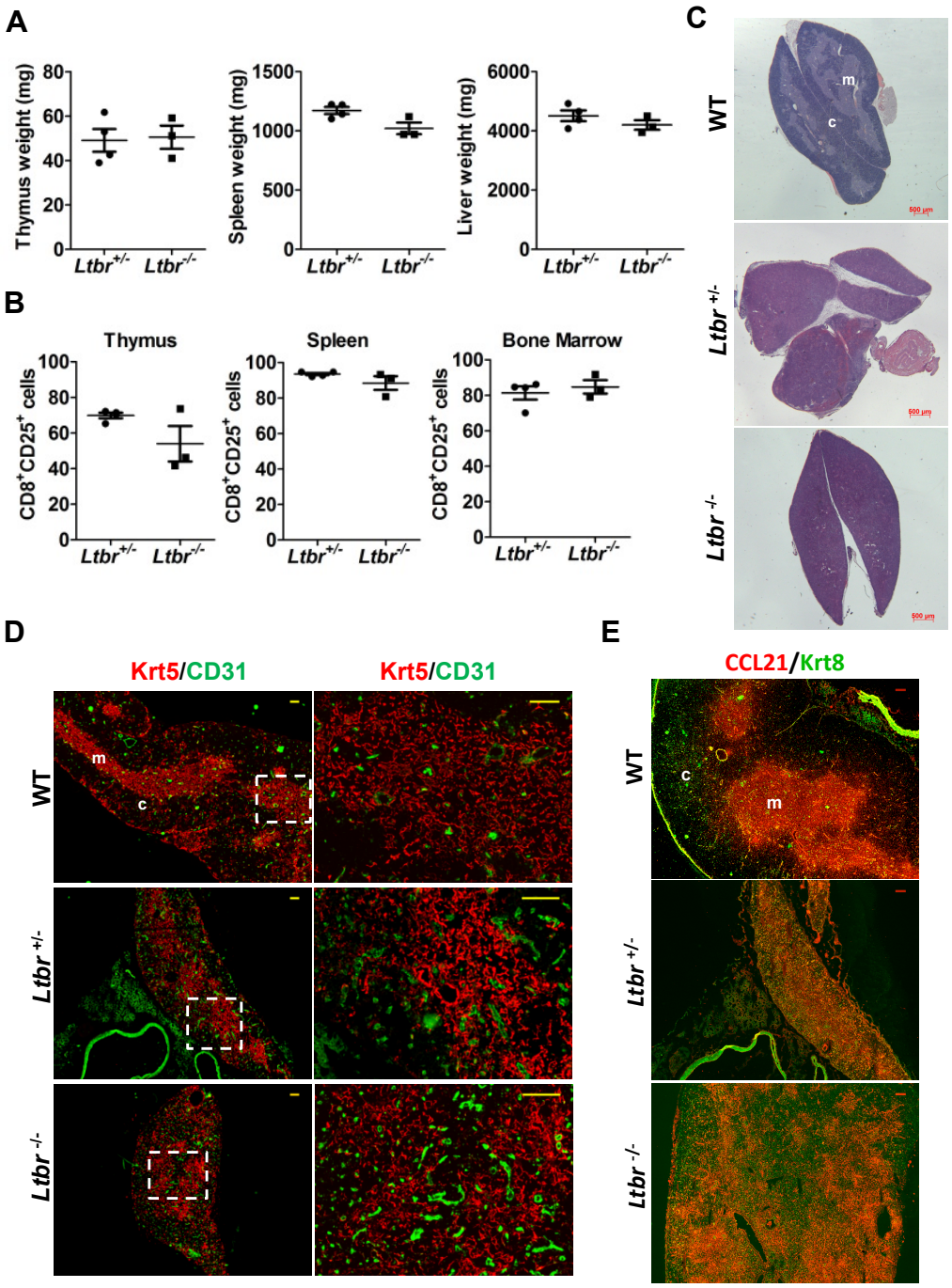


Figure 3.15. LTβR is not required for TJ2-Tg leukemia engraftment and dissemination in recipient mice. (**A**) Weight of organs collected from terminally ill *Ltbr*$^{+/-}$ (n=4) and *Ltbr*$^{-/-}$ (n=3) mice, 3 weeks post-inoculation with TJ2-Tg leukemic cells. Representative results of 4 independent experiments are presented as mean ± SEM. (**B**) Percentage of CD8^{+}CD25^{+} leukemic cells in organs from *Ltbr*$^{+/-}$ and *Ltbr*$^{-/-}$ recipient mice, as assessed by flow cytometry. (**C**) Hematoxylin and eosin staining of thymi collected from wild-type mice, and *Ltbr*$^{+/-}$ and *Ltbr*$^{-/-}$ mice injected with TJ2-Tg leukemic cells. Scale bar 500 µm. (**D**-**E**) Immunostaining of (**D**) CD31 and keratin 5 (Krt5), and (**E**) CCL21 and keratin 8 (Krt8) in representative thymi from leukemic *Ltbr*$^{+/-}$ and *Ltbr*$^{-/-}$ recipient mice. Scale bars, 100 µm. m, medulla; c, cortex.

3.3.6. LTβR pharmacological block impacts on TJ2-Tg leukemic cell export from the thymus and dissemination

Since *Ltbr* knockout mice are born with no lymph nodes and other specific lymphoid defects, we sought to inhibit lymphotoxin signaling in phenotypically normal young, pre-leukemic TJ2-Tg mice through the administration of neutralizing LTβR-Fc fusion protein. Substantiating our previous results in which LTβR was genetically inactivated, LTβR-Fc treatment delayed leukemogenesis, with half of the mice outliving control-treated mice (Figure 3.16A). The median survival in treated mice was 15 versus 13 weeks in control mice; therefore, we could delay the median survival of TJ2-Tg mice in 2 weeks by blocking LTβR signaling from 5 until 10 weeks of age (Figure 3.16A). In addition, acute inhibition of lymphotoxin signaling resulted also in increased tumor burden in the thymus (Figure 3.16B) and a tendency for reduced infiltration in other organs such as the spleen, LNs (Figure 3.16B), and BM (Figure 3.16C), suggesting that leukemic cell export from the thymus was reduced. Moreover, white blood cell counts in peripheral blood were generally lower in treated mice (Figure 3.16D). This finding suggests that less leukemic cells were circulating in the peripheral blood, the main route for T-ALL dissemination.

In synthesis, although the low number of mice analyzed may have precluded the finding of statistically significant differences, LTβR signaling abrogation through pharmacological means may influence bloodborne leukemic cell dissemination to distant metastatic sites from the thymus, where it originates.

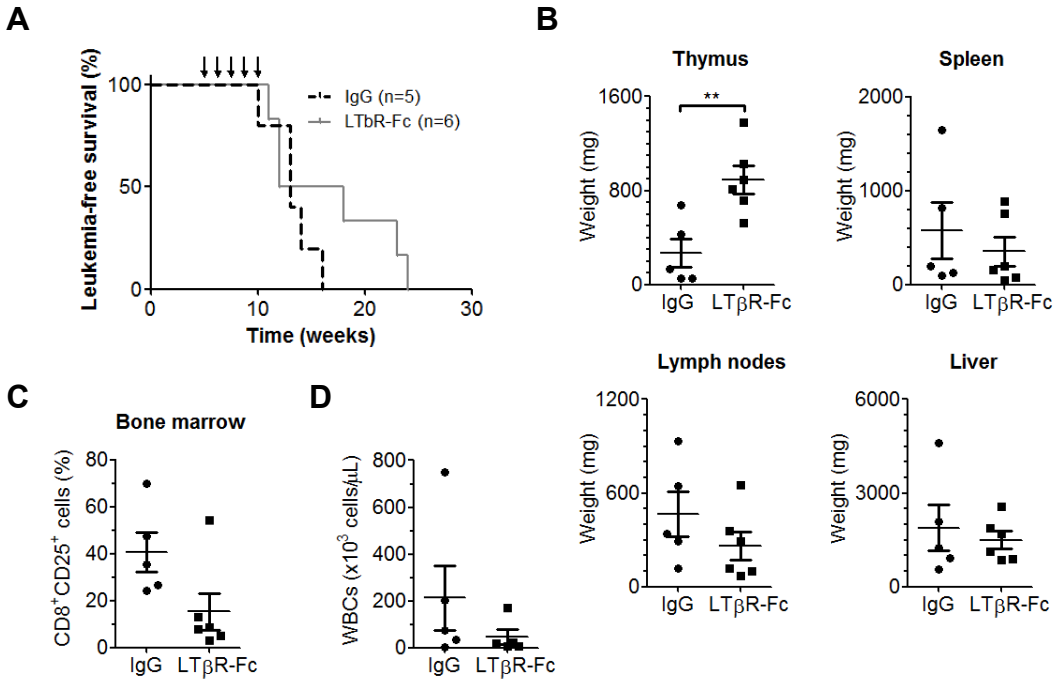


Figure 3.16. Pharmacological inhibition of LTβR signaling impairs leukemogensis in TJ2-Tg mice. TJ2-Tg mice received intraperitoneal injections of LTβR-Fc (or mouse polyclonal IgG as control) once a week for 5 weeks, starting at 5 weeks of age (arrows). (**A**) Kaplan-Meier survival curves for LTβR-Fc- and control-treated mice (P = 0.2693, log-rank Mantel Cox test). (**B**) Weights of thymus, spleen, liver and lymph nodes, (**C**) white blood cell (WBC) counts, and (**D**) leukemic cell ($CD8^+CD25^+$) bone marrow infiltration (P = 0.0553) in terminally ill LTβR-Fc- and control-treated TJ2-Tg mice. P-value determined using two-tailed, unpaired Student´s t-test.

3.4. LYMPHOTOXIN EXPRESSION AND REGULATION IN HUMAN T-ALL

3.4.1. Human T-ALL cell lines express lymphotoxin through an NF-κB-dependent mechanism

To determine if LTβR ligands are also expressed in human T-ALL, *LTA* and *LTB* mRNA levels were first assessed in a panel of established human cell lines. These genes were expressed at variable levels across all T-ALL cell lines analyzed (Figure 3.17A). Furthermore, these were found to be highly expressed in the HUT-78 cutaneous T-cell lymphoma cell line and weakly expressed in the Nalm6 B-cell precursor leukemia cell line

(Figure 3.17A). In contrast to LT genes, no significant *LIGHT* expression was detected in any cell line (data not shown).

By performing flow cytometry analysis, variable expression of LTβR ligands was also detected at the surface of all T-ALL cell lines studied, but not on the Nalm6 cell line (Figure 3.17B). Although we did not have tools to analyze which LTβR ligand proteins were expressed in T-ALL cell lines, these were most likely $LT\alpha_1\beta_2$ heterotrimers due to the lack of detection of *LIGHT* mRNA. Importantly, none of the T-ALL cell lines analyzed expressed LTβR as determined by western blotting (data not shown).

To study the mechanism(s) of LTα and LTβ expression in human T-cell leukemia/lymphoma, we determined the impact of NF-κB activation using PMA, and NF-κB inhibition using an IKK inhibitor. Similarly to TJ2-Tg leukemic cells, PMA stimulation induced cell surface LTβR ligand expression in an IKK-dependent manner in T-ALL cell lines but not in the Nalm6 B-cell line (Figure 3.17D). In addition, *LTA* and *LTB* mRNA expression was found to be upregulated by PMA activation and downregulated by IKK inhibition in T-ALL cell lines (Figure 3.17C). Collectively, these experiments show that LTβR ligand expression can be found in both mouse and human leukemic T cells and these are similarly regulated. Furthermore, these data confirmed previous reports obtained with other T-ALL cell lines (Ware et al., 1992) indicating that the lymphotoxin genes and $LT\alpha_1\beta_2$ heterotrimers are expressed in human T-ALL cells and regulated by the activation of signaling pathways including the IKK/NF-κB pathway.

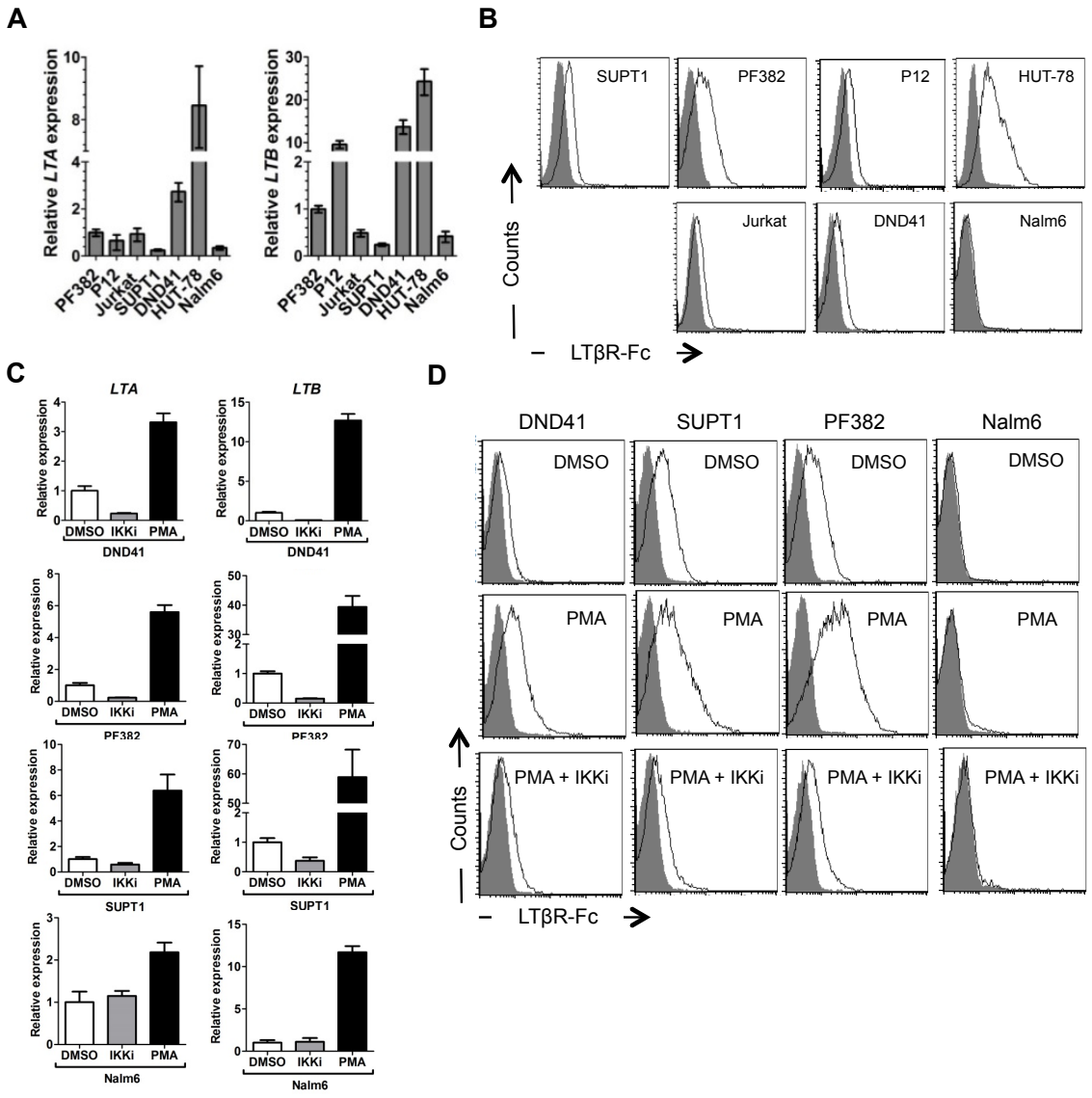


Figure 3.17. LTβR ligand expression in human T-ALL cell lines is induced by PMA stimulation and inhibited by IKK inhibitor treatment. (**A**) RT-qPCR quantification of *LTA* and *LTB* (normalized to *GAPDH* expression) in the indicated human T-ALL cell lines and non-T-ALL (HUT-78 and Nalm6) cell lines. Results are represented as the mean ± range. (**B**) Cell surface immunostaining with LTβR-Fc (solid line) and the secondary antibody alone (gray shading) by flow cytometry on the indicated cell lines. (**C**) *LTA* and *LTB* mRNA quantification by RT-qPCR on DND41, SUPT1, PF382, and Nalm6 cell lines treated for 10 h with either IKK inhibitor (IKKi), PMA or DMSO (control). (**D**) Flow cytometry cell surface immunostaining with LTβR-Fc (solid line) and secondary antibody alone (gray shading) on DND41, SUPT1, PF382, and Nalm6 cell lines treated for 10 h with either PMA, PMA plus IKKi, or DMSO.

3.4.2. Human primary patient samples express lymphotoxin

LTβR ligands have been previously shown to be expressed in malignant cells and contribute to chronic lymphocytic leukemia (CLL) and B-cell lymphoma development (Heinig et al., 2014; Rehm et al., 2011). Moreover, analysis of a publically available large multicenter dataset of 2022 leukemia patient and 74 nonmalignant BM samples (Haferlach et al., 2010), showed that *LTA* and *LTB* genes were significantly more expressed in T-ALL and other lymphoid malignancies than in myeloid malignancies or nonmalignant BM (Figure 3.18A).

To determine whether *LTA* and *LTB* were also expressed in primary T-ALL cells, we performed RT-qPCR on pediatric patient samples collected at diagnosis from either BM or PB and on age-matched human control thymocytes (Table 3.1). *LTA* and *LTB* mRNA expression was found in normal thymocytes (as expected (Wolf and Cohen, 1992)) and patient samples, albeit heterogeneously in the latter. Some patient samples expressed similar or higher, while others lower levels than thymocytes (Figure 3.18B). Conversely, no significant *LIGHT* expression was detected in T-ALL primary samples (data not shown).

To determine whether lymphotoxin gene expression was associated with particular T-ALL molecular subgroups (Graux et al., 2006), *LTA* and *LTB* gene expression was assessed in a larger cohort of 49 primary T-ALL patients by microarray analysis followed by RT-qPCR validation in a subset of 33 patients. Molecular subtypes were classified by Hierarchical Clustering Analysis (HCA) according to the expression of genes previously associated with each subtype (Table 3.2) (Homminga et al., 2011). The *LTA* and *LTB* genes were expressed at variable levels in the different molecular subtypes, but expression of *LTA* and most strikingly *LTB* was significantly higher in the TAL/LMO molecular subgroup than in the remaining samples (Figure 3.18C). RT-qPCR analyses corroborated that *LTA* and *LTB* were on average more expressed in samples from the TAL/LMO than other subgroups (Figure 3.18D and Table 3.2). No further correlations between LT gene expression and clinical or molecular data were found.

Together, these data indicate that lymphotoxin expression is a common feature in T-ALL with higher levels associated with TAL/LMO T-ALL. Furthermore, either LT expression does not correlate with any clinical characteristic of the disease studied, or our cohort is not large enough to account for the variability found when studying these potential effects.

Table 3.1. Clinical and immunophenotypical characteristics of primary T-ALL samples (Portuguese cohort).

Patient	Age at diagnosis (years)	Sample[a]	Gender	Immunophenotype[b]												Relative expression[c]	
				CD1a	CD2	CD3	CD4	CD5	CD7	CD8	CD10	CD33	CD34	CD117	TdT	*LTA*	*LTB*
46	4	BM	F	88%	14%	90%	78%	90%	88%	85%	n.d.	3%	0.3%	n.d.	88%	0.06	0.44
97	4	PB	M	66%	+	n.d.	-	+	+	-	n.d.	n.d.	66%	n.d.	66%	0.47	4.42
111	6	BM	F	-	+	n.d.	-	-	n.d.	-	n.d.	-	+	+	+	0.05	0.53
112	10	BM	M	-	n.d.	n.d.	-	-	+	-	n.d.	n.d.	n.d.	+	-	0.70	4.56
115	4	BM	M	+	+	n.d.	-	+	+	+	+	n.d.	-	+	+	0.06	0.10
123	4	BM	F	-	+	-	-	+	+	-	+	n.d.	25%	n.d.	+	0.22	0.22
178	13	PB	M	-	+	-	-	+	+	-	+	-	-	-	+	0.99	0.83
179	4	PB	M	n.d.	+	-	-	+	+	-	-	-	-	-	+	1.54	2.47

[a] Samples collected from bone marrow (BM) or peripheral blood (PB).
[b] All cases were positive for cytoplasmic CD3. n.d., no data. Percentage of positive cells indicated when available.
[c] *LTA* and *LTB* expression by RT-qPCR relative to expression in normal human thymocytes, which was arbitrarily set to 1 (normalized to *GAPDH* expression).

Table 3.2. Clinical, immunophenotypical and genetic characteristics of primary T-ALL samples clustered in molecular subtypes (Brazilian cohort).

	Cohort	%	HOXA	%	Immature	%	Proliferative	%	TAL/LMO	%	TLX1/3	%
	33		3		4		4		14		8	
Clinical												
Total (n)	22		2		4		4		13		7	
Gender												
Male	15	68	2	100	3	75	1	25	11	85	4	57
Female	7	32	0	0	1	25	3	75	2	15	3	43
Age[a]												
Median	11.0		12.5		9.1		7.8		12.0		8.7	
Range	1.7-17.6		11.5-13.5		1.7-22.6		2.9-16.2		3.4-17.6		6.7-15.6	
WBC (^10^4/mm^3)												
Median	14.55		21.845		15.48		33.495		26.5		13.2	
Range	1.79-98		1.99-41.7		6.51-62.1		2.02-98		5.28-61.2		1.79-36.8	
Immunophenotype[b]												
CD34 (n)	30		1		4		3		14		8	
Positive	10	33	0	0	2	50	0	0	6	43	2	25
CD33 (n)	29		2		3		4		13		7	
Positive	2	7	0	0	0	0	0	0	1	8	1	14
CD1a (n)	20		1		3		1		10		5	
Positive	12	60	1	100	1	33	1	100	4	40	5	100
CD2 (n)	32		2		4		4		14		8	
Positive	29	91	1	50	3	75	3	75	14	100	8	100
CD3 (n)	32		2		4		4		14		8	
Positive	32	100	1	50	4	100	4	100	13	93	4	50
CD4 (n)	20		1		3		1		10		5	
Positive	16	80	1	100	1	33	1	100	7	70	5	100
CD8 (n)	20		1		3		1		10		5	
Positive	16	80	0	0	2	67	1	100	9	90	4	80
CD4/8 (n)	10		1		1		1		5		2	
Positive	7	70	0	0	1	100	1	100	5	100	0	0
CD5 (n)	32		2		4		4		14		8	
Positive	31	97	2	100	3	75	4	100	14	100	8	100
CD7 (n)	32		2		4		4		14		8	
Positive	32	100	2	100	4	100	4	100	14	100	8	100
CD10 (n)	31		1		4		4		14		8	
Positive	11	35	1	100	1	25	3	75	2	14	4	50
TdT (n)	11		0		2		1		7		2	
Positive	11	100			2	100	1	100	7	100	2	100
Genetic alterations												
NOTCH (n)	23		2		1		4		9		6	
Positive	11	48	2	100	0	0	4	100	2	22	3	50
WT1 (n)	22		2		1		4		9		5	
Positive	1	5	0	0	0	0	0	0	0	0	1	20
PTEN (n)	23		2		1		4		9		6	
Positive	2	9	0	0	0	0	0	0	1	11	1	17
IL7R (n)	22		2		1		4		9		5	
Positive	5	23	0	0	0	0	0	0	1	11	3	60
Lymphotoxin expression												
Total (n)	33		3		4		4		14		8	
LTA												
Median	5.1		1.8		5.3		2.4		7.9		1.5	
Mean	6.1		7.8		5.0		3.5		8.2		2.6	
Range	0-20.7		0.9-20.7		0.6-8.8		0.0-9.3		0-20.1		0-8.5	
LTB												
Median	179.6		194.6		142.8		115.6		376.7		65.6	
Mean	264.7		179.1		165.0		121.6		403.9		106.4	
Range	9.7-796.5		128.1-214.7		45.6-328.8		9.7-245.5		101.7-796.5		9-291.7	

[a]Age at diagnoses in years.
[b]Positivity was considered when 20% or more cells were positive for each marker.

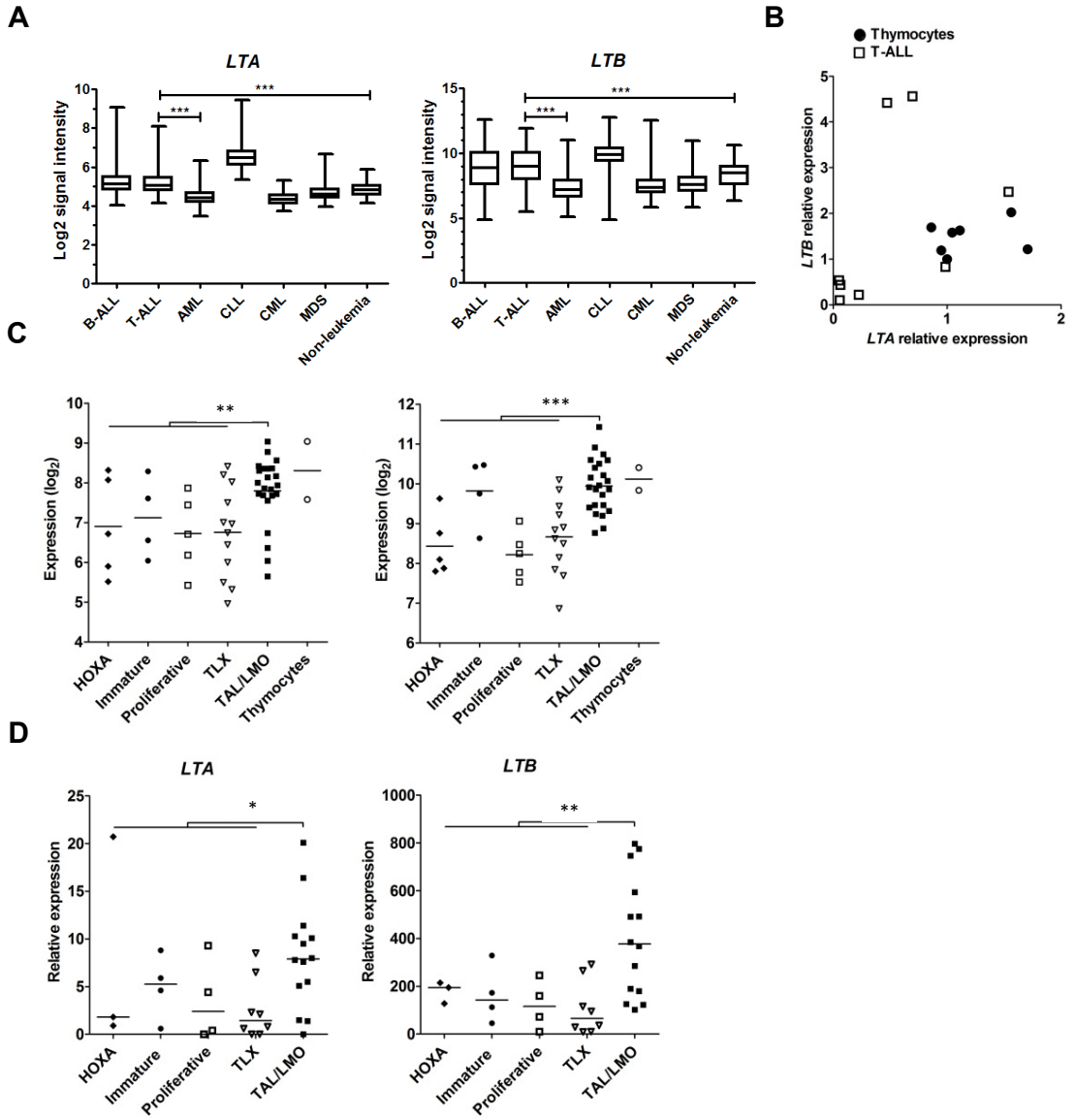


Figure 3.18. T-ALL patient samples express lymphotoxin genes. (**A**) Microarray (GEO GSE 13159) analysis of *LTA* and *LTB* gene expression levels in several human samples: B-ALL, B-cell acute lymphoblastic leukemia; T-ALL, T-cell acute lymphoblastic leukemia; AML, acute myeloid leukemia; CLL, chronic lymphocytic leukemia; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; Non-leukemia, non-leukemia and healthy bone marrow samples. Lines, bars, and whiskers represent the median, quartiles, and minimum and maximum values, respectively. (**B**) RT-qPCR quantification of *LTA* and *LTB* mRNA levels in human thymocytes (n=7) and T-ALL samples (n=8). Results were normalized to 18S rRNA expression and are expressed as the mean of three technical replicates. (**C**) *LTA* and *LTB* gene expression, as determined by microarray analysis, in 49 T-ALL samples, classified in the HOXA (n=5), immature (n=4), proliferative (n=5), TLX (n=12), and TAL/LMO (n=23) molecular subtypes, and 2 human thymocyte samples. Results are represented as the mean (bar). (**D**) RT-qPCR quantification of *LTA* and *LTB* in 33 T-ALL patient samples (n=8) subdivided according to molecular subtype, normalized to *ABL1* expression and relative to expression in Jurkat cells. Results are expressed as the mean (bar). For (A), (C) and (D): *P*-values determined using two-tailed, unpaired Student´s *t*-test with Welch's correction.

3.4.3. TEL-JAK2 transgenic mice model human T-ALL with cortical/mature immunophenotype

The TAL/LMO molecular subtype of T-ALL has been associated with TCRαβ differentiation, cell surface expression of TCRαβ and CD3, and cortical/mature immunophenotype (Asnafi et al., 2004; Ferrando et al., 2002; Graux et al., 2006; Soulier et al., 2005). Moreover, TAL/LMO samples were shown to cluster preferentially with post-β-selection human thymocyte subsets, including immature CD4 SP, CD4/CD8 DP, and mature CD4 and CD8 SP cells (Soulier et al., 2005). TJ2-Tg mouse leukemias are characterized by the presence of CD4/CD8 DP and CD8 SP leukemic cells expressing surface TCRαβ, CD3 and CD5, and responsive to TCR stimulation (Carron et al., 2000; dos Santos et al., 2007). Furthermore, unsupervised clustering analysis of four TJ2-Tg leukemic samples and nine thymocyte subpopulations revealed that TJ2-Tg cells clustered with double negative stage 4 (DN4), CD8 ISP and DP thymocytes (Figure 3.19). These results indicate that TJ2-Tg mouse leukemias, like most TAL/LMO T-ALL cases, are malignancies of late cortical, post-β-selection thymocytes, and suggest that lymphotoxin signaling contributes for TAL/LMO T-ALL development as it does for TJ2-Tg-induced leukemias.

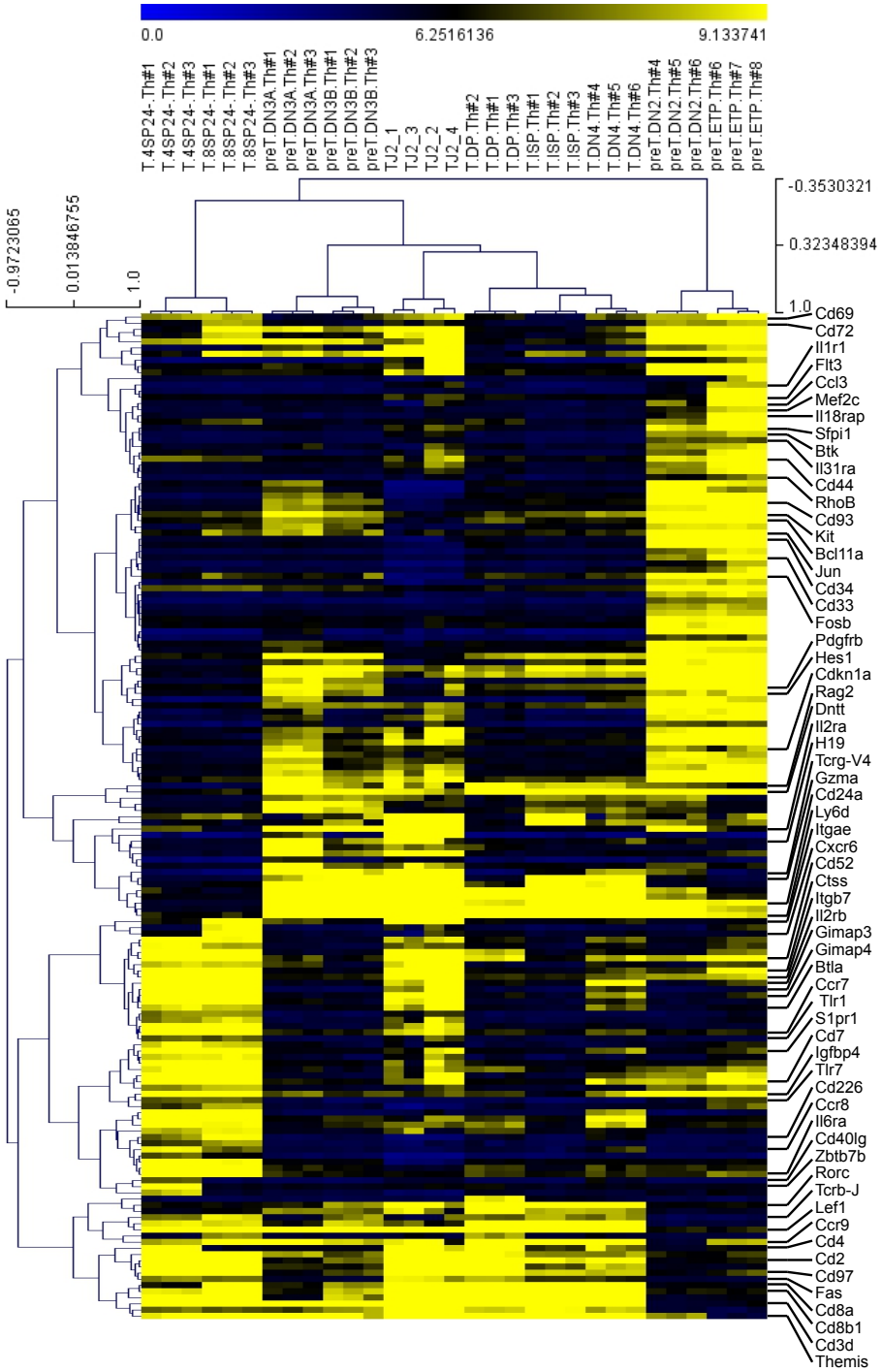


Figure 3.19. TJ2-Tg leukemic cells clustered with post-β-selection immature thymocytes. Unsupervised hierarchical clustering of four TJ2-Tg samples (TJ2) (GEO GSE51243), and nine wild-type thymocyte subpopulations, in triplicate, from the ImmGen consortium (GSE15907) using the top 1 % most varying probe sets (n=163) across all 31 datasets (mainly immune-related membrane-bound proteins). Hues of yellow and blue indicate higher to lower levels of relative fluorescence signal. Thymocyte subpopulations starting from the most immature are: preT.ETP, early T lineage precursor; preT.DN2, DN2 thymocytes; preT.DN3A, DN3A thymocytes; preT.DN3B, DN3B thymocytes; T.DN4, double negative (DN) 4 thymocytes; T.ISP, intermediate SP, $CD3^{-}$ $CD8^{+}$; T.DP, CD4/CD8 double positive; $T.4SP24^{-}$, CD4 single-positive (SP), mature; $T.8SP24^{-}$, CD8 SP, mature.

3.5. ROLE OF RANK SIGNALING IN T-ALL: PRELIMINARY FINDINGS

TNF superfamily receptors expressed in thymic stromal cells, including LTβR, CD40 and RANK, and their cognate ligands, expressed in developing T cells, are essential players in thymocyte-stroma molecular crosstalk. In some processes, these receptors cooperate, while in others, show redundant functions due to their shared ability to activate the alternative NF-κB pathway (Akiyama et al., 2012). The above results suggest that disruption of lymphotoxin signaling dalays TJ2-Tg-induced leukemogenesis by hampering crosstalk between leukemic and stromal cells. As such, other TNFR superfamily members may be involved in this crosstalk. As a first approach to determine if CD40 or RANK may cooperate with LTβR signaling in T-ALL development, we addressed whether the respective ligands are also expressed in TJ2-Tg leukemic cells. RANK ligand gene (*Rankl*) was significantly more expressed in TJ2-Tg leukemic cells that in wild-type thymocytes (Figure 3.20A). In contrast, no CD40 ligand gene (*Cd40l*) expression was detected in TJ2-Tg leukemic cells (data not shown). Interestingly, a correlation between *Lta* and *Rankl* expression was found in TJ2-Tg leukemic cells (Figure 3.20B), suggesting that these may be activated by the same mechanisms. It was previously reported that in the mouse thymus, RANK is mainly expressed in mTECs, while mature CD4 SP thymocytes are the main source of RANK ligands (Hikosaka et al., 2008). Although TJ2-Tg leukemic cells include immature CD8 SP and DP cell populations in variable proportions, we found that these cells expressed high levels of *Rankl* mRNA, comparable to those in CD4 SP thymocytes (Figure 3.20C). Furthermore, *Rank* was detected in whole TJ2-Tg thymic lymphomas as it was in whole wild-type thymi (Figure 3.20D), despite the accentuated decrease in mTEC proportion observed as the lymphoma develops (data not shown). These observations suggest that, similarly to what was previously reported in the wild-type thymus (Hikosaka et al., 2008), RANK and its ligand are present in the microenvironment where TEL-JAK2-induced leukemia originates.

To determine the mechanism(s) underlying *Rankl* expression in TJ2-Tg leukemic cells, these were treated *in vitro* with pharmacological inhibitors as described before. Similarly to *Ltb* and *Light*, *Rankl* expression in leukemic cells was reduced by IKKi treatment (Figure 3.21A), while the pan-JAK inhibitor had no effect (Figure 3.21B). These results indicate that NF-κB activation is an important mechanism underlying TNF ligand family

gene expression (comprising *Lta*, *Ltb*, *Light*, and *Rankl*) in TJ2-Tg leukemic T cells, as well as in wild-type thymocytes (data not shown).

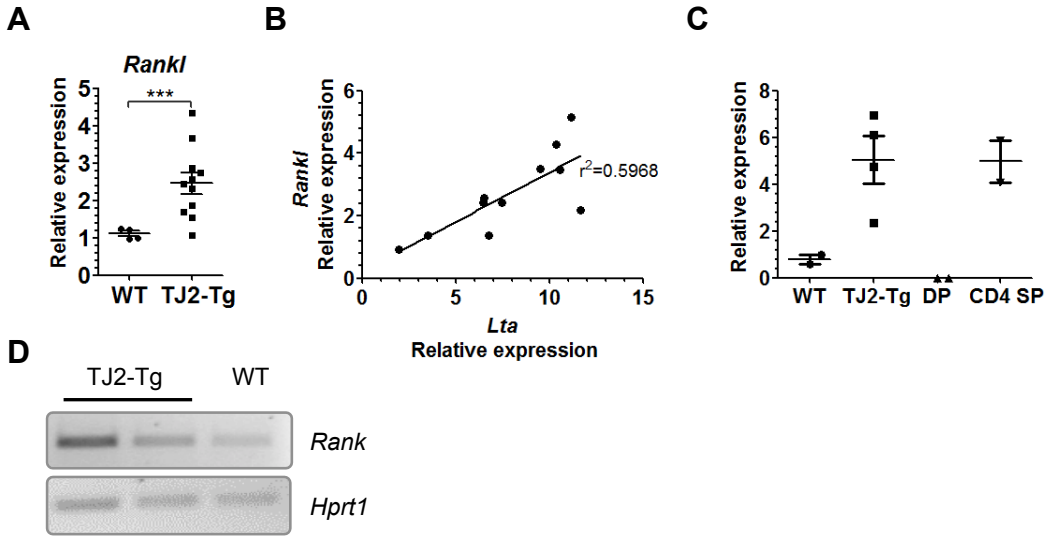


Figure 3.20. RANK and RANK ligand expression in TJ2-Tg mice. (**A**) RT-qPCR quantification of *Rankl* in thymocyte-enriched fractions collected from wild-type thymi (WT; n=4) and leukemic cell-enriched fractions collected from TJ2-Tg thymic lymphomas (n=11). *P*-value determined using two-tailed, unpaired Student´s *t*-test with Welch's correction. (**B**) *Rankl* relative expression plotted with *Lta* relative expression. r^2 is a measure of goodness-of-fit of linear regression. The existence of a significant correlation was assessed by determining the Spearman coefficient and the *P* value ($P = 0.0440$). (**C**) *Rankl* RT-qPCR analysis in WT thymocytes (n=2), TJ2-Tg leukemic cells (n=4), and sorted CD4/CD8 double-positive (DP; n=2) and CD4 single-positive (CD4 SP; n=2) thymocytes. (**D**) *Rank* expression detection by RT-PCR in whole thymic lymphomas from TJ2-Tg mice (n=2) and whole wild-type thymi (n=1). *Hprt1* expression was detected as loading control.

Further supporting a role for NF-κB activity in induction of *Rankl* expression in leukemic cells, this was upregulated in an NF-κB-dependent manner by PMA and ionomycin treatment (Figure 3.21C,D). Conversely, *Rankl* expression was similar in TJ2-Tg;*Rag2*$^{-/-}$ and pre-TCR/TCR-proficient TJ2-Tg leukemic cells (Figure 3.21E), supporting the notion that either TCR expression in leukemic cells may not contribute to *Rankl* gene regulation, unlike normal thymocytes (Hikosaka et al., 2008), or that other regulatory mechanisms operating in leukemic cells induce *Rankl* expression in the absence of pre-TCR or TCR signaling, possibly those underlying the high *Rankl* expression in DN1 thymocytes (Hikosaka et al., 2008).

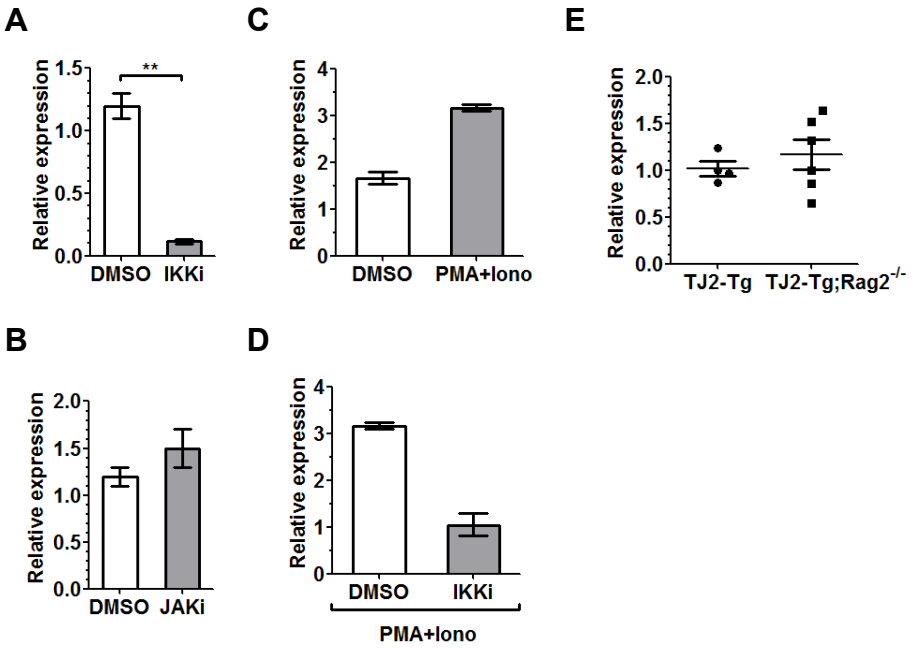


Figure 3.21. RANK ligand gene expression in TJ2-Tg leukemic cells depends on IKK activity. (**A**-**D**) *Rankl* mRNA quantification by RT-qPCR for three independent TJ2-Tg leukemic cell samples treated *ex vivo* for 10 h with either DMSO (control) and (**A**) an IKK inhibitor (IKKi), (**B**) a pan JAK inhibitor (JAKi), or (**C**) PMA and ionomycin (PMA+Iono). (**D**) *Rankl* expression was also determined in samples treated with PMA+Iono and PMA+Iono in combination with IKKi and one representative sample is shown. (**E**) RT-qPCR quantification of *Rankl* expression (normalized to *Gapdh* expression) in leukemic *Rag2*-deficient TJ2-Tg leukemic cells (TJ2-Tg;*Rag2*$^{-/-}$; n=6) and control leukemic cells (TJ2-Tg; n=4). Results are expressed as mean ± SEM. *P*-value determined using two-tailed, paired Student´s *t*-test.

Although *Rankl* mRNA was consistently expressed in TJ2-Tg leukemic cells, RANKL protein expression was absent or weak at the cell surface of freshly collected cells (*in vivo*), as detected by flow cytometry (Figure 3.22A). Upon *ex vivo* culture, TJ2-Tg leukemic cells showed slightly higher RANKL surface levels (Figure 3.22B), which were dramatically upregulated following PMA and ionomycin stimulation (Figure 3.22C).

The observed RANKL cell surface expression following *ex vivo* culture and PMA and ionomycin stimulation could result from a combination of *de novo* protein synthesis and protein subcellular relocalization. By blocking mRNA translation in leukemic cells with CHX, we found that RANKL cell surface induction upon either *ex vivo* culture or PMA/ionomycin stimulation depended on *de novo* protein synthesis (Figure 3.22B-C). Supporting our previous findings, we detected *Rankl* mRNA upregulation in leukemic cells

upon *ex vivo* culture (Figure 3.22D). Therefore, RANKL expression at the surface of TJ2-Tg leukemic cells upon *ex vivo* culture, and probably also after PMA plus ionomycin treatment, likely results from enhanced *Rankl* transcription and translation.

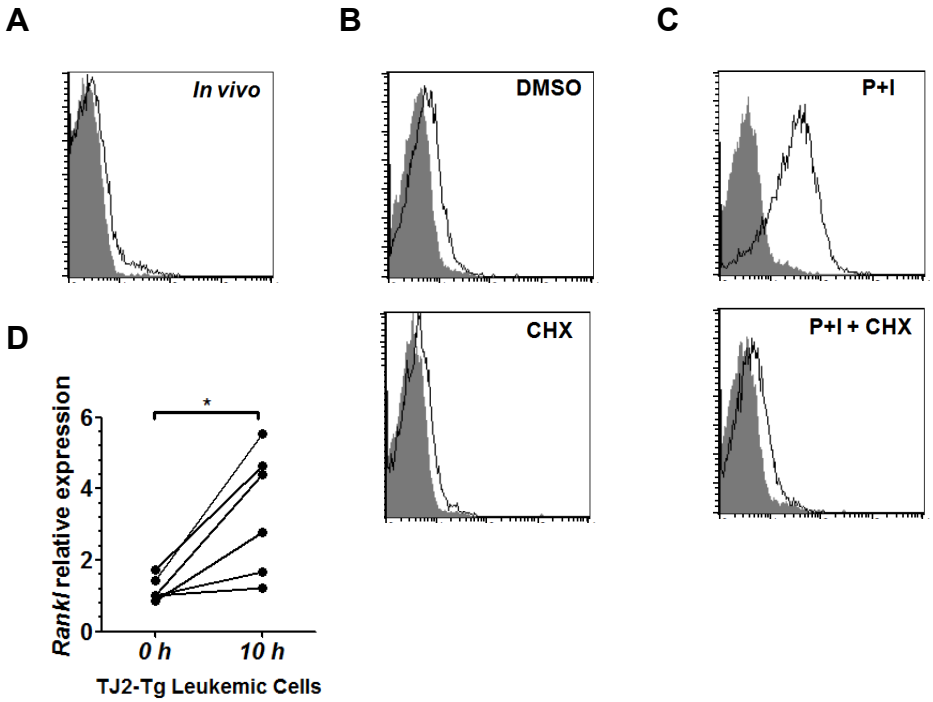


Figure 3.22. RANK ligand cell surface expression in TJ2-Tg leukemic cells is induced through NF-κB activation and depends on *de novo* protein synthesis. Flow cytometry immunostaining of RANKL (solid line) or isotype control (gray shading) of TJ2-Tg leukemic cells (**A**) freshly collected (*in vivo*), (**B**) cultured *ex vivo* with vehicle (DMSO) for 22h, and (**C**) treated with PMA+Iono, alone or in combination with cycloheximide (CHX). (**D**) RT-qPCR analysis of *Rankl* mRNA levels in TJ2-Tg leukemic cells freshly collected (0 h) or after *ex vivo* culture for 10 h (n=6). Results for each sample were normalized to *Gapdh* mRNA expression and are represented as the mean of triplicates. *P*-value determined using two-tailed, paired Student´s *t*-test.

CHAPTER 4

DISCUSSION
&
FUTURE PERSPECTIVES

4.1. TNFSF SIGNALING IN THE THYMUS OF TEL-JAK2 TRANSGENIC MICE

Under physiologic conditions, intercellular communication between developing thymocytes and the thymic stroma is critical for both thymocyte differentiation, maturation and selection, and thymic stroma development and organization. This crosstalk is largely mediated by ligand/receptor interactions between members of the Tumor Necrosis Factor Superfamily (TNFSF), such as $LT\alpha_1\beta_2$/LTβR, RANKL/RANK, and CD40L/CD40 (Akiyama et al., 2012; Boehm et al., 2003; Lkhagvasuren et al., 2013; Zhu et al., 2007). Notably, unlike prototypical TNF receptors, which activate exclusively the classical NF-κB pathway, LTβR, RANK, and CD40 activate both the classical and alternative NF-κB pathways using common signaling transducers (Akiyama et al., 2012). Through the alternative NF-κB pathway, these signaling proteins activate RelB, which is essential for the formation of the thymic medulla (Akiyama et al., 2012; Derbinski and Kyewski, 2005). Importantly, it was shown that RelB expression in non-hematopoietic stromal cells, most likely in the thymus, favored TEL-JAK2-induced murine T-ALL and a more severe malignant phenotype (dos Santos et al., 2008) in this animal model of human T-ALL.

In recent years, a wide body of evidence has shown that T-cell leukemogenesis originates due to genetic aberrations in combination with microenvironmental stimuli such as cytokines, growth factors, or adhesion molecules (Medyouf et al., 2011; Silva et al., 2011; Uzan et al., 2014; Van Vlierberghe and Ferrando, 2012). In addition, it is currently believed that the pro-leukemogenic effects caused by the interaction between malignant and non-malignant cells in the tumor microenvironment is bidirectional, resembling at least in part, the interactions occuring between normal lymphoid cells and their microenvironment in lymphoid organs (Herreros et al., 2008). Therefore, we hypothesized that similarly to normal thymocytes, T-ALL cells may also establish interactions with non-malignant stromal cells through TNFSF/RelB signaling, modulating the microenvironment into a pro-leukemogenic niche.

To study the role of TNFSF signaling in T-cell leukemogenesis, we used the TJ2-Tg mouse model of cortical/mature T-ALL. These mice are characterized by the presence of CD4/CD8 DP and CD8 SP leukemic cells expressing surface TCRαβ, CD3 and CD5, and responsiveness to TCR stimulation (Carron et al., 2000; dos Santos et al., 2007). Such

cortical/mature immunophenotype is also frequent in TAL/LMO T-ALL cases. We first assessed the expression of TNFSF ligands in primary TJ2-Tg leukemic cells from diseased mice. LTβR ligand-encoding genes (*Lta*, *Ltb* and *Light*) and the RANK ligand-encoding gene (*Rankl*) were highly expressed in leukemic cells, as compared to wild-type thymocytes. Conversely, CD40 ligand (*Cd40l*) expression was not detected in leukemic cells, even though it was expressed in wild-type thymocytes. *Lta*, *Ltb*, *Light* and *Rankl* gene expression in TJ2-Tg leukemic cells may reflect either the differentiation stage of the cell of origin or result from gene expression alterations linked to the oncogenic process itself. On the other hand, the lack of *CD40l* expression, which is highly expressed in CD4 SP but not in CD8 SP and DP thymocytes, likely reflects the differentiation stage of leukemic cells (Desanti et al., 2012; Hikosaka et al., 2008). In addition, the LTβR and RANK genes were found to be expressed in whole thymic lymphomas. Therefore, the presented data suggests that the pattern of LTβR and RANK and their cognate ligand expression in thymic lymphomas is similar to that found in the normal thymus. Furthermore, similarly to the physiological crosstalk, TJ2-Tg leukemic cells expressing the ligands can also potentially interact with their cognate receptors in stromal cells and thus modulate the thymic lymphoma microenvironment. The detection of low LTβR protein expression in TJ2-Tg leukemic cells and in wild-type thymocytes may be derived from a small subset of DN and γδ thymocytes (Silva-Santos et al., 2005), or contamination with few DCs that may permeate the strainer used to separate TJ2-Tg leukemic cells from thymic lymphoma stromal cells. Nonetheless, the level of *Ltbr* mRNA expression in whole WT thymocytes and TJ2-Tg cells was undetectable like previously shown for the former (Seach et al., 2008).

Despite the high levels of *Lta*, *Ltb* and *Light* expression in TJ2-Tg leukemic cells, surface LTβR ligands were barely detectable by flow cytometry, although LTβ protein was detected upon leukemic cell permeabilization. Thus, these results suggest that either the ligand surface levels are tightly regulated *in vivo*, or upon LTβR activation on stromal cells, the surface level of $LT\alpha_1\beta_2$ in thymocytes is downmodulated possibly due to an autoregulatory mechanism to dampen LTβR activation. The fact that we could not detect high cell surface levels of LTβR ligands in freshly collected leukemic cells does not exclude the possibility that these cells can activate LTβR in thymic lymphomas. In fact, Boehm and coworkers (2003) showed that LTβR ligands were not detectable by flow cytometry LTβR-Fc staining neither in wild-type nor in $Ltbr^{+/-}$ thymocytes despite the important role the LTβR signaling pathway plays in the normal mouse thymus, as demonstrated by the loss of the three-dimensional thymic medullary epithelial cell network by systemic administration of

LTβR-Fc fusion protein (Boehm et al., 2003). However, LTβR ligands were clearly expressed at the surface of mature CD4 SP and CD8 SP thymocytes from *Ltbr*$^{-/-}$ mice. These findings suggest that when $LT\alpha_1\beta_2$ does not interact with LTβR, it is either induced to supraphysiological levels or retained in the membrane of leukemic T cells or normal thymocytes.

According with the notion that $LT\alpha_1\beta_2$ heterotrimers are lost from the cell surface upon interaction with LTβR, those proteins were found to be upregulated at the leukemic cell surface upon *ex vivo* culture in the absence of stromal cells. Furthermore, these were also detected at the surface of leukemic cells collected from TJ2-Tg;*Ltbr*$^{-/-}$diseased mice, but not TJ2-Tg;*Ltbr*$^{+/-}$. Since transplantation of TJ2-Tg leukemic cells into *Ltbr*$^{+/-}$ or *Ltbr*$^{-/-}$ mice showed that $LT\alpha_1\beta_2$ expression on the surface of these cells were detectable only in a *Ltbr*-deficient background, we confirmed that the LTβR-expressing cells interacting with leukemic cells, resulting in loss of $LT\alpha_1\beta_2$ from the membrane, are microenvironmental cells.

Supporting the notion that the effects on surface lymphotoxin expression was directly linked to LTβR expression and not due to systemic effects, nor structural defects found in LTβR-deficient mice, we used simple co-culture systems to study $LT\alpha_1\beta_2$ cell surface expression. We detected surface $LT\alpha_1\beta_2$ on leukemic cells in direct contact with LTβR-deficient MEFs, but not on leukemic cells in contact with LTβR-expressing MEFs or MS5 stromal cells. Furthermore, surface lymphotoxin downmodulation depended on direct contact with LTβR-expressing cells, since weak downmodulation occurred in co-cultured leukemic cells that did not adhere to stromal cells and in leukemic cells cultured with conditioned medium collected from co-cultures. The fact that we used two-dimensional (2D) co-cultures, which do not mimic the three-dimensional (3D) thymic stromal structure where thymocytes relocate through chemotaxis and passive inward flow contacting with virtually all mTECs (Hikosaka et al., 2008), may have precluded the downmodulation of $LT\alpha_1\beta_2$ ligands at the surface of all TJ2-Tg leukemic cells, specially those non-adhering to stromal cells. Furthermore, our results have shown that the interaction of $LT\alpha_1\beta_2$ with soluble extracellular region of LTβR (fused to human immunoglobulin Fc) is not sufficient to induce ligand downmodulation. This result suggests that $LT\alpha_1\beta_2$ downmodulation may rely on specific conformational alterations or on the activation of signaling pathways downstream of LTβR. Alternatively, $LT\alpha_1\beta_2$ downmodulation may depend on LTβR immobilization at the surface of another cell or adsorbed to plastic. Supporting this notion, it was previously shown that the use of soluble or immobilized LTβR as well as other TNFSF member agonists resulted in diverse effects, presumably by creating an higher level of receptor clustering in the latter case

(Bazzoni and Beutler, 1995; Beyaert and Fiers, 1994; Browning et al., 1996; Degli-Esposti et al., 1997; Grell et al., 1995).

Although the mechanism of surface lymphotoxin downmodulation remains to be determined, our data show that it depends on LTβR interaction. It is therefore possible that $LT\alpha_1\beta_2$ may be continuously expressed and removed from thymocyte/leukemic cell surface by shedding (Young et al., 2010) or internalized as a mechanism used by these cells to detach from LTβR-expressing stromal cells after signaling initiation, or to terminate activation of the receptor that could potentially lead to deleterious effects on stromal cells, including apoptosis (Heikenwalder et al., 2008; Liepinsh et al., 2009). An alternative explanation is that LTβR activation in stromal cells induces the expression of specific proteins that may be associated with a negative feedback loop acting *in vivo* to repress the expression of the ligands in T cells (Boehm et al., 2003). In addition, it is also possible that low ligand expression at the cell surface and decreased valency of the extracellular trigger is compensated by cooperation of the LTβR-induced intracellular signaling assemblies. In this model, minimal extracellular ligand-induced LTβR clustering would be sufficient to trigger the intracellular signal (Sudhamsu et al., 2013). To test this hypothesis, further analysis of the events stemming from different levels of receptor oligomerization and the varying lifetime of the oligomerized states would be required to understand LTβR activation. Furthermore, the mechanisms leading to ligand downmodulation need to be studied using for instance pharmacological inhibitors of protein shedding and endocytosis.

Regarding the alternative LTβR ligand, LIGHT expression at the cell surface of TJ2-Tg leukemic cells was never detected neither in freshly collected leukemic cells, nor in cells cultured *ex vivo*. Therefore, either *Light* mRNA is translated and the protein is stored in intracellular compartments, is shed from the cell surface (Morel et al., 2000), or it is not even translated. The fact that LIGHT surface expression was not induced after *ex vivo* culture may also be explained by the observed *Light* mRNA downregulation under these conditions.

Likewise, RANKL expression at the surface of TJ2-Tg leukemic cells was also hardly detectable by flow cytometry using a RANKL monoclonal antibody. These results can be explained by RANKL cleavage from the cell surface by proteases (Lum et al., 1999) and/or intracellular storage and restriction of cell surface RANKL presentation only upon RANK binding, like previously shown for osteoblasts and osteocytes (Aoki et al., 2010; Honma et al., 2013). As proposed above for LTβR, it is possible that intracellular signaling assemblies would compensate for the reduced extracellular ligand-induced RANK clutering and trigger the intracellular signal.

The fact that both LTβR ligands and RANK ligand were expressed in TJ2-Tg cells leaves open the question whether LTβR and RANK signaling share redundancy, cooperate and/or have synergistic effects, as shown in other contexts (Brunetti et al., 2014; Hemingway et al., 2013; Mouri et al., 2011; Rossi et al., 2007). Interestingly, a correlation between *Lta* and *Rankl* expression was found in primary TJ2-Tg leukemic cells suggesting that these may be activated by the same mechanism(s). Furthermore, it raises the possibility that LTβR and RANK signaling may cooperate in T-cell leukemogenesis like they cooperate in mTEC development and organization (Akiyama et al., 2012; Mouri et al., 2011).

4.2. MECHANISM OF TNFSF LIGAND EXPRESSION IN TJ2-TG LEUKEMIC CELLS

Several stmuli may underlie LTβR ligand expression in TJ2-Tg leukemic cells. So, we sought to determine the mechanism of lymphotoxin gene expression in these cells. Treatment of T lymphocytes with PMA and ionomycin leads to T cell activation, resulting in cytokine production, cytokine receptor expression, and ultimately cell proliferation. These two agents constitute a pharmacological means of activating signaling pathways downstream of pre-TCR/TCRαβ and other receptors by producing an elevation of intracellular free calcium with a calcium ionophore (ionomycin) combined with activation of protein kinase C (conventional and novel isoforms) by the PMA phorbol ester. Following stimulation with PMA and ionomycin, NF-κB and calcineurin signaling pathways are activated in leukemic T cells (Medyouf et al., 2007; dos Santos et al., 2008; Serwold et al., 2010). Importantly, pre-TCR/TCRαβ signaling transmits either survival or death signals throughout T-cell development, and pre-TCR has been shown to cooperate with the TEL-JAK2 fusion protein as well as other oncoproteins to induce T-cell leukemia (Bellavia et al., 2002; Campese et al., 2006; dos Santos et al., 2007; Talora et al., 2006). In agreement with previous reports showing that TCR signaling induces lymphotoxin genes in mature thymocytes (Hikosaka et al., 2008), PMA and ionomycin treatment induced lymphotoxin gene transcription and surface $LT\alpha_1\beta_2$ expression in primary TJ2-Tg leukemic T cells. By treating TJ2-Tg leukemic cells with pharmacological inhibitors, we verified that both basal and PMA/ionomycin-induced lymphotoxin expression was dependent on IKK/NF-kB activity, and that *Lta* expression was also dependent on JAK kinase activity, which is in line with previous reports

on T cells (Kuprash et al., 1996; Lu et al., 1998; Paul et al., 1993; Voon et al., 2004). NF-κB signaling in T cells can be activated by TCRαβ or pre-TCR signaling (Aifantis et al., 2001; Cheng et al., 2011; Moore et al., 1995). Supporting the notion that these receptor complexes may upregulate lymphotoxin genes in T-ALL, we observed that *Ltb* mRNA levels were reduced in TJ2-Tg leukemic cells lacking pre-TCR/TCRαβ (*Rag2* deficiency). It was previously shown that in contrast to *Ltb,* which is constitutively expressed, *Lta* expression is inducible in thymocytes (Browning et al., 1993). So, our findings support the notion that LTβ gene expression in T-ALL may be induced by TCR or pre-TCR signaling in an NF-kB-dependent manner. On the other hand, LTα may be induced in TJ2-Tg leukemic cells due to constitutive TEL-JAK2 activation, in addition to NF-κB activation. Since JAK/STAT signaling is often active in T-ALL, this signaling pathway may also upregulate *LTA* expression in these cells.

Light mRNA basal levels in TJ2-Tg leukemic cells were also shown to depend on IKK/NF-kB activity, but not to be consistently downmodulated by an IKK inhibitor following stimulation with PMA and ionomycin. Since calcineurin/NFAT signaling was shown to be inactivated *ex vivo* in TJ2-Tg leukemic cells (Medyouf et al., 2007) and our results showed that *Light* expression was often downregulated or unchanged when leukemic cells were cultured *ex vivo* for 10h, this observation supports the possibility that calcineurin/NFAT signaling may underlie *Light* expression *in vivo*. This notion is in accordance with a previous study showing that calcineurin/NFAT signaling activates *Light* expression in T cells (Castellano et al., 2002). In addition, *Light* downregulation in *ex vivo*-cultured leukemic cells was reversed by PMA and ionomycin stimulation, probably due to ionomycin-induced calcineurin/NFAT reactivation (Medyouf et al., 2007) and not NF-κB activation. Nevertheless, LIGHT protein expression at the surface of TJ2-Tg cells was not detected even after stimulation.

Similarly to lymphotoxin, RANKL cell surface expression was induced following PMA and ionomycin stimulation**.** Moreover, further supporting a role for NF-κB in *Rankl* expression induction, treatment with the IKK inhibitor could reduce *Rankl* mRNA expression in cultured cells treated or not with PMA and Ionomycin. Together, these experiments define IKK kinases as key regulators of *Lta*, *Ltb*, *Light*, and *Rankl* expression in TJ2-Tg leukemic cells. The mechanisms identified are not exclusive of these leukemic cells since we identified the same mechanisms in normal thymocytes. However, the observed gene and protein expression levels indicate that their activation is exacerbated in leukemic cells.

Besides NF-κB and JAK/STAT signaling pathways, others have been described to induce the expression of TNF ligand family genes. As mentioned before, the study of calcineurin/NFAT signaling pathway in these mechanisms has been hindered by the fact that when TJ2-Tg leukemic cells are cultured *ex vivo*, NFAT proteins are inactivated by hyperphosphorylation and, as a result, become insensitive to the effect of the calcineurin inhibitor cyclosporine A (CsA) (Medyouf et al., 2007). Similarly, the study of Notch signaling pathway and its possible impact on LTβR ligand expression is hindered by the presence of diverse Notch-activating point mutations (unpublished results), which can render TJ2-Tg leukemic cells resistant to Notch inhibition. Furthermore, considering potential signaling pathway crosstalk, it would be also worthy to study other signaling pathways frequently activated in T-ALL, like the MAPK or PI3K/AKT pathways (Zhao, 2010) in the regulation of TNFSF gene expression.

In future studies, it would also be interesting to study the impact of the activation of other receptors known to be expressed in T-ALL (e.g., IL-7R, TNFα, and CCR7) on LTβR ligand expression in TJ2-Tg leukemic cells (Buonamici et al., 2009; Scupoli et al., 2007; Voon et al., 2004).

4.3. IMPACT OF LTβR SIGNALING ABROGATION ON T-CELL LEUKEMOGENESIS

Although LTβR is known to be important for microenvironment establishment in solid and hematopoietic tumors (Ammirante et al., 2010; Haybaeck et al., 2009; Heinig et al., 2014; Rehm et al., 2011), its role in T-cell leukemia has never been explored. Notably, this study reveals a non-redundant role for LTβR in TEL-JAK2-induced leukemia.

In human T-ALL, leukemic transformation was reported to require at least three successive mutations that occur during hematopoietic differentiation and drive normal T-cells into uncontrolled cell growth and clonal expansion (Ferrando and Look, 2003). Similarly, TJ2-Tg mice develop fatal, oligoclonal leukemia after variable latency (Carron et al., 2000). Furthermore, the same TEL-JAK2 fusion protein has been shown to be capable of inducing a CML-like disease, B-ALL or a T-ALL disease in other experimental mouse models (Ihle and Gilliland, 2007), depending on the cellular compartment where it is expressed. These two findings suggest that the basis for the disease heterogeneity may be related to the acquisition

by hematopoietic target cells of secondary transforming events and/or microenvironmental factors supporting leukemogenesis in addition to the TEL-JAK2 kinase activity.

Although the absence of LTβR was not sufficient to prevent leukemia progression and leukemia-related death, it significantly delayed T-cell leukemia onset in TJ2-Tg;*Ltbr*$^{-/-}$ mice as compared to TJ2-Tg;*Ltbr*$^{+/-}$ littermates. This delay likely reflects the notion that leukemic cells require more time to acquire additional transforming events and/or that microenvironment assistance to these cells is affected when the receptor is absent. TJ2-Tg;*Ltbr*$^{-/-}$ mice presented a mean survival time of 23 weeks while TJ2-Tg;*Ltbr*$^{+/-}$ mice displayed a mean survival of 15 weeks. Therefore, interference with LTβR signaling resulted on average in an 8-week delay in leukemia-derived TJ2-Tg mouse death. These inhibitory effects prompt us to reason whether interventions that prevent LTβR signaling after disease onset may delay leukemia-related mouse death. For this purpose, it would be interesting to use targeting molecules like the LTβR-Fc fusion protein in pre-clinical trials.

Despite the delayed onset of leukemia in LTβR-deficient mice, the tumor load in lymphoid organs and the cell surface marker phenotype of leukemic cells were not significantly altered. Although global differences in gene expression may exist, we can conclude that the tumors from the two mouse groups under study were phenotypically similar.

As *Ltbr* knockout mice do not develop lymph nodes and have altered thymic and splenic structures, it was conceivable that the delay in fatal leukemia/lymphoma development could be linked with a decreased tumor burden due to impaired lymphoma development in these lymphoid tissues, especially in lymph nodes. To discard this possibility, we inhibited lymphotoxin signaling in young pre-leukemic TJ2-Tg mice through the administration of neutralizing LTβR-Fc fusion protein. Substantiating our previous genetic results, LTβR-Fc treatment delayed leukemogenesis, and additionally induced accumulation of leukemic cells in the thymus and reduced tumor load in the bone marrow and peripheral blood.

It was previously reported that leukemia was delayed and thymic tumor load was reduced in TJ2-Tg mice without RelB expression (dos Santos et al., 2008). LTβR-deficient mice did not present such reduction in thymic tumor load, supporting the idea that receptors other than LTβR may activate RelB in stromal cells to support leukemogenesis. Alternatively, the similar thymic lymphoma weights could be explained by confounding factors such as the absence of lymph nodes or the disturbed export of thymocytes from the thymus in LTβR-deficient mice, as compared to LTβR-proficient mice (Boehm et al., 2003).

Recently, some studies shed light on LTβR and RANK redundancy in osteoclastogenesis (Brunetti et al., 2014; Hemingway et al., 2013). In addition, both receptors activate RelB in mTECs (Akiyama et al., 2012). As such, some degree of LTβR and RANK redundancy in TEL-JAK2-induced leukemia may explain the fact that the differences found in LTβR-deficient TJ2-Tg mice and controls do not differ as much as we expected from the results obtained by RelB activation (dos Santos et al., 2008). Consequently, it would be important to study if RANK activation is involved in TEL-JAK2-induced leukemia in combination with LTβR.

4.4. TIMING OF LTβR SIGNALING ACTION IN TEL-JAK2-INDUCED LEUKEMOGENESIS

To understand how LTβR supports TEL-JAK2-induced leukemogenesis, thymi from pre-leukemic transgenic mice were analyzed. It was found that before thymic enlargement, a population of aberrant, immature T cells characterized by CD8, CD24 and CD25 expression expands in the thymus. This cell population is malignant since they engraft and expand in Nude recipient mice following transplantation. Conversely, few or no $CD8^{+}CD25^{+}$ T cells were detected in other organs such as the spleen and BM in early stages of leukemia development, consistent with the notion that in TJ2-Tg leukemic mice, leukemic cells originate in the thymus. Notably, the expansion of $CD8^{+}CD25^{+}$ T cells in the thymus was associated with increased expression of *Lta* and *Ltb*.

Given that LTβR is expressed in thymic stromal cells, including epithelial cells and fibroblasts, but not thymocytes (Seach et al., 2008), our results indicate that emerging LT-expressing leukemic cells require LTβR-expressing thymic stromal cells for rapid disease onset. At terminal phases of TJ2-Tg leukemogenesis, we observed no significant differences in tumor burden, indicating that LTβR activation is not absolutely required beyond the initial stages of the disease. Also discarding an essential role for LTβR in late stages of T-ALL development, engraftment and dissemination of TJ2-Tg leukemic cells transplanted to recipient mice through i.v. injection was not LTβR-dependent. However, leukemic cell infiltration is variable depending on the transplantation approach, so this experiment should be repeated by transplanting TJ2-Tg leukemic cells through intra-thymic injections in order

to evaluate the role of LTβR in leukemic cell engraftment in the thymus and dissemination from this to other organs. Furthermore, secondary tumors derived from the seeding of a large number of full-fledged, highly malignant cells possibly grows more autonomously than tumors resulting from a small number of spontaneously transformed cells. In addition, ectopic transplantation of tumor cells does not mirror the process of multistage carcinogenesis from the normal cells over an extended period of time.

The analysis of pre-leukemic mice showed that LTβR-deficiency specifically impairs leukemic cell expansion in the thymus in the early stages of disease development, when other organs do not present a significant degree of infiltration. Consequently, we conclude that the lack of lymph nodes in *Ltbr*$^{-/-}$ mice does not account for the delayed leukemia onset in TJ2-Tg mice.

Altogether our results indicate that stimulation of LTβR-expressing microenvironmental cells by emerging malignant thymocytes accelerates early oncogenesis. LTβR expression and activation in thymic stromal cells constitutes therefore a non-cell-autonomous mechanism promoting T-ALL alongside with cell-autonomous mechanisms such as pre-TCR signaling (dos Santos et al., 2007). Whether LTβR-expressing microenvironmental cells from other organs are also involved in interaction with TJ2-Tg leukemic cells should be addressed in future studies.

4.5. MECHANISM MEDIATING LTβR SUPPORTIVE ROLE IN T-ALL

T-ALL develops from a complex interplay between genetic and microenvironmental factors. Indeed, the microenvironment where T-ALL develops may be modulated by these cells to gain a selective advantage. Several microenvironmental factors participating in T-ALL progression have been identified, including homeostatic cytokines, adhesion molecules, and pro-inflammatory mediators (Silva et al., 2011; Uzan et al., 2014; Winter et al., 2001). In addition, a link between pro-inflammatory factors and T-ALL development is illustrated by a recent report in which IL-18 pro-inflammatory cytokine expression by stromal cells was shown to support T-ALL progression (Uzan et al., 2014). Another interleukin essential for T-cell development and homeostasis, IL-7, has been implicated in T-ALL development through

expression by T-ALL microenvironmental cells (Silva et al., 2011). Interestingly, IL-7 and IL-18 are both expressed by thymic epithelial cells (Ito et al., 2006; Ribeiro et al., 2013) and were shown to act in synergy to promote *ex vivo* expansion of T lymphoid progenitor cells (Gandhapudi et al., 2015), but the significance of these synergistic effects *in vivo* remains to be investigated. Nevertheless, the previous findings support the tumor-promoting effects of pro-inflammatory microenvironments in T-ALL.

The mechanism(s) by which LTβR signaling contributes to T-ALL development is not yet completely understood. Nevertheless, it is clear that the LTβR is linked to its expression in stromal cells, most likely from the thymic microenvironment. $LT\alpha_1\beta_2$ reverse signaling, as found for other TNFSF ligands (Sun and Fink, 2007), has never been reported, so we infer that lymphotoxin expression in leukemic T cells promotes leukemogenesis through activation of LTβR in microenvironmental cells.

$LT\alpha_1\beta_2$-LTβR signaling was shown to be responsible for generating inflammatory microenvironments leading to carcinogenesis (Ammirante et al., 2010; Bjordahl et al., 2013; Haybaeck et al., 2009). Furthermore, LTβR may support carcinogenesis either by regulating the development and organization of supportive tumor microenvironmental cell networks (Heinig et al., 2014; Rehm et al., 2011), or by inducing the expression of LTβR target genes in stromal cells that stimulate tumor cell growth and survival (Lau et al., 2014). One of these mechanisms or even both may be on the basis of the LTβR-expressing stromal cell supportive role in T-ALL development. Therefore, we reason that $LT\alpha_1\beta_2$ expression in emerging malignant thymocytes may either be important for mTEC or fibroblastic cell network development and/or activate an NF-kB-dependent transcriptional program in thymic microenvironmental cells that promotes T-cell leukemogenesis. Cytokines and chemokines are relevant candidates, since they are induced by LTβR in stromal cells (Dejardin et al., 2002; Seach et al., 2008). A possible collaboration between LTβR and RANK, as described in other cellular systems (Akiyama et al., 2012), may be involved in T-ALL microenvironmental modulation and/or in target gene expression induction. Such LTβR/RANK redundancy regarding NF-κB/RelB activation and common target gene induction may explain the failure to detect LTβR target gene differential expression in LTβR-deficient leukemias.

Another question that remains unanswered is whether LTβR signaling in the thymic microenvironment promotes the survival and/or proliferation of the bulk leukemic cell population involved in T-ALL progression, or supports the rare, putative leukemia-initiating cell population (Konopleva et al., 2009) underlying T-ALL initiation and maintenance. So

far, only one stroma-derived factor has been described to sustain T-ALL LICs, insulin-like growth factor-1 (IGF-1) (Medyouf et al., 2011). This growth factor binds and activates insulin-like growth factor-1 receptor (IGFR1), which was shown to be induced by Notch and is on its own required for LIC activity in T-ALL (Armstrong et al., 2009).

NOTCH1 protein is activated in more that 60% of T-ALL cases (Weng et al., 2004), and has been reported to induce the expression of receptors that integrate signals from the microenvironment in addition to IGFR1 (Medyouf et al., 2011). These include cytokine receptors such as CCR7 (Buonamici et al., 2009; Mirandola et al., 2012), and IL-7R (González-García et al., 2009). Notably, these receptors have also been implicated in T-ALL development (Buonamici et al., 2009; Qiuping et al., 2004; Silva et al., 2011). Therefore, Notch activation enhances the responsiveness of T-ALL cells to microenvironmental factors. The importance of these factors is further supported by the identification of mutations constitutively activating the cognate receptors or downstream signaling mediators (e.g., IL-7R, JAK1, JAK3, PI3K and AKT) that eliminate or reduce dependency on stromal cells (Van Vlierberghe and Ferrando, 2012; Zenatti et al., 2011; Zhang et al., 2012). Therefore, the possibility that Notch may also modulate the expression of other factors involved in crosstalk between T-ALL and stromal cells, like lymphotoxins proteins and RANK ligand is worth studying.

An important aspect of the clinical progression of T-ALL is the dissemination of transformed T cell progenitors from the organ of origin, likely the thymus, through the bloodstream, leading to high white blood cell counts in the peripheral blood and large tumor burden in infiltrated organs, including the bone marrow, spleen, and brain. Although the mechanism is not completely understood, some studies have identified few important players responsible for leukemic cell export from the thymus and consequent dissemination through the bloodstream, including S1P1 and ICAM-1 (Feng et al., 2010). Importantly, the confinement of leukemic cells to the thymus, originating large thymic lymphomas, was shown to lead to a milder disease phenotype in the T-ALL zebrafish model (Feng et al., 2010). A more recent study, implicated also TCR, and to a lesser extent common gamma chain (γ_c)-containing receptor signaling in T-ALL egress from the thymus (Hagenbeek et al., 2014). Although our observations on the impact of LTβR signaling inhibition using LTβR-Fc requires further investigation, it is noteworthy that we found a tendency for higher tumor burden in the thymus and lower leukemic cell number both in the peripheral blood and infiltrating the bone marrow, in combination with improved survival. In accordance with these results, LTβR was previously implicated in mature T-cell export from the thymus

(Boehm et al., 2003) presumably trough activation of NF-κB-inducing kinase (NIK) (Mouri et al., 2011).

Finally, the immune system was reported to negatively affect T-ALL development (Schneider et al., 2010; Uzan et al., 2014). Since *Ltbr* knockout mice present signs of immunity due to defective cental tolerance (Boehm et al., 2003), we reasoned the immune system could have anti-ALL effects in TJ2-Tg mice, similarly to what has been shown for prostate cancer in mice (Zhou et al., 2009). However, this possibility seems unlikely because evident signs of auto-immunity were detected only in 24-week-old mice (Boehm et al., 2003), an age when almost all LTβR-proficient TJ2-Tg mice have already died.

Although the LTβR-dependent mechanism supporting T-cell leukemogenesis in TJ2-Tg mice requires further investigation, the hypothesis that leukemic T cells upregulate $LT\alpha_1\beta_2$ and engage in crosstalk with LTβR-expressing stromal cells to generate a pro-leukemogenic thymic stromal microenvironment seems the most likely to explain this mechanism. However, it remains unclear if the microenvironment favors the occurrence of transforming events and/or sustains specifically the survival or proliferation of transformed cells over non-transformed cells.

4.6. ROLE OF LYMPHOTOXIN IN HUMAN T-ALL

Supporting a role for $LT\alpha_1\beta_2$-LTβR signaling in human T-ALL, *LTA* and *LTB* mRNA (but not *LIGHT*), as well as lymphotoxin cell surface expression, were detected at variable levels in human T-ALL cell lines. Surface lymphotoxin was upregulated by PMA treatment in all T-ALL cell lines analyzed, in agreement with published data (Browning et al., 1997). Moreover, IKK inhibition not only downregulated *LTA* and *LTB* mRNAs but also blocked PMA-induced lymphotoxin surface expression, thus confirming that lymphotoxin genes are regulated by IKK/NF-κB signaling in both mouse and human T-ALL.

Importantly, *LTA* and *LTB* mRNA was also detected in primary patient samples. Despite the low number of patient samples analyzed in a preliminary study, two cases presented relatively high levels of both *LTA* and *LTB* genes, as compared to normal human

thymocytes, suggesting that increased lymphotoxin expression may occur in a subset of T-ALL patients.

By performing unsupervised clustering analysis of transcriptomic data, we found that TJ2-Tg leukemia presents features of cortical/mature T-cell leukemia. Although no increased expression of the TAL1 or LMO1/2 proto-oncogenes was found in TJ2-Tg leukemic cells, these cells present molecular features similar to those present in TAL/LMO T-ALL (e.g., TCR loci rearrangements, TCR/CD3 surface expression, and CD4/CD8 DP immunophenotype). Interestingly, lymphotoxin gene expression was found in T-ALL cells from a large cohort, being more pronounced in TAL/LMO T-ALL. Conversely, no significant associations were found between *LTA*/*LTB* expression and the clinical features of human T-ALL.

Although lymphotoxin expression in human T-ALL cell lines has long been known, the present study is the first to our knowledge showing lymphotoxin expression in human T-ALL primary samples. These findings call for further studies on the role of the $LT\alpha_1\beta_2$-LTβR signaling axis in human T-ALL development.

4.7. LTβR AS A POSSIBLE THERAPEUTIC TARGET IN HUMAN T-ALL

Cell-autonomous alterations are heavily implicated in the origin of T-ALL and accordingly have been widely studied. However, only relatively few studies explored the microenvironmental factors that contribute to T-ALL expansion and progression. Moreover, emerging evidence indicates that solid and hematopoietic malignancies manipulate components of the $LT\alpha_1\beta_2$-LTβR signaling axis to modify their tumor microenvironment. These alterations create tumor-permissive microenvironments that provide strong selective pressure for tumor evolution and growth, protection from immune assaults, or even resistance to therapeutic approaches. In this context, the novelty of our study resides in the identification of the $LT\alpha_1\beta_2$-LTβR signaling axis as a modulator of the T-cell leukemia microenvironments.

Notably, LTβR signaling abrogation by LTβR-Fc (baminercept) affected leukemia development and dissemination in TJ2-Tg mice, suggesting that inhibition of crosstalk between leukemic and stromal cells through this signaling pathway may be involved in

different aspects of T-ALL. Moreover, suggesting that $LT\alpha_1\beta_2$-LTβR signaling axis may also be involved in human T-ALL development, we found that lymphotoxin genes are expressed in human T-ALL cell lines and primary samples, prominently in TAL/LMO T-ALL samples.

Altogether, this study contributes to a better understanding of the mechanisms of T-ALL pathogenesis. Accordingly, the gained knowledge reveals that inhibition of the $LT\alpha_1\beta_2$-LTβR signaling axis is as a potential novel therapeutic opportunity to target pro-oncogenic mechanisms not intrinsic to leukemic cells, which often display high mutation rates leading to secondary resistance to therapy.

To this end, uncovering the precise mechanism(s) by which this axis supports leukemogenesis will be of great value. First, future studies should determine which microenvironmental cells and LTβR-derived signals play a role in T-ALL pathogenesis. Moreover, the question on how $CD8^+CD25^+$ malignant thymocytes expand in LTβR-proficient thymi should be addressed. In addition, it should be determined if $CD8^+CD25^+$ malignant thymocytes arising early in the thymus constitute leukemia-initiating cells or bulk leukemia cells. Second, we should further address whether lymphotoxin signaling is involved in later phases of T-ALL progression in addition to the initial steps in the thymus. Our results show a tendency of reduced bloodborne leukemic cell dissemination to distant metastatic sites in the absence of LTβR signaling, which should be further examined. Consequently, LTβR signaling may be involved in multiple aspects of leukemia development just like it is in thymocyte development and export from the thymus. In this line, it would be interesting to evaluate if it is also involved in leukemic cell engraftment and growth in peripheral organs like it has been shown for malignant B cells in lymph nodes (Rehm et al., 2011) and spleen (Heinig et al., 2014). In addition, an in-depth scrutiny on the effects of LTβR-Fc block in TJ2-Tg leukemic mice should be considered in larger cohorts of mice. This would contribute to evaluate the possibility of interfering with this signaling axis in different phases of the disease as a therapeutic approach, or as a preventive strategy. Third, we found some shared features between TEL-JAK2-induced leukemia and human T-ALL that support the importance of further investigation on the impact of LTβR signaling inactivation. To this end, and as a preliminary approach, xenograft models consisting of mice transplanted with human primary T-ALL leukemias could be used as a model. Finally, a potential collaboration between LTβR and RANK signaling in T-ALL development should be further explored either using double knockout mice or simultaneous pharmacological block of both signaling axes.

The answer to all these questions may contribute to the development of rational therapies targeting the communication between leukemic and stromal cells namely by inhibition of $LT\alpha_1\beta_2$-LTβR signaling alone or in combination with RANKL-RANK signaling. Importantly, inhibitors for both pathways are already used to treat other pathologies or in clinical trials (Denosumab, Amgen; Baminercept, Biogen Idec).

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