UNIVERZA V LJUBLJANI
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KAVŠEK TJAŠA

THE ROLE OF CHEMOKINE RECEPTOR CXCR4 IN T-CELL LEUKEMIA MURINE MODEL

MASTER'S THESIS

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Univerza *v Ljubljani*
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SAPIENZA
UNIVERSITÀ DI ROMA

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VLOGA KEMOKINSKEGA RECEPTORJA CXCR4 V MIŠJIH MODELIH T CELIČNE LEVKEMIJE

MAGISTRSKA NALOGA

Ljubljana, 2016

My work was performed at the Laboratory of Molecular Pathology, Sapienza University of Rome, under supervision of prof.ssa dr. Maria Pia Felli and written at the Faculty of pharmacy, University of Ljubljana, under supervision of prof. dr. Irena Mlinarič Raščan.

Statement

I hereby declare that I have performed and written this master's thesis by myself under the supervision of prof. dr. Irena Mlinarič Raščan and co-supervision of prof.ssa dr. Maria Pia Felli.

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ABSTRACT

Constitutive activation of Notch signaling is one of the leading causes of acute T-cell lymphoblastic leukemia in mice and people. Transgenic mouse model of this disease, over-expressing active Notch3 intracellular domain in immature thymocytes, was created in our laboratory (Laboratory of Molecular Pathology, La Sapienza, Università di Roma) and it summarizes the important human acute T-cell lymphoblastic leukemia characteristics: constitutive activation of NF-κB pathway signaling, continuous expression of CD25 marker and the α chain of pre-TCR, deregulated proliferation and maturation of T-cells. Differentiation of T-cells alone depends on multiple signals by thymic environment (expressing chemokines) and the cells themselves (expressing chemokine receptors). Any pathological modification of this dynamic organization can subsequently result in an abnormal T-cell development.

Cancer cells often express deregulated profiles of chemokine receptors, especially chemokine receptor CXCR4. Notch signaling is also known to regulate cancer cell fate in many types of tumors. In fact, immature thymocytes for their transition from pre-T into T-cells essentially require chemokine receptor CXCR4, pre-T-cell receptor and Notch cooperation. This combination suggests that trans-membrane receptors CXCR4 and Notch3 could play an *in vivo* role in acute T- lymphoblastic leukemia, where these pathways are frequently altered. In our study, flow cytometry experiments have shown a decrease in the percentage of CXCR4 positive cells in double negative (DN) thymocytes, possibly associated to CXCR4 gene transcriptional down-modulation, contrary to increased double positive (DP) cells of pathological mice model compared to wild-type (WT). This shows that CXCR4 down-regulation allows Notch3 to interfere with the properties of differentiation and migration of immature DN thymocytes. The negative regulation of CXCR4 on DN T-lymphocytes may result in impaired progressive T-cell differentiation, in contrast to enhanced CXCR4 cell surface expression in DP T-cells, probably associated to increased egress from the thymus, positively affecting metastasis and thus the cancer aggressiveness.

This observation for the first time highlights that, in contrast to Notch1, which does not affect CXCR4 in cell lines of human T-ALL, Notch3 is able to regulate the CXCR4 gene transcriptionally *in vivo* in a mouse model of T-ALL.

Keywords: T-lymphocytes, thymocytes, T-cell leukemia, Notch3, CXCR4

POVZETEK

Akutna T-celična limfoblastna levkemija (T-ALL) je ena najbolj agresivnih oblik krvnega raka. Akutne levkemije so najpogostejša vrsta otroškega raka (25% teh je T-celičnih, druge so B-celične), pri katerih pa se je stopnja preživetja v zadnjih letih precej izboljšala. Bolj kritična populacija so dojenčki ter odrasli s to diagnozo ter vsi bolniki z relapsom bolezni, pri katerih je izid še vedno slab. Klinično se bolezen kaže kot močno povečano število limfoblastov in celotno število levkocitov, pogosto sovpada s splenomegalijo (povečano vranico), povečanimi bezgavkami ter inflitracijo in okvaro drugih perifernih organov. Povečana proizvodnja nezrelih T-limfocitov oz. timocitov ima vzrok nekje v procesu dozorevanja le-teh. Razvoj T-celic primarno poteka v priželjcu, kamor limfoblasti pripotujejo iz kostnega mozga. V začetku timocite imenujemo dvojno negativni (DN), ker ne izražajo ne CD4 ne CD8 antigena. Te DN celice se delijo na 4 stopnje razvitosti, razlikujejo se glede na izražanje celičnih označevalcev CD25 in CD44. Prva prelomnica v priželjcu je β-selekcija, ki omogoča prehod iz DN3 v DN4 le timocitom s funkcionalnim pred-T-celičnim receptorjem. V nadaljevanju te izrazijo dozorel T-celični receptor in postanejo dvojno pozitivne (DP); izražajo tako CD4 kot CD8 antigen. Sledeča pozitivna selekcija je proces, ko se te celice vežejo na poglavitni histokompatibilnostni kompleks (MHC) tipa I ali II; nevezane celice pa so odstranjene. Celice, ki vežejo MHC I, se diferencirajo v enojno pozitivne celice $CD8^+$, tiste, ki vežejo MHC II pa v enojno pozitivne celice $CD4^+$. Zadnja ovira pred izstopom T-limfocitov je t.i. negativna selekcija, v kateri so celice, ki premočno vežejo MHC, odstranjene zaradi možnosti, da kasneje povzročijo avtoimunske reakcije. Vsi ostali enojno pozitivni, zreli T-limfociti nato zapustijo priželjc in migrirajo v periferne organe.

Poskusi na mišjih modelih z inducirano hiperaktivacijo intracelične domene Notch3 receptorja, ustvarjeni v našem laboratoriju (Laboratorij za molekularno patologijo, Univerza La Sapienza, Rim), so dokazali nedvomno vpletenost tega receptorja v bolezen; miši so namreč po 5-7 tednih zbolele za akutno T-celično levkemijo / limfomom. Značilnosti njihovega fenotipa so: povečano število DN timocitov, nenehno izražanje označevalca CD25 in α-oblike pred-T-celičega receptorja ter stalna aktivacija NF-κB signalne poti. Notch3 je transmembranski receptor družine Notch, ki ima poglavitno vlogo v razvoju T-celic, predvsem v koraku prehoda iz DN3 v stopnjo DN4, β-selekciji. Tu tesno sodeluje s signalizacijo pred-T-celičnega receptorja ter kemokinskim receptorjem CXCR4, zato smo

se odločili, da preverimo, ali med receptorjema Notch3 in CXCR4 obstaja povezava in kakšen je njen mehanizem.

CXCR4 je receptor, član velike družine kemotaktičnih citokinov oz. kemokinov. Ti so hiter in učinkovit način sporazumevanja med celicami, udeleženimi v imunskem odzivu, in skrbijo za nadzor nad proizvodnjo ter migracijo potrebnih celic. Nekateri so izraženi konstitutivno in urejajo normalne procese v organizmu, medtem ko so drugi prisotni le ob akutnih fazah, kot so alergije, vnetje, rak, ipd. Znano pa je tudi, da kemokini promovirajo tumorsko angiogenezo in pripomorejo h komunikaciji med rakavimi celicami. Receptor CXCR4 ter njegov ligand SDF-1α (CXCL12) sta vpletena v več kot 23 različnih oblik neoplazem; rak jajčnikov, levkemije, itd. Povečano izražanje receptorja CXCR4 tako pripomore ne le h komuniciranju, temveč tudi k rasti in metastaziranju tumorjev, poleg tega pa ima vlogo pri pojavu rezistence na standardno farmakoterapijo. Raziskavo smo se odločili izvesti na prej omenjenih transgenih mišjih modelih s povečano ekspresijo Notch3 receptorja v primerjavi z zdravimi živalmi. Uporabili smo priželjce ter vranice 6-12 tednov starih živali, iz katerih smo izolirali T-limfocite. Te smo označili z različnimi protitelesi ter jih analizirali s pretočno citometrijo. Protitelesa proti specifičnim antigenom so bila označena s fluorescirajočimi označevalci različnih barv, s katerimi smo razlikovali med populacijami.

Za analize smo potrebovali več različnih protokolov tretiranja celic. Najprej smo populacijo timocitov razdelili na osnovne štiri podskupine (DN, DP ter enojno pozitivne $CD4^+$ in $CD8^+$) z inkubacijo vzorcev z anti-CD4 in anti-CD8 protitelesi. Za dodatno ločitev DN celic (stopnje DN1-4) smo inkubirali s protitelesi anti-CD25 ter anti-CD44. S tem barvanjem smo dobili sliko distribucije celic v patološkem in ne-patološkem modelu. Za še bolj natančno določitev smo prav tako s protitelesi iz populacije DN celic izključili celice ubijalke (NK), ki v majhnih količinah izražajo receptor CXCR4, ter se tako prepričali o pravilnosti dobljenih rezultatov.

Za določanje izražanja receptorja CXCR4 smo poleg osnovnih delitev dodali še inkubacijo s protitelesom proti CXCR4 ter kontrolno proti IgG2b. Za določanje izražanja receptorja Notch3 smo inkubirali s protitelesom proti Notch3 ter kontrolo s protitelesom proti IgG.

Enako smo ekspresijo teh receptorjev določali na DP celicah vranic patološkega modela, le da smo celice najprej inkubirali s protitelesom anti-Thy-1 ter s tem izločili vse razen celic T. Poleg izražanja teh receptorjev smo določili tudi ekspresijo epitelijske celične adhezijske molekule (EpCAM), znanim celičnim označevalcem za mnoge vrste raka, s protitelesoma proti EpCAM ter IgG2a (kontrola).

Ekspresijo genov za CXCR4 in Bcl2A1 smo določali z metodo RT-PCR. mRNA smo izolirali iz priželjcev že prej uporabljenih mišjih modelov, nato pa jo z reverzno transkripcijo prepisali v komplementarno DNA, ki smo jo lahko uporabili v verižni reakciji s polimerazo (PCR). Kot normalizator smo uporabili β-aktin, ki smo ga skupaj s produkti PCR reakcije analizirali na 1,5% agaroznem gelu z vstavljenim Midori barvilom, ki omogoča detekcijo nukleinskih kislin pod UV svetlobo.

Zadnji poskus je bil test migracijske sposobnosti DN in DP celic pod vplivom naravnega liganda SDF-1α Za kontrolo specifičnosti testa smo uporabili ligand SLC, ki se veže CCR7. Rezultate lahko razdelimo v dve skupini, ki izkazujeta popolnoma obraten vzorec izražanja receptorja CXCR4 pod vplivom receptorja Notch3.

Prvi del analiz kaže, da je prisotnost receptorja CXCR4 znatno zmanjšana na membranah dvojno negativnih timocitov in dokazuje, da Notch3, v nasprotju z receptorjem Notch1, dejansko vpliva na ekspresijo kemokinskega receptorja CXCR4. V DN populaciji se to še posebej kaže v zmanjšani porazdelitvi predvsem timocitov DN4 in tudi DN3. To nakazuje motnje v selekciji β, v kateri igrata pomembni vlogi prav receptorja Notch3 in CXCR4. Dodatne analize te populacije so pokazale, da Notch3 v priželjcu povzroči tudi popolno spremembo epitelija in 'ugasne' izražanje molekule EpCAM. Ta dva dogodka imata velik vpliv na razvoj T-limfocitov, saj motita njihov prehod skozi faze in normalne interakcije med celicami, z analizo anti-apoptotičnega Bcl2A1 faktorja pa smo opazili, da Notch3 še dodatno ovira normalne pro-proliferativne mehanizme.

Po drugi strani se izražanje CXCR4 v populaciji dvojno pozitivnih celic močno poveča. Opazili smo tudi pogosto pojavljanje obeh receptorjev na membranah celic tako priželjca kot perifernih organov in predpostavljamo, da njuna ko-eksistenca močno poveča agresivnost bolezni. Slednjo prav tako stopnjuje visoka prisotnost receptorjev CXCR4 na površini celic, ki še posebej favorizira migracijo teh nezrelih DP celic iz priželjca v periferne organe in metastaziranje.

Vse to dokazuje, da Notch3 preko deregulacije receptorja CXCR4 negativno vpliva na diferenciacijo nezrelih timocitov, kar v povzetku pomeni, da pozitivno vpliva na nastanek in hiter napredek akutne T-limfoblastne levkemije.

Ključne besede: T-limfociti, timociti, T-celična levkemija, Notch3, CXCR4

Thank you prof. Mlinarič-Raščan for your patience and guidance through the whole process. This work was only made possible thanks to prof. Screpanti and prof. Felli of Laboratory of Molecular Pathology (La Sapienza University). Thank you for accepting me into your group. And it was only finished thanks to mie principesse Fra, Ta, Cla, Silvia and Giulia, as well as caffè macchiato al vetro. Thank you for accepting me into your lives.

"You know what happens when you dream of falling? Sometimes you wake up. Sometimes the fall kills you. And sometimes, when you fall, you fly." - *Neil Gaiman*

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LIST OF ABBREVIATIONS

AA	Amino Acid
ADAM	A Disintegrin and Metalloprotease
ANK	Ankyrin Domain
APC	Allophycocyanin
Bcl2-A1	Bcl2-related protein A1
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
cDNA	Complementary DNA
CI	Cubitus Interruptus
CSL	CBF1, Suppressor of Hairless, Lag-1
CLP	Common Lymphoid Progenitor
CXCL12	CXC Chemokine Ligand 12
CXCR4	Chemokine CXC Receptor 4
Cys	Cysteine
DAG	Diacylglycerol
DMEM	Dulbecco's Modified Eagle's Medium
DN	Double Negative
DP	Double Positive
EGF	Epidermal Growth Factor
EpCAM	Epithelial Cell Adhesion Molecule
ERK	Extracellular signal-regulated Kinase
FACS	Fluorescence Activated Cell Sorting
FITC	Fluorescein Isothiocyanate
GPCR	G-Protein Coupled Receptor
GDP	Guanosine Diphosphate
GTP	Guanosine Triphosphate
HA	Hemagglutinin
HD	Heterodimerization Domain
HEPES	4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid
HES	Hairy Enhancer of Split
hGH	human Growth Hormone

IP3	Inositol triphosphate
ISP	Immature SP cell intermediate
MAML	Mastermind-like Protein
MAPK	Mitogen Activated Protein Kinase
MHC	Major Histocompatibility Complex
N3-IC Tg	Notch3 Intracellular Transgenic (mice)
NF-κB	Nuclear Factor Kappa-light-chain-enhancer of activated B cells
NK	Natural Killer
PBS	Phosphate-Buffered Saline
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll protein complex
PEST	proline, glutamate, serine, threonine
PIP2	Phosphatidylinositol-(4, 5)-bisphosphate
PIP3	Phosphatidylinositol-3-phosphate
PKB	Protein Kinase B
PKC	Protein Kinase C
PLC-β	Phospholipase C-β
RAM	RbP-jκ Associated Molecule
SDF-1α	Stromal Derived Factor-1α
SHAP	Serum-derived Hyaluronan-associated Protein
SMRT	Silencing Mediator for Retinoid or Thyroid-hormone receptors
SP	Single Positive
T-ALL	T-Cell Acute Lymphoblastic Leukemia
TAD	Trans-Activation Domain
TCR	T-Cell Receptor
TM	Transmembrane Region
WT	Wild-Type (mice)

1. INTRODUCTION

1.1. T-LYMPHOCYTES

1.1.1. The Immune System

The most important role of immune system is to protect the organism from foreign invasions without causing autoimmunity. It consists of lymphoid organs, tissues, and cells that clear and protect organism. In central lymphoid organs (bone marrow, thymus), these cells are produced and in others - peripheral (lymph nodes, tonsils, spleen...), they are only stored after maturation. Innate immunity is the first answer to foreign invasion, through physical barriers, inflammation and defense cells (nature killer cells, phagocytes…). Adaptive immunity is specific and enduring, but its response is slower. There, a part of the insulter (*antigen*) first binds to a major histocompatibility complex (MHC) class I or II and is eliminated by T–lymphocytes or antibodies, produced by B-lymphocytes (1).

1.1.2. T-lymphopoiesis

Differentiation into a T-cell is a multi-step process, beginning in the bone marrow (2). The pluripotent hematopoietic stem cell can differentiate into either common myeloid (leading to myeloid lineages; *erythrocytes, platelets, mast cells, myeloblasts*) or lymphoid progenitor cell. Further maturation of the latter leads to lymphocytes T, B and natural killer (NK) cells. B-cells remain and mature in the bone marrow, while the developing T-cells migrate to the thymus and are called thymocytes (3, 4). In the thymus, thymocytes undergo a series of processes, before becoming fully developed T lymphocytes. Cell type can be defined by specific receptors on cell surface, epitopes, or shorter CD (*Cluster of Differentiation*) - see Figure 1. T-cells express many markers, among them CD45 and CD3, CD4 and/or CD8, depending on the stage of maturation; which we can determine with the use of specific anti-CD antibodies.

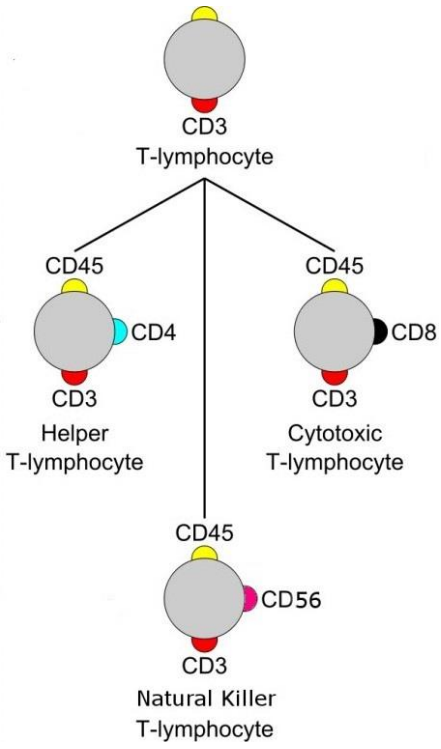

Figure 1: Cell markers on T-cells (5)

1.1.3. Thymic development

In thymus, T-lymphocytes turn from immature, double negative (DN) to mature, single positive (SP). At starting point, they express neither CD4 nor CD8 ($CD4^-$ $CD8^-$) markers (6). They progress through four different DN stages and develop distinct cell markers on the way: DN1 ($CD44^+$ $CD25^-$), DN2 ($CD44^+$ $CD25^+$), DN3 ($CD44^-$ $CD25^+$), DN4 ($CD44^-$ $CD25^-$), before finally reaching the double positive (DP) stage ($CD4^+$ $CD8^+$). During these stages, a specific T-cell receptor (TCR) is forming. At late DN2 and early DN3 stages, genes are rearranged in a way that TCR will have consisted of two protein chains (7); either αβ or γδ. Normally (in over 90%), precursors transform into αβ lineage. β-chain is rearranged to connect with pre-Tα chain at DN3 stage, forming a so-called pre-TCR, which allows the thymocytes to progress to DN4 stage. Cells, unable to form a working pre-TCR, are at that point removed by apoptosis (8). This process is called β-selection and ensures a functional precursor for the mature receptor. For a successful progression, all Notch3, CXCR4 and pre-TCR signals are needed (see chapters 1.3.4 and 1.5.4 for more detailed descriptions of CXCR4 and Notch3 receptor signaling in T-cell development). When a pre-TCR signaling occurs, a massive proliferation and differentiation of cells to DP stage follow. Only αβ-DP cells undergo positive and negative selections), in the end yielding a fully set TCR receptor.

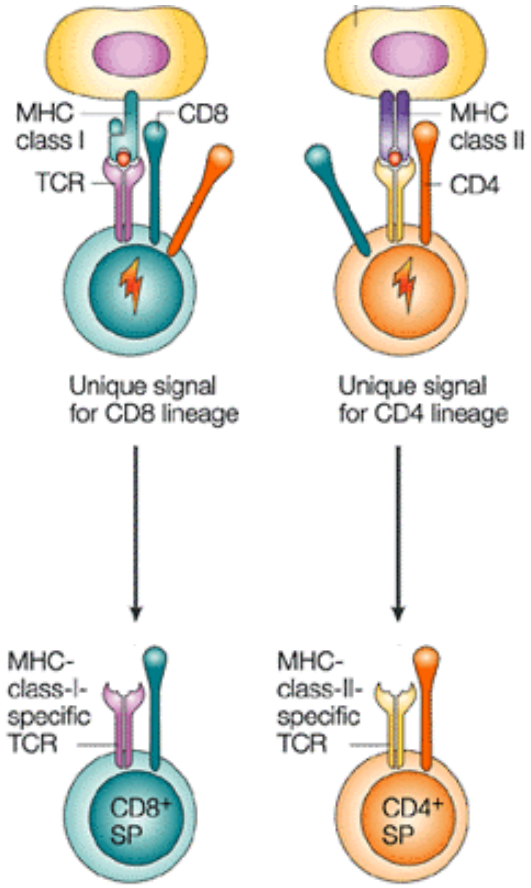

Figure 2: T-cell positive selection (adapted from 12)

This 'thymic education' starts with positive selection, where TCR needs to recognize and bind the molecules of self-MHC to receive a survival (positive) signal. T-cells can proceed, but the cells without the signal die due to no stimulation (9). The positive selection also determines T-cells' further commitment either $CD4^+$ or $CD8^+$ line (Fig. 2). DP cells interacting with MHC class I mature into SP $CD8^+$ cytotoxic T-cells (*Tc*), by down-regulating CD4 surface expression. MHC class II binding generates $CD4^+$ SP helper cells (*Th*) and down-regulates CD8

expression (4,10). After surviving positive selection, SP cells migrate into medullar area of the thymus. The cells that bind to self-MHC with a too high affinity are subject to apoptosis due to their high self-reactivity potential. This is called the negative selection and it removes the cells that could later on cause autoimmunity (11). Following this, mature SP thymocytes in their naïve form are released to peripheral (predominantly lymphoid) tissues, where they are ready to play their role as effectors in immune response.

1.1.4. Thymocytes localization in the thymus

DN1 and DN2 cells begin to migrate from cortico-medullary to the sub-capsular area, where DN3 are located and where β-selection starts (Fig. 3). This process, responsible for a productive rearrangement of pre-TCR complex, starts at DN3 stage and finishes at DN4. In DN4 stage, thymocytes begin migrating from the cortex back to medullar zone.

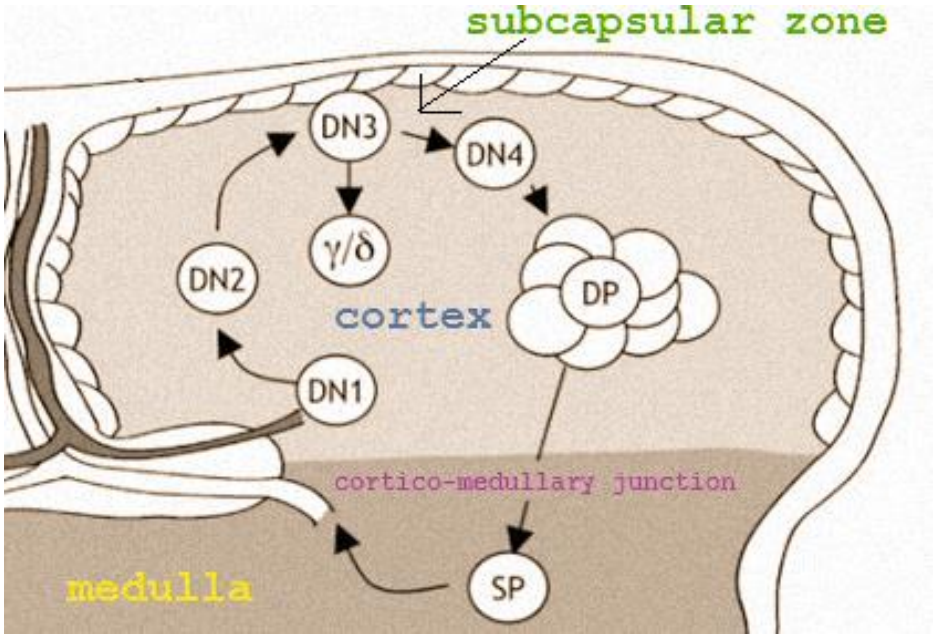

Figure 3: T-cell maturation from DN to DP and localization of these events in the thymus (adapted from 11)

1.2. T-CELL ACUTE LYMPHOBLASTIC LEUKEMIA

T-cell acute lymphoblastic leukemia (T-ALL) is a form of highly aggressive blood cancer, characterized by over-production of immature thymocytes (13). Clinically it shows as high white blood and blast cell counts (Fig. 4) and it often coincides with splenomegaly (enlarged spleen), enlarged lymph nodes and infiltration to other organs (14), including the central

nervous system in later stages. Among all acute lymphoblastic leukemia patients, there are about 15 – 25 % T-ALL cases (15 % pediatric, 25 % adult) (15). While many genetic factors and mutations may be involved in onset of human T-ALL, it has been determined that continuous Notch signaling is one of the biggest causes for this malignancy.

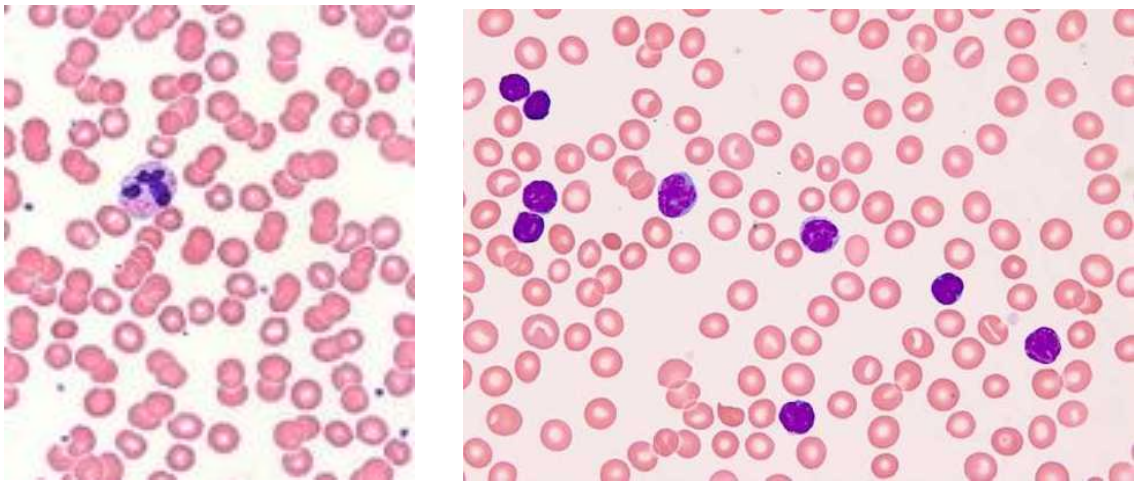

Figure 4: Normal (left) vs. T-ALL (right) blood smear (18, 19)

The oncogenic capability of Notch3 in T-ALL has already been shown at Department of Molecular Pathology, Experimental Medicine and Pathology of University La Sapienza in Rome with mice models. Function of Notch3 was displayed through forced Notch3 active form expression in immature thymocytes in transgenic mice (16). Furthermore, there is a connection between Notch3 signaling, CXCR4 cell surface over-expression and normal T-lymphopoiesis deregulation, which results in a rise of DP T-cell spreading via blood circulation (17). The migration to periphery of these immature cells increases the T-ALL aggressiveness.

New therapies with intense and toxic anti-neoplastic agents have shown an improvement in survival rate through the last decade - especially in children, but the disease is very prone to relapse and these patients are likely to develop a resistance to chemotherapy. Thus, the overall outcome is still very poor (20, 21).

1.3. NOTCH

1.3.1. Notch family

The Notch gene family is evolutionarily conserved and found in different organisms. It was first identified in *Drosophila melanogaster*, the fruit fly, by geneticist Thomas Hunt Morgan

and his colleagues in 1917. There is a direct correspondence between receptors and ligands of mammals, *D. melanogaster* and *Caenorhabditis elegans*. However, the number of paralogs differs among species:there are four Notch genes in vertebrates (NOTCH 1, 2, 3, 4), two in *C. elegans* (LIN-12 and GLP-1) and a single gene in *D. melanogaster*. The same connection applies to the ligands; in mammals there are five different ligands (Delta-like 1, 3, 4 and Jagged 1 and 2), while *D. melanogaster* and *C. elegans* both have two (Delta, Serrate and Lag-2, Apx-1, respectively) (22).

The four receptor homologs are expressed in a variety of tissues, often overlapping in their function, but sometimes having completely different roles (23). In many cases, this is due to the diverse tissue distribution and the amount of ligands or Notch receptor itself. It has become evident over the years that Notch regulates the cell fate early from embryonic development on, as well as in adult tissue homeostasis. Even more, the highly conserved Notch signaling pathway is the central mechanism in generating several mature cell lineages from their precursors, regulating cell differentiation, proliferation and apoptosis. It is essential in T-cell development and haematopoiesis and plays an important role in peripheral lymphoid cell regulation, therefore affecting the immune response (24, 25).

1.3.2. Structure of Notch receptors and their ligands

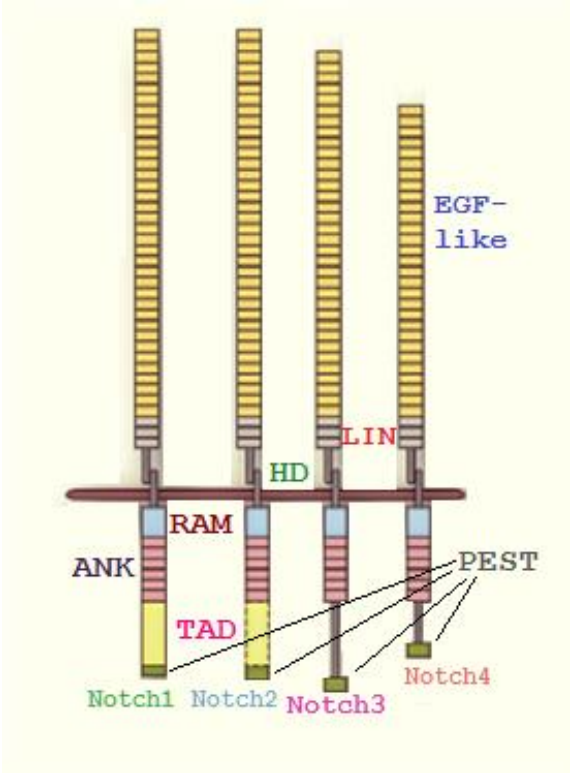

Figure 5: Notch receptors 1- 4 structure (adapted from 25)

The Notch genes encode a single-helix trans-membrane receptor, which is synthesized as a protein precursor. It then undergoes a cleaving in the Golgi apparatus, producing a heterodimeric receptor that consists of extra- and intra-cellular subunits, non-covalently connected through the heterodimerization domain (HD) (26). The extracellular domain, 17000 AA long, is involved in ligand interaction. It contains series of EGF-like (*Epidermal Growth Factor*) repeats; the number depends on species and receptors. Each repeat with 6 Cys

residues forms three disulfide bridges. The structure continues with three Cys rich LIN repetitions that prevent the Notch signaling cascade activation in the absence of ligands, and a transmembrane (TM) region (27). The intracellular domain (1000 AA) contains RAM (*RbP-jκ Associated Molecule*) region, followed by seven ankyrin domains (ANK). Next, there is a trans-activation domain (TAD), which is in its complete form found solely in Notch 1 and 2, and finally a PEST (proline, glutamate, serine, threonine) domain. PEST domain is involved in turnover of the receptor through proteosomal degradation, ensuring a short lived Notch signal - a key to avoiding the oncogenic receptor properties (28). This has recently become of great importance with identification of new mutations with deleted PEST domain, associated with T-ALL.

Notch3 detailed structure: 34 EGF-like repeats, 3 LIN repeats, TM region; HD; RAM, 7 ANK, a significantly shorter TAD and PEST (29) (Fig. 5).

1.3.3. Notch Signaling

Notch signaling is a complex process, as multiple pathways can lead to its activation, which can then be further modulated by numerous intra- and extracellular proteins; and finally, the processing of receptor itself is interconnected with its own pathway. Signaling is triggered by a receptor-ligand interaction, inducing a conformational change that leads to two consecutive proteolytic cleavages. First cut is mediated by metalloproteases of the ADAM (*A Disintegrin and Metalloprotease*) family and second one occurs at the TM level by a γ-secretase of a multi-protein membrane complex (30, 31).

Following these events, extracellular domain is endocytosed by the signaling cell (E3 dependent mono-ubiquintinylation process) and intracellular domain is free to enter the nucleus, where it transmits the signal dependently or independently of a DNA transcription factor CSL (*CBF1, Suppressor of Hairless, Lag-1*). In heterodimerization of intracellular domain and CSL, a short-lived complex is formed, regulating gene expression and converts the repressor CSL into a transcriptional activator (32). They bind weakly through ANK, but form a stronger connection through RAM domain, which, however, is not essential for the complex formation, while ANK is (23, 32, 33, 34). Once ANK region docks to CSL transcription factor, it recruits mastermind-like proteins (MAML1-3), recruiting additional co-activators (SMRT, SHAP, CI) (25). Not all signaling pathways require the CSL intervention. An alternative route involves Deltex, a cytoplasmic protein, binding to intracellular Notch region (35). In the end of this Notch signaling process, PEST domain

promotes the E3 polyubiquitinilation of intracellular domain and its degradation. The scheme of Notch signaling is represented in Figure 6.

There is a remarkable number of genes regulated by Notch, mainly important ones that decide the cell fate, and there is data suggesting that Notch can cooperate with other signaling pathways as well. Some major transcriptional targets include Hes (Hairy enhancer of split), Hey or Hrt genetic family members, especially HES1 and HES5 (36). HES1, in particular, is responsible for the expansion of immature thymocytes during T-cell differentiation, as well as silencing the CD4 expression in CD8 destined cells. Other genes can be tissue-specific. Some of Notch effects are provided by molecules that are not directly involved in the signaling pathway, but they influence it. Among these modulators there are factors such

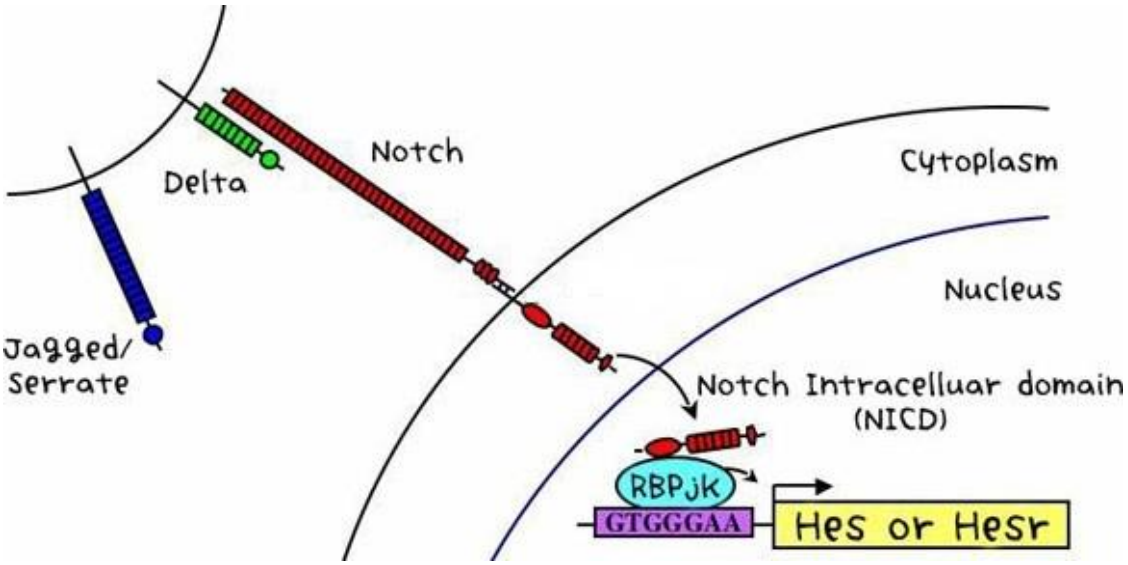

Figure 6: Schematic model of Notch signaling (39)

as Fringe, Numb and Itch, which modify its interaction with the ligands, its activity and the amount of Notch available, respectively (27).

Notch signaling disruptions are often associated with diseases. Notch has been implicated in various human diseases, in particular many different cancers, including T-ALL, where in 50% of the patients, sporadic mutations in the Notch receptor locus have been observed.

1.3.4. Notch receptor and its role in T-cell differentiation

Early in lymphopoiesis, Notch1 already regulates the T versus B cell fate decision (37, 38). Several studies have shown that in case of blocking Notch-mediated signal, T-cell differentiation is stopped at the initial stages, and there is an accumulation of B-cells (40, 41, 42). But the main events involved in T-cell differentiation occur in the thymus;

proliferation, specification of cell fate, or death of progenitor cells. These processes are regulated by several different factors, from pre-TCR and mature TC-αβ receptor, T-cells themselves, to other bio-regulators, such as the Notch family, particularly Notch 1 and 3 (43). Notch3 receptor expression increases in later DN thymocyte stages (DN3 and DN4) and during the conversion of cells from DN to the DP stage (44). *In vitro* experiments have shown that Notch3 is important for advancing from DN to DP. It is vital particularly for passing the β-selection in cooperation with CXCR4 and pre-TCR, and it promotes the progression beyond that checkpoint as well (45).

Notch1 expression is high in early DN thymocytes (DN1 and DN2). At later stages of development, its signaling is proposed to favor CD4 over CD8 T-lineage commitment and the αβ over γδ (46); moreover, αβ T-cell development in contrast to γδ requires constant Notch signaling.

The role of Notch3 receptor in thymocytes differentiation was confirmed in characterization of transgenic mice, over-expressing the IC form (16). Constitutive expression of this domain leads to specific alterations in differentiation of thymocytes, resulting in development and early onset of an aggressive form of T-cell leukemia/lymphoblastic lymphoma. The in-depth analysis of pre-tumoral thymocytes and the lymphoma cells allowed to identify the initial target in the immature DN thymocytes; alterations caused by Notch3 deregulation. In particular, a severe persistence of immaturity characters was observed: CD25 expression, presence of pre-TCR chain (pTα) and constitutive NF-κB activation, suggesting that Notch3 receptor can control crucial events in the late stages of DN thymocyte development (8, 47). These are the stages in which normally CD25 expression ceases and pTα allows for the pre-TCR formation.

Finally, it has been shown that the signaling pathway of Notch is required to sustain growth and survival of T-ALL leukemic cell lines, making it a very important and attractive target in therapy (36, 48).

1.4. TRANSGENIC MICE

1.4.1. Transgenic mice model creation

The term ‘transgenic’ is used to describe organisms with stable copies of one or more genes, originating from other species or genes that are not normally present in the host organism genotype (49). Mainly generated transgenic animals are mice. Following the insertion of

transgenes, we can either inspect the influence of oncogene on neoplasia, or over-expression of a cytokine on immune system or even design completely different transgenic models, specific to various diseases.

Transgenic models can be generated from different animals by microinjecting the cloned gene DNA fragment genes into fertilized eggs (oocytes) (50). Those are then transferred into the animal uterus and transferred to the children according to Mendel's law. This gene transfer is an important introspect into molecular pathways and as a way to uncover underlying pathological mechanisms offers an important base for all biomedical research and most importantly, new disease managing and therapy concepts.

However, it is important to be aware that sometimes, transgenic mouse models may have completely different outcomes than the same disease in humans.

1.4.2. Notch3-IC transgenic mice

To assess Notch3 effects on T-cell development, transgenic mouse model with over-expression of active intracellular form of Notch3 receptor (N3-IC) was generated as previously described in the reference Bellavia et al, 2000 (16).

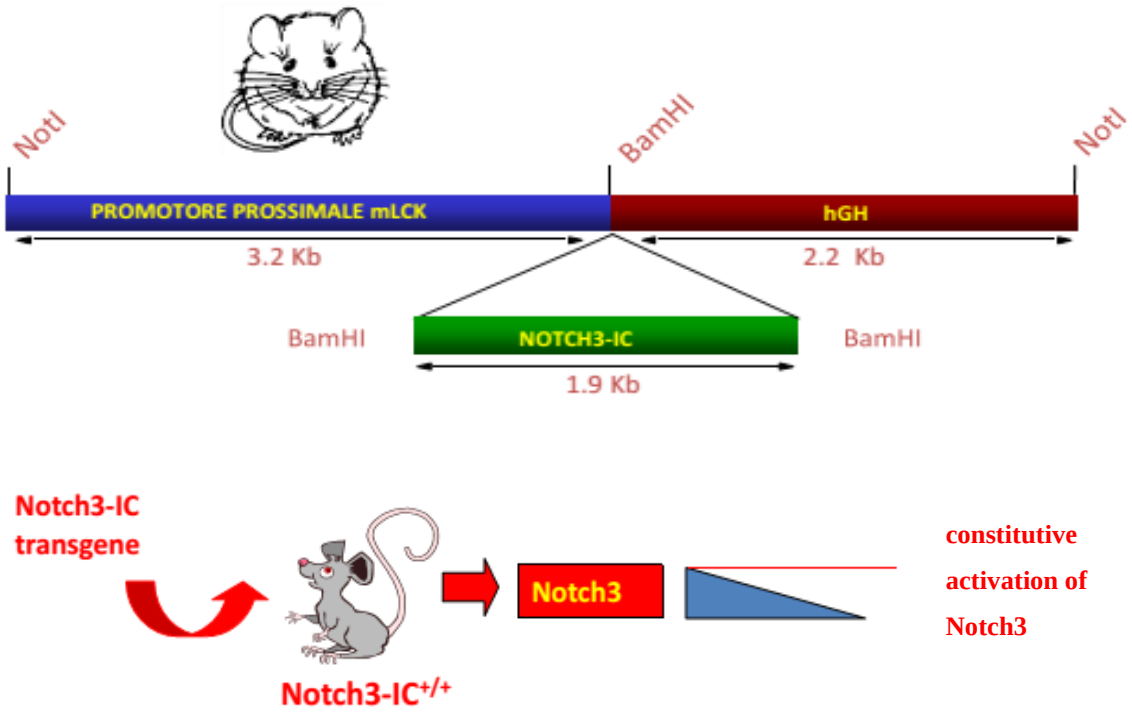

Figure 7: Generation of Notch3 transgenic mice: N3-IC encoding cDNA, tagged with the HA tag was ligated into BamHI vector, following the proximal *lck* promoter. This Notch3-IC transgene was inserted in mice, which caused a constitutive Notch3 activation.

The modified *lck*-Notch3-IC-hGH *Sac*II fragment was created by ligating the N3-IC cDNA into the BamHI vector, containing hGH (*human growth hormone*), using the proximal *lck* promoter (specific for thymocytes) (16, 51). It was subsequently inserted into mice, inducing a perpetual activation of Notch3 signaling - independently of ligand interaction (Figure 7). N3-IC was tagged with hemagglutinin (HA) epitope, to differentiate between endo- and exogenous expressions.

Immunophenotype of the thymus shows an increased absolute number of thymocytes, with altered differentiation (increased DN2 and DN3 subpopulations rather than DN4). The constitutive activation of Notch signaling in transgenic mice is demonstrated by increased expression of target genes HES1, HES5 and Deltex.

At 5-7 weeks of age N3-IC HA Tg mice already developed fast evolving aggressive T-cell lymphoblastic leukemias/lymphomas, prevailing in the spleen and lymph nodes, causing up to 6-fold increased size and weight of the organs.

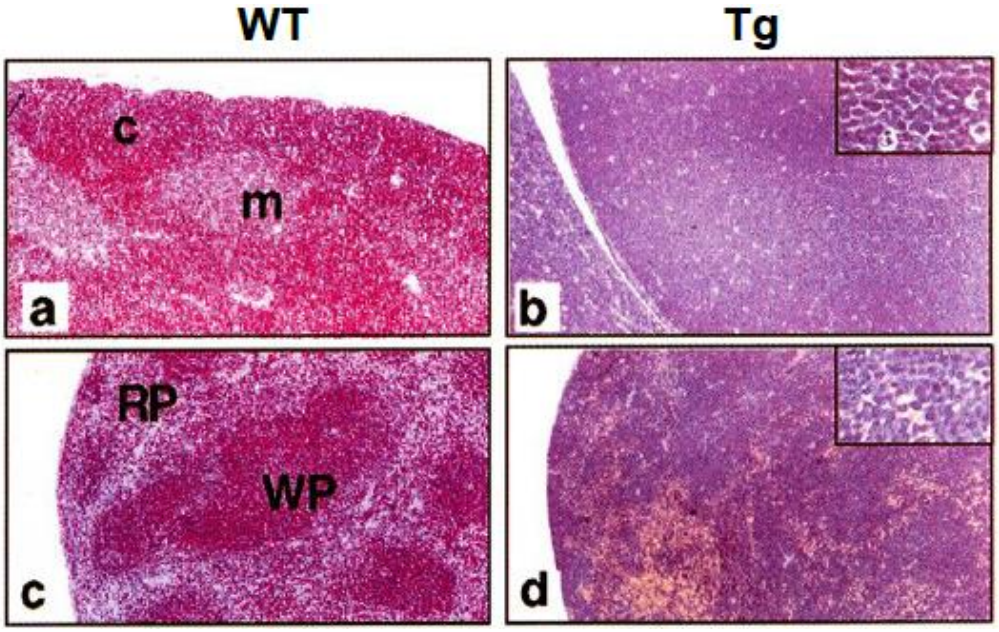

Figure 8: Histology of 12-weeks old mice; thymus (a, b) and spleen (c, d) show disrupted architecture in Tg animals; C=cortex, M=medulla, RP=red pulp, WP=white pulp (16).

Histology of these tissues and thymus showed an enormous disruption of normal architecture and invasion of lymphoblastic cells. The tumors retained the characteristics of immature T-cells; persistent CD25 and pTα expressions, constitutive activation of NF-κB and its anti-apoptotic function (17). By 16 weeks, 95% animals have died.

1.5. CHEMOKINE SYSTEM

1.5.1. Chemokines

The chemokine system is a very efficient and fast way of cell-migration management and a perfect means of communication between all cells involved in the immune response. The term ‘chemokine’ or chemotactic cytokine was introduced in 1992 to define a big family of cytokines with chemotactic activity. Chemotaxis is the act of inducing and controlling selective leukocyte movement towards the tissues (53). The cellular recruitment is regulated via interaction with different G-protein transmembrane receptors. Many chemokines have a high level of expression only during acute phases, inflammation, allergies, cancer, while others maintain a constant level and regulate normal homeostatic processes.

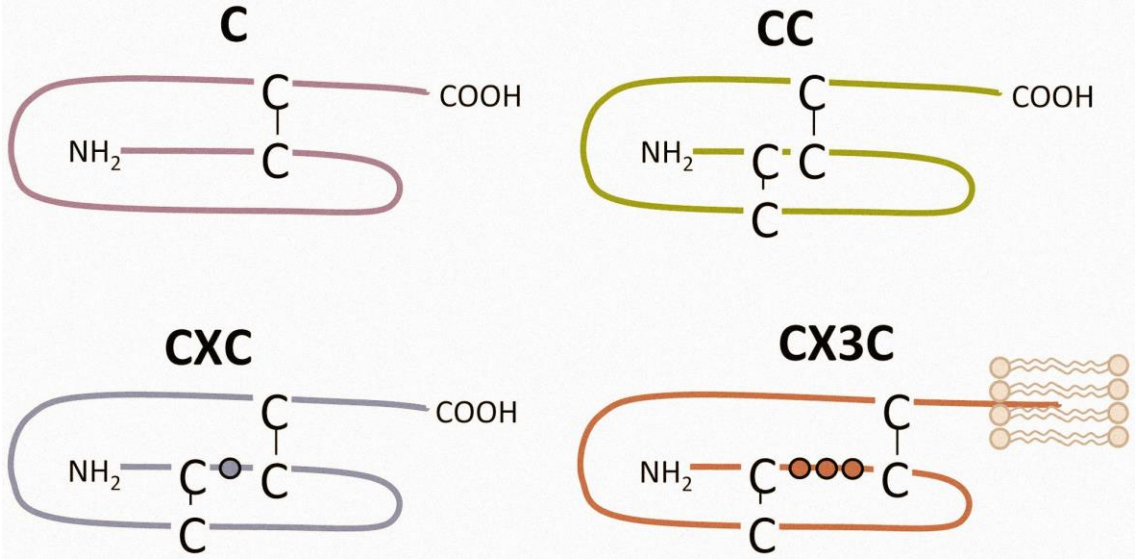

Figure 9: Cytokine subfamilies; showing Cys residues and AA (adapted from 56)

Structurally, they are small signaling proteins, consisting of up to 127 AA (8 – 10 kDa), usually with four highly conserved cysteine (Cys) residues (54). First two Cys are close to N-terminal end, third in the middle, and the fourth at C-terminal end and through Cys-Cys disulphide bridges they form a common and specific tertiary structure.

Chemokines can be divided into four subfamilies, depending on their Cys residues position and the number of AA separating them at the N-terminal end: CXC (α): two Cys residues, separated by one AA, CC (β): two Cys residues that are next to each other, XC (C or γ): only 1 Cys residue, CX3C (CXXXC or δ): two Cys residues separated by three AA (55) (Fig. 9). Based on their expression profile, we can divide them in two groups. Inducible or inflammatory chemokines are typically expressed by leukocytes, after a foreign stimulation

triggers the movement of defense cells towards the site of inflammation. Constitutive (homeostatic) chemokines on the other hand have a crucial role in organ development, tissue reparation etc. and are expressed all the time. They normally participate in lymphocyte migration and other cellular movement in haematopoiesis. Nevertheless, some chemokines cannot be classified in any of the two groups, since they are able to function as both. This group is believed to be highly selective for lymphocytes, particularly for T-cell development and recruitment.

1.5.2. Chemokine receptors and signaling

The signaling is transmitted by surface cell receptors, belonging to a larger family of G-protein coupled receptors (GPCRs). They form a single polypeptide chain with seven trans-membrane helical domains; three intra- and three extracellular loops. N-terminal end of the peptide lies on the outside of the cell and is the part that binds the ligand – therefore it establishes the specificity, while C-terminal end on the inside is important for the intracellular signaling process (57). The receptors are also divided in subgroups, depending on the chemokine family they bind: CCR (binds CC chemokines), CXCR (CXC), XCR (XC) and CX3CR (CX3C). Unusual characteristic is their selectivity for chemokines, as it varies widely among receptors and ligands. The receptors can recognize several chemokines - and *vice versa* (58). Current data accounts for about 20 chemokine receptors and about a double number of their ligands.

Heterodimeric G-proteins are composed of three units – G_α, G_β and G_γ, as depicted in Figure 10. α and β units are covalently bound to lipid tails, anchoring G-protein to cellular membrane. In the inactive form, G_α subunit is bound to a GDP (*guanosine diphosphate*)

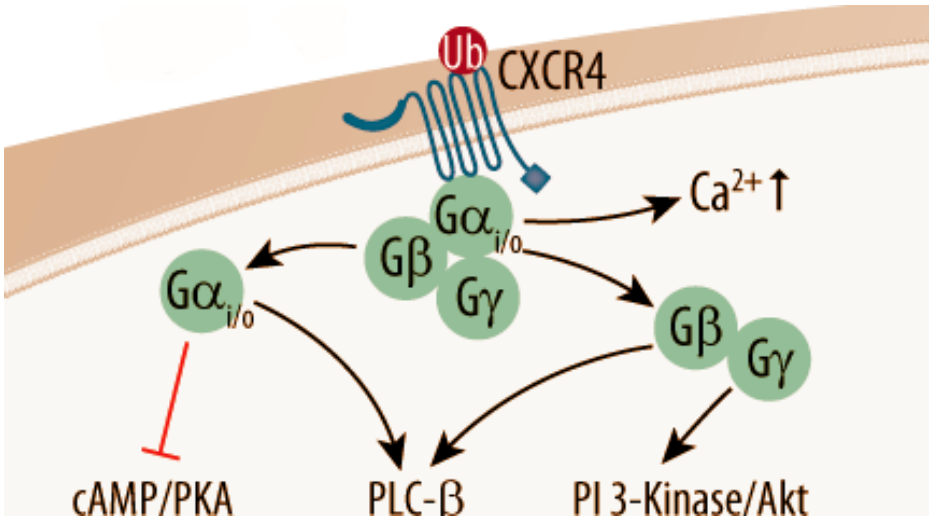

Figure 10: Schematic model of CXCR4 GPCR receptor and its effects (adapted from 61)

molecule. Upon binding of a specific ligand, chemokine receptors are activated. At that point, GDP is released and GTP (*guanosine triphosphate*) is bound instead. This substitution causes dissociation of G_{α} and $G_{\beta\gamma}$ dimers from the receptor. Both components then interact directly with effector proteins and activate different signaling pathways (59, 60). $G_{\beta\gamma}$ starts the following cascade: phospholipase C-β (PLC-β) enzyme cuts PIP2 (*phosphatidylinositol-(4, 5)-bisphosphate*) into two smaller parts, one forming IP3 molecule (*inositol triphosphate*) that causes an increase of intracellular Ca^{2+}. The other part, DAG (*diacylglycerol*), activates protein kinase C (PKC), which through Erk 1 and 2 (*extracellular signal-regulated kinases 1, 2*) induces chemotaxis. Reactive oxygen species production is also encouraged through nitric oxide induction by DAG and Ca^{2+}. Meanwhile, G_{α} unit causes an increase in levels of PIP3 (*phosphatidylinositol-3-phosphate*), activation of protein serine kinases PKB (*Protein Kinase B*) and Akt, PKC and MAPK (*mitogen activated protein kinases*). Duration of GPCR signaling depends on half-life of GTP-bound G_{α}. GTP hydrolysis leads to inactivation of this α unit and its re-association with βγ dimer, terminating all interactions with effectors.
Chemokine signaling is complicated, and it has to be mentioned that all of these pathways are not yet completely understood. Therefore they are still a subject of research.

1.5.3. CXCR4

CXC chemokine receptor 4 (CXCR4) is a protein, until recently thought to be the only chemokine receptor to bind stromal derived factor-1α (SDF-1α) or CXCL12. CXCR4 is expressed in a wide range of different tissues, although predominantly in hematopoietic and immune cells. Also known as fusin, it is considered a modulator in many physiological processes, forming intricate axes together with CXCL12 and CXCR7, that will be a subject to many researches in near future. It is renowned for its role as a HIV infection mediator and promoter of cancer metastasis. Recent evidence has demonstrated that CXCR4 is also capable of promoting the anti-apoptotic factor Bcl2A1 expression, since mRNA expression of Bcl2A1 is directly controlled by the p65 subunit of the NF- κB (62). The latter is activated by Notch signaling pathways and pre-TCR receptor in a ligand-independent way.

1.5.4. CXCR4 in thymocytes

Based on the literature, CXCR4 surface expression on DN thymocytes differs in in various maturation stages in different thymic areas; it is highest in DN2 and DN3, while completely absent in the SP cells (63, 64). Thymic microenvironment consists of various anatomical

compartments that promote T-cell progenitors' differentiation and proliferation from the bone marrow on (65). T-cell progression through stages of development is influenced by two factors: one is the location of cells inside the thymus; molecules of the thymic epithelium. Second are the receptors on thymocytes themselves. Particularly SDF-1α, expressed highly in the cortex, and CXCR4 on thymocyte surface are believed to have a crucial role in regulation of $CD25^+$ thymocytes stable localization in the subcapsular zone rather than in the lower part of the cortex. It has been demonstrated on animal models that mice without expression of either CXCR4 or SDF-1α have a defective DN cell migration from subcapsular to cortical region and subsequently have an impaired T-cell transition from DN to DP stage (66). The thymocytes migration ability has an important target in CXCR4 signaling - phosphorylation of Erk; since Erk inhibitors induce the blocking of migration, as observed in DN thymocytes in response to SDF-1 (67). CXCR4 also acts as a co-stimulator with pre-TCR to provide survival signals essential during the maturation of thymocytes and therefore promotes optimal proliferation of DN thymocytes (45). There is a lot of evidence supporting the idea that SDF-1/CXCR4 axis has a fundamental role in tissue development – that is, in fact, essential for progenitor cell migration during embryonic haematopoiesis and organogenesis, vascularization and later on organ homeostasis and tissue regeneration (68, 69).

1.5.5. The role of CXCR4 in cancer

The importance of these polypeptides shows also the fact that they play an important role in different pathological processes, such as inflammation, infections, allergies, tissue damage, cardiovascular diseases and also malignancies. It has already been established that chemokines promote tumor angiogenesis, facilitate the communication between cancerous and non-cancerous cells in tumor microenvironment and are therefore probably favoring the tumor survival (58, 68). Over-expression of CXCR4 receptor has been shown in over 23 various types of cancers; from kidney, lung, brain, ovaries to leukemias (Figure 11). Due to this evidence, it is considered a poor prognostic predictor. In T-ALL, CXCR4 over-expression in periphery severely increases T-ALL aggressiveness. And in addition to communication, it also aids tumor growth, metastasis and has a role in occurrence of therapeutic resistance (58).

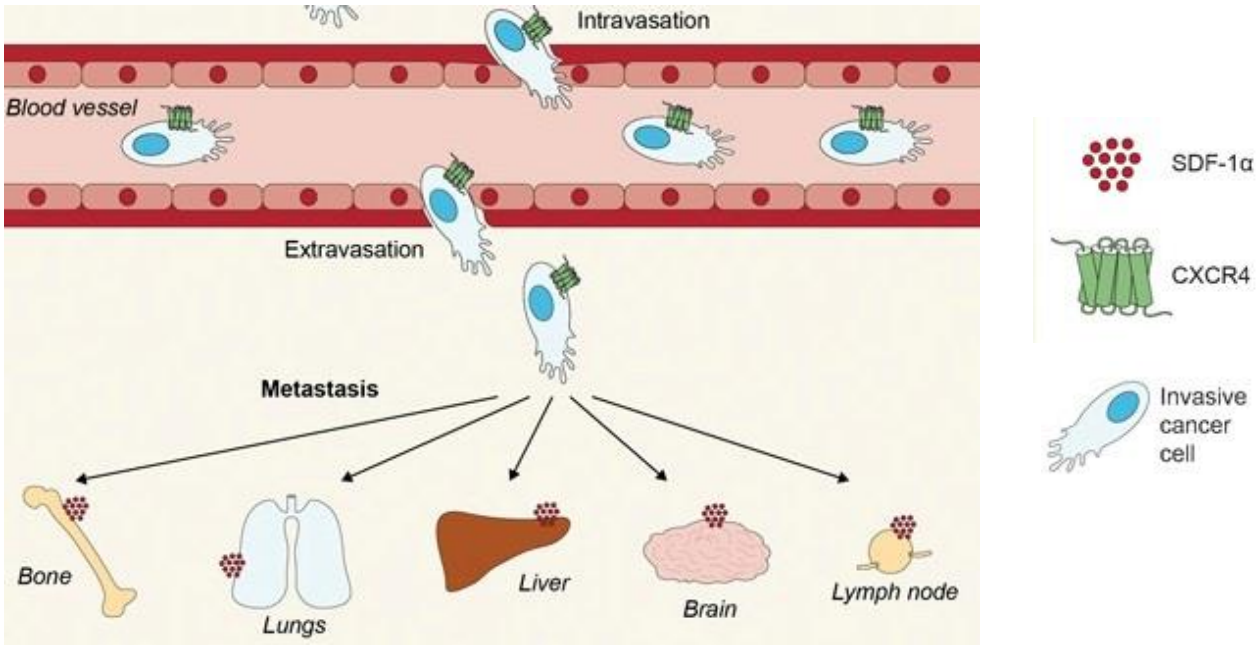

Figure 11: CXCR4 over-expression favors cancer spreading via blood circulation (adapted from 58)

All these characteristics make them an attractive target for a research of inhibiting agents of both the tumor growth and metastasis. But due to its essential role in normal organism functioning as well, its targeted therapy should be well considered. It is thus very important to fully understand its biology, its physiological and pathological role.

2. THE AIM OF OUR WORK AND A WORKPLAN

Notch signaling plays an important role in the early stages of T-cell differentiation and leukemogenesis, and for this reason we decided to use our laboratory created Notch3-IC transgenic (N3-IC Tg) mice for the research. Prominent features of these mice are activation of pre-TCR and constitutive activation of NF-κB, both indispensable to leukemia development (43). In thymus of young N3-IC transgenic mice (5 – 7 weeks), an increased number of DN thymocytes ($CD4^-CD8^-$) can be observed, followed by a failure of turning off the CD25 expression, thus deregulating the normal pre-T / T-cell transition (transformation from immature into fully mature T-cell; see chapter 1.1.3.). In the next phase, mice develop an aggressive T-cell lymphoma with a high level of penetrance. The tumor cells show a persistent expression of CD25 on the cell surface; they are over-expressing the pTα / pre-TCR (and its signaling) and show a constitutive activation of NF- κB, a transcription factor that promotes signals for the survival of immature thymocytes by prompting the anti-apoptotic (Bcl2-A1) and proliferative mechanisms. These three factors are among the most significant of T-lymphoma features (17). Inside the lymphoid organs (thymus, spleen, lymph nodes) complete tissue architecture subversion is observed, thus sustaining a deregulated T-cell differentiation. This data acknowledges Notch3 as an important inducer of tumorigenesis in T-cells. But, it has also been noted that signaling of Notch3 alone is insufficient for these β-selection-associated metabolism alterations.

Indeed, we were particularly interested in the chemokine receptor CXCR4 due to the fact that CXCR4/CXCL12 axis in combination with pre-TCR and Notch defines the β-selection; they enable cellular changes in DN3 cells and their differentiation into $CD4^+CD8^+$ (DP) cells (63, 64). Also supporting our premise is the data showing CXCR4 expression on the surface of all DN1 to DP thymocytes, and none at all on the SP cells (62). This suggests that Notch3 by modulating the expression of CXCR4 during migration of T-cell precursors in different thymic compartments, may interfere with normal development and can, in fact, induce T-ALL.

The main aim of the study is to first, find if there is; and then to understand the possible crosstalk between Notch3 and CXCR4 pathways, possibly impairing early deregulated T-cell differentiation and leading to T-ALL.

For these reasons, we will analyze expression of CXCR4 receptor in wild-type (WT) and pathological model N3-IC Tg mice, over-expressing the gene for intracellular Notch3 receptor form, using flow cytometry.

- First, through a specific staining with different fluorochrome conjugated antibodies we will evaluate the distribution of four main T-cell groups in the thymus; DN ($CD4^-$ $CD8^-$), DP ($CD4^+CD8^+$), SP $CD4^+$ and SP $CD8^+$.

- Then we want to investigate the CXCR4 surface expression on:
  - DN thymocytes, excluding the NK-positive population (DN and NK negative cells)
  - DP thymocytes and splenocytes and
  - anomalous represented DP splenocytes in N3-IC Tg mice.

  Indeed, DN-NK cells can express low levels of CXCR4. Using appropriate anti-NK antibodies, we will need to make the analysis in order to exclude possible results misinterpretation due to dilution effect on the account of the NK cells.

- Moreover, we will evaluate the CXCR4 expression of every DN stage separately, using additional antibodies, anti-CD44 and anti-CD25, to sort the DN T-cells in four more groups (DN1-DN4).

- We also wish to see if there is a co-expression of Notch3 and CXCR4 receptors in DP cells of thymocytes and then splenocytes, since we assume this adds to the cancer aggressiveness and metastasis.

- With the use of PCR, we will evaluate the mRNA expressions of:
  - CXCR4 and
  - anti-apoptotic Bcl2A1,

  to understand the up- or down-regulation mechanisms. The Bcl2A1 mRNA was already reported to increase with CXCR4 increase (62).

- To further explore the mechanisms, we will analyze EpCAM (*Epithelial Cell Adhesion Molecule*) surface expression, a known homotypic adhesion molecule, whose deregulation may be associated to lymphoid architectural disorganization (71).

- In addition, we will perform *in vitro* migration testing to determine the modulatory effect of Notch3 in SDF/CXCR4 chemotactic properties of DN and DP T-cells.

3. MATERIALS AND METHODS

3.1 . MATERIALS

3.1.1. Mice models

Experiments were performed using animal models with different genotypes, but all coming from the same genetic background: C57B1/6 (BK8) wild type (WT) and transgenic (N3-IC Tg) mice.

Generation of this model was done at Department of Experimental Medicine and Pathology of University La Sapienza in Rome and is described in the reference Bellavia et al, 2000 (16). All mice we used for the experiments were between 6 and 12 weeks old.

3.1.2. Antibodies, chemokines, reagents

Antibodies were conjugated with fluorophores:

- fluorescein isothiocyanate (FITC)
- R-phycoerythrin (R-PE)
- allophycocyanin (APC)
- peridinin-chlorophyll protein complex in tandem with Cyanine 5.5 (PerCP-Cy5.5)

Antibodies were specific against:

- CD4 APC (rat anti-mouse, clone RM4-5, 553051, BD Bioscience Pharmingen),
- CD4 PerCP-Cy5.5 (rat anti-mouse, clone RM4-5, 550954, BD Bioscience Pharmingen),
- CD8 APC (rat anti-mouse, clone 53-6.7, 553035, BD Bioscience Pharmingen),
- CD8 PerCP-Cy5.5 (rat anti-mouse, clone 53-6.7, 551162, BD Bioscience Pharmingen),
- CD44 PE (rat anti-mouse, clone Pgp-1 Ly-24 M7, BD Bioscience Pharmingen),
- CD25 FITC (rat anti-mouse, clone 7D4, BD Bioscience Pharmingen),
- CD45R/B220 PE (rat anti-mouse, clone RA3-6B2, BD Bioscience Pharmingen),
- CD90.2/Thyl FITC (rat anti-mouse, clone 53-2.1, 553004, BD Bioscience Pharmingen),
- NK1.1 FITC (rat anti-mouse, clone PKl36, 553164, BD Bioscience Pharmingen),
- EpCAM APC (rat anti-mouse, clone G8.8, ab95641 Abcam)
- CXCR4 (CD184, clone 2Bl1, eBioscience)

Isotype controls:

- IgG2b PE (rat anti-mouse, clone RTK4530, BD Bioscience Pharmingen)
- IgG2a APC (rat anti-mouse, clone RMG2a-62, BD Bioscience Pharmingen)
- IgG FITC (donkey anti-goat, sc-2024, Santa Cruz Biotechnology)

Chemokines:

- SDF-lα (Protech)
- SLC (Protech)

Chemokines were reconstituted in Milli-Q® water to the concentration of 1mg/mL to a 'STOCK' solution and then aliquoted as needed

Other reagents:

- DMEM (Dulbecco's Modified Eagle's Medium) High Glucose without L-Glutamine (EuroClone)
- D-PBS (Dulbecco's Phosphate-Buffered Saline) without Ca^{2+} and Mg^{2+} (EuroClone)
- Milli-Q® Type I ultrapure water (Merck KgaA)
- ACK/ammonium chloride lysis buffer (NH_4Cl 0.15 M, $KHCO_3$ 1 mM, Na_2EDTA x $2H_2O$ 0.1 mM)
- RPMI 1640 Medium (Thermo Fisher Scientific)
- BSA (Bovine Serum Albumin) (Thermo Fisher Scientific)
- 25 mM HEPES (4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid) (Thermo Fisher Scientific)

3.1.3. Utensils, machines and software

- Automatic Pipettes - pipettors: P2 (0.2-2µL), P10 (1-10µL), P20 (2-20µL), P100 (20-100µL), P200 (50-200µL), P1000 (200-1000µL), (Gilson, Inc.)
- 15 mL, 50 mL Falcon Tubes (BD Biosciences)
- Eppendorf Tubes® 1.5 mL, 2.0 mL (Eppendorf AG)
- 2, 5, 10, 25 mL Falcon pipettes (BD Biosciences)
- Cell filters 70µm Falcon; (BD Biosciences)
- Transwell plates with 24 wells; 5 µm pores (Corning Costar Corp)
- Syringes, needles (BD Biosciences)
- LD column, coated with anti-CD8a Ly-2 microbeads 130-049-401 (Milenyi Biotec)

- NanoDrop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific)
- Quantity One® 1-D analysis software (Bio-Rad Laboratories)
- FacsAria Cell Sorter (FACS) (BD Biosciences)
- FACSCalibur (BD Biosciences)
- CellQuest (BD Biosciences)
- RNeasy Micro Kit – RNA extraction kit (Quaigen)
- Light Microscope

3.2. METHODS

3.2.1. Cell extraction from the organs

All tissues and organs we used for experiments (spleen and thymus) were from both WT and N3-IC Tg mice, sacrificed by cervical dislocation The animals were between six and twelve weeks of age.

Thymuses were stripped of their connective tissue capsule and blood capillaries, but the spleens did not need any preparation. Next, we mashed all the organs (thymuses and spleens) separately into a clear suspension and filtered it in a tube using 70-μm cellular filters. This way we obtained lymphocytes in form of cell-suspension in a DMEM medium. After that, we preformed one suspension wash with DMEM, centrifuging each sample at 1250 rpm at + 4° C for 7 minutes, to eliminate the debris. Following that, we re-suspended the samples in new DMEM solutions. To count the cells, erythrocytes then had to be removed from the samples by room-temperature incubation with ACK lysis buffer.

3.2.2. $CD4^-/CD8^-$ thymocytes purification

Thymocytes, obtained through extraction from WT and N3-IC Tg mice were depleted of $CD8^+$ cells by marking them with anti-CD8 antibody, conjugated to magnetic beads, and passing them through an LD column. LD column was used following the manufacturer's instructions.

The eluted cells, enriched with $CD4^+/CD8^-$ and $CD4^-/CD8^-$ populations, were labeled using specific antibodies; anti-CD4 PE and anti-CD8 PE. PE negative cells (DN cells) were sorted using 'Fluorescence Activated Cell Sorting' type of flow cytometry by FACSAria Cell Sorter. We used cells with purity greater than 95%.

3.2.3. Phenotype analysis by flow cytometry

Flow cytometry principle

Flow cytometry (FC) uses light to count and characterize cells. Cells enter the system in a fluid medium and go one-by-one through a beam of light. When passing this laser, they scatter its light, and any present fluorescent molecule emits fluorescence (70). These events are detected by sensors, translating them into electronic signal and saving onto computer for specific analyses. Parameters measured with FC are physical properties (size and internal complexity) and fluorescence intensity. Forward-scattered light (FSC), detected in the direction of the laser beam is proportional to cell size. Side-scattered light (SSC), detected at 90° to the laser, reflects cell complexity; granulated cells (neutrophils) produce more SSC than simpler cells (71). Gating is a way of defining subpopulations within the data by manually defining numerical parameters to limit the samples; e.g. with FSC/SSC plot we defined the lymphocyte population and further divided it by fluorescence intensity of fluorophores, bound to cell markers CD4 and CD8 (Fig. 12).

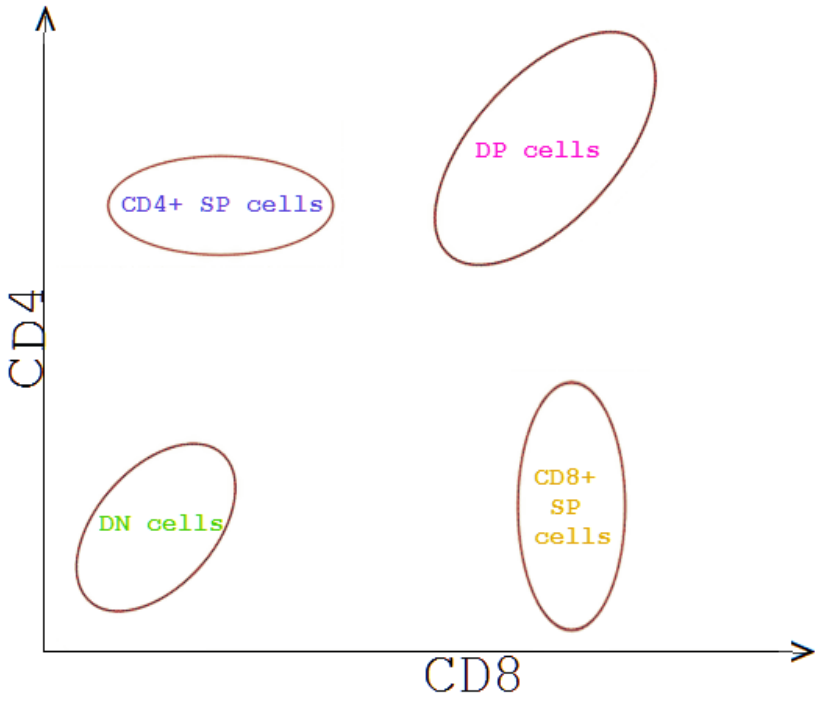

Figure 12: DN, DP and SP populations, separated with FC, based on their CD4 and CD8 surface expression

A special type of FC, Fluorescence Activated Cell Sorting (FACS), can be used for cell sorting and purification. The machine separates selected cells by rerouting them out of the stream to a special tube for further use instead of going to waste.

Flow cytometry experiments

The expression of cell-surface molecules was determined using standard flow cytometry experiments. For staining against extracellular antigens in each tube (sample), approximately $1x10^6$ cells were used. This short process summary was almost identical for all FACS experiments: first, thymocytes isolated from thymus or spleen were washed in fresh PBS 1X and centrifuged. Cells were then incubated in the dark with appropriate specific antibodies, conjugated with fluorescein proteins (FITC, APC, R-PE, PerCP-Cy5.5) for 15 minutes on ice, except for CXCR4 and its isotype control (45 minutes at room temperature).

All antibodies were used at the final concentration of 1: 200, in a multi-parametric analysis with four sample colors. The monoclonal antibodies used for samples were anti-CD4 PerCP, anti-CD4 APC, anti-CD8 APC and anti-CXCR4 PE. The monoclonal antibody anti-IgG2b PE was used as a control for anti-CXCR4 immunoglobulin. At the end of incubation times, 1 ml of PBS 1X was added to each tube of cell samples to remove the unbound antibodies, centrifuged again and removed. Finally, the samples were assessed by 2-laser FACSCalibur flow cytometer and analyzed using the CellQuest software. Dead cells were excluded from the analysis by gating the viable cells, manipulating the parameters of size and granularity (FSC/SSC).

Immunofluorescence intensity of the samples was normalized in relation to fluorescence of their corresponding isotype control, which was stained with the same protocol. For each sample tube, at least 10,000 positive events (cells) were analyzed with FACS.

To characterize different T-lymphocyte populations, we used anti-CD4 PerCP-Cy5.5 and anti-CD8 APC antibodies. In order to define the DN subpopulations (DN1-DN4), we used anti-CD44 FITC and anti-CD25 APC antibodies.

Details of the experiments:

I. Dividing the subpopulations

For both genotypes (WT and N3-IC Tg), we prepared two samples with approximately $8x10^5$ cells and colored them with specific anti-CD4 PerCP-Cy5.5 and anti-CD8 APC antibodies to allow for selective analysis of DN cells.

II. CXCR4 expression on DN cell surface

Thymocytes ($8x10^5$) were stained with anti-CD4 PerCP and anti-CD8 APC as in analysis I. Additionally, they were incubated with specific anti-CXCR4 PE antibody or alternatively with IgG2b PE as the isotype control. Staining with this antibody is necessary for normalization of CXCR4 expression; to eliminate the background 'noise'. The triple staining allowed us to evaluate the expression of CXCR4 receptor and its control only in DN cells population; CD4 and CD8 negative. The CXCR4 positivity rate of these cells was calculated by subtracting the control sample value from the results.

III. CXCR4 expression on DN cell surface, exclusion of NK cell population

Each sample ($8x10^5$ cells) was stained with specific anti-CD4 PerCP, anti-CD8 APC and anti-NK1 FITC. After that, they were incubated with specific anti-CXCR4 PE and IgG2b PE control. This quadruple staining allowed for exclusion of DN/NK^+ cells and evaluation of CXCR4 receptor or its control expression in the $DN/NK1.1^-$ population.

IV. Expression profile of CXCR4 in different DN maturation stages

Approximately $1x10^6$ thymocytes from both genotypes were stained with specific anti-CD4 PerCP, anti-CD8 APC, anti-CD44 FITC, anti-CD25 APC and anti-CXCR4 PE or its isotype control IgG2b PE antibodies. After excluding all $CD4^+$ and $CD8^+$ containing subsets, we divided the remaining DN cells by CD44 and CD25 expression to evaluate each individual DN1-4 stage CXCR4 and control expressions. The percentage of positivity was calculated by subtracting from the value of CXCR4 that of its IgG2b isotype control.

V. Surface expression of EpCAM molecule on DN cells

About $1x10^6$ thymocytes of WT and N3-IC Tg mice were stained with anti-CD4 PerCP, anti-CD8 APC antibodies, and incubated with specific anti-Ep-CAM APC antibody or its isotype control IgG2a APC. The triple staining allowed for limiting the evaluation of EpCAM or its control expression only to the DN population. Anti-IgG2a, normalizing the expression of antibody of interest, was necessary to measure the actual expression. The positivity rate was calculated by subtracting the value of control from EpCAM-positive values.

VI. CXCR4 expression on DP cell surface

The same triple staining as in analysis II, except we gated the DP cells and evaluated the positivity rate of CXCR4 expression on these. Final value was obtained by subtracting the value of control expression from CXCR4 results.

VII. Notch3 and CXCR4 co-expression on DP cell surface

Quadruple staining: anti-CD4 PerCP-Cy5.5, anti-CD8 APC, both with anti-CXCR4 PE and anti-Notch3 FITC. As controls, we used anti-IgG2b PE for CXCR4 and anti-goat IgG FITC for Notch3. This way we will be able to detect the cells with presence of both receptors.

VIII. CXCR4 and Notch3 (co-)expression on DP cells in the spleen

For CXCR4 expression profile of splenic DP cells, samples were prepared with about $1x10^6$ cells. Each sample was stained with specific anti-CD4 APC, anti-CD8 PerCP and anti-FITC Thy1 antibodies. The last antibody is a specific marker for T-cells, used to exclude all non-T-cells in the sample from analysis. Then we incubated with specific anti-CXCR4 PE or IgG2bPE isotype control. Second analysis for Notch3 and CXCR4 co-expression was similar, except we added the anti-Notch3 FITC. As a control, we used anti-IgG2b PE for CXCR4 and anti-goat FITC for Notch3. The use of markers CD4 and CD8 allowed for specific selection of DP spleen cell population, and then a simultaneous detection of CXCR4 and Notch3 presence on these cells.

3.2.4. RNA extraction

Total RNA of T-cell was extracted from thymuses of both WT and N3-IC Tg mice, using an RNeasy extraction kit (Micro Kit Quiagen) following the manufacturer's protocol. All samples were kept on ice after isolation and tips with filter were used.

3.2.5. RT-PCR

Expressions of CXCR4 and Bcl2-A1 were determined using the RNA obtained with isolation (3.2.4 RNA extraction), in a process of reverse transcription polymerase chain reaction (RT-PCR), followed by 1.5% agarose gel analysis. A quantity of 1 μg of the RNA extracted was subjected to retro-transcription (first step in RT-PCR), as previously described (44). That way we generated a stable complementary DNA (cDNA) from freshly isolated RNA with the use of enzyme reverse transcriptase. This cDNA transcript is necessary for

the coupled PCR reaction, as the enzyme Taq polymerase used in the process is a DNA polymerase, meaning it only amplifies the DNA chains.
Semi-quantitative PCR was performed at appropriate annealing temperatures and using the following primers for different samples:

Mouse CXCR4:	Forward 5'-TCCTCATCCTAGCTTTCTTTGC-3'
	Reverse 5'-TTGCATAAGGGTTAGCTGGAGT-3'
	62° C
Mouse β-actin:	Forward 5'-GTGGGCCGCTCTAGGCACCAA-3'
	Reverse 5'-CTCTTTGATGTCACGCACGATTTC-3'
	60° C
Bcl2-A1:	Forward 5'-ATTCCAACAGCCTCCAGATATG-3'
	Reverse 5'-GAACAAAATATCTGCAACTCTGG-3'
	57° C

Sample loading was monitored with the use of β-actin transcript; a normalizing reference control. To quantify the expression levels, all, CXCR4, Bcl2-A1 and β-actin products were analyzed at cycles 25 and 35 (the reaction was stopped; the samples were frozen and afterwards loaded together at the end of the reaction).

All PCR products (CXCR4 cycles 25 and 35, Bcl2-A1 cycles 25 and 35, β-actin cycles 25 and 35) were analyzed together, using 1.5% agarose gel with Midori green nucleic acid stain solution (for UV detection of DNA), and all the samples were loaded using loading buffer.

3.2.6. Migration testing

Chemotaxis analysis *in vitro* was performed with CXCL12 (SDF-1α), a natural and specific ligand for CXCR4. It was tested at two different concentrations, 20 and 200 ng/ml. To verify the migration specificity, we prepared a parallel test with another ligand, SLC, which stimulates the receptor CCR7 rather than CXCR4, at concentration of 1 μg/ml. We set up migration tests using $1x10^6$ thymocytes, taken from thymuses of N3-IC Tg and WT mice.

The cells were subjected to *in vitro* migration in response to a physiological signal; treatment with the specific ligand.

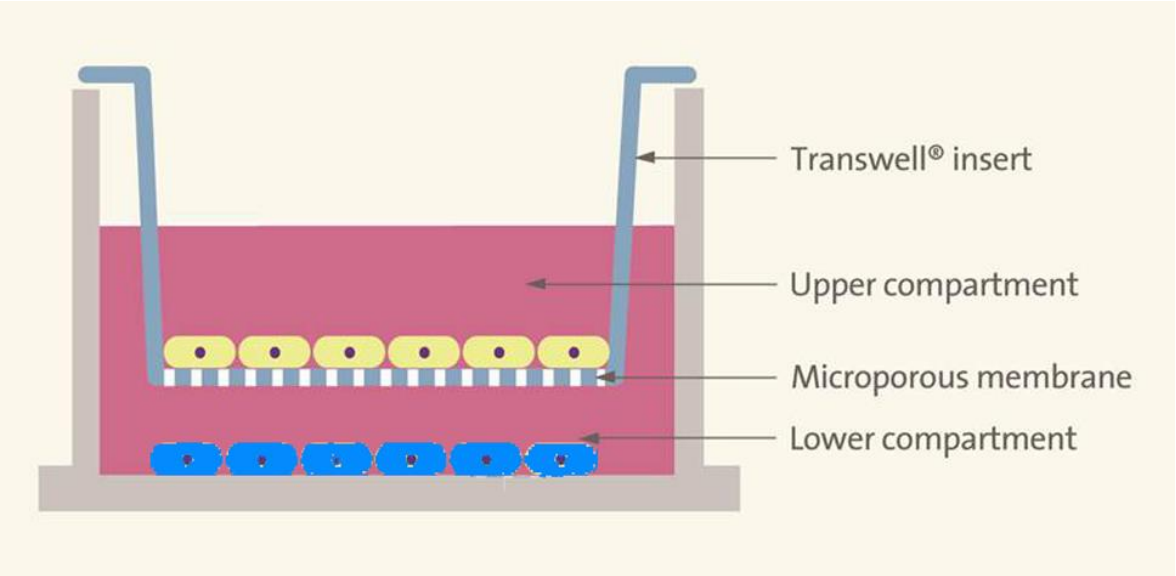

Figure 13: Transwell system (73)

We used a special trans-well culture system (inserts with 5 μm pores, 24 wells), which generated an upper and lower chamber, divided by a membrane insert – see Figure 13. This membrane still allowed passage of the cells from upper to the bottom space. CXCL12 and SLC cytokines were added in the lower chamber with the medium (RPMI + 0.5% BSA + 25mM HEPES) and thymocytes from WT and N3-IC Tg mice ($1x10^6$ cells) were put in the upper chamber. We were observing cell migration towards the lower chamber, therefore, the migration of cells in response to chemokine stimulus.

Each sample was evaluated and duplicated, and the wells filled with the medium only, served as a negative control. After 90 minutes, contents of the lower chamber were transferred into a tube and centrifuged for 10 minutes at 300 rpm. The cells were re-suspended in 20μl of the staining solution, containing fluorescent anti-CD4 and anti-CD8 antibodies. At this point, the cells of duplicates for each sample were joined. The totals of $2x10^6$ cells were incubated on ice for 10 minutes and were added the appropriate antibodies. After that, they were incubated again for 30 minutes on ice, washed with 2 ml of the staining solution and resuspended in 200 μl of the same solution. Cell migration was assessed by flow cytometry (FACS).

The results are expressed as a percentage of migrated cells, calculated using the following formula: sample acquired events (number of migrated cells towards the chemokine)/control acquired events (cells in no-chemokine chamber) x 100.

3.2.7. Statistical analysis

The data was analyzed using Microsoft Office Excel 2007. All charts in the work have been based on average value of all the data obtained from the experiments and made statistically significant with the standard deviation.

Many *in vitro* data of the surface expression was analyzed by calculating the absolute number of positive cells to the antibody of interest. We calculated that based on the percentage of fluorescent cells and the actual number of the cells examined, making the data more visible and that way even further significant.

4. RESULTS AND DISCUSSION

I. T-cell populations in the thymus characterization

First of all, we have characterized the four main subpopulations in the thymus, using flow cytometry experiments (FACS) and specific anti-CD4 and anti-CD8 conjugated antibodies, only binding the surface CD4 and CD8 markers, therefore only the cells expressing these.

From the FACS charts (Fig. 14) it is possible to observe the subthymic population distribution; the x-axis shows the increasing fluorescence of anti-CD4 PerCP and y-axis the one of anti-CD8 APC; corresponding to increasing quantities of CD4 and CD8 markers.

Based on the positivity/negativity of the two different antibodies, it is possible to distinguish four subpopulations; DP, SP CD4+, SP CD8+ and DN. The largest group of cells in WT mice is composed of DP cells, which make up for roughly 85 % of the total population. DN cells represent a small fraction, consisting of only 3 - 4%.

With the disease progression, in Tg mice, there is a decrease in the percentage of DP thymocytes (down to 76.5%, Fig. 14).

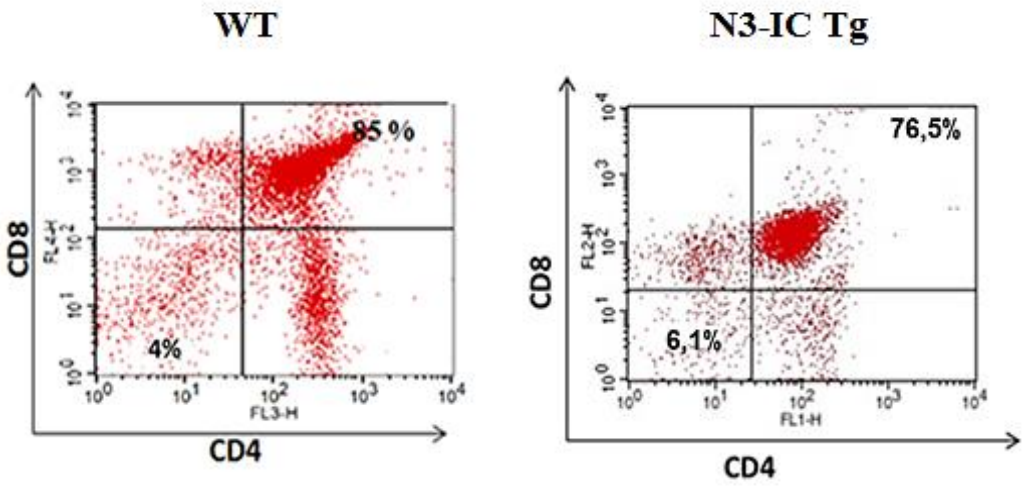

Figure 14: Distribution of thymocyte subpopulations in WT and N3-IC Tg mice. Freshly isolated T-lymphocytes (approx. $8x10^5$ cells) from WT and transgenic mice thymuses. Flow cytometry charts of FSC/SSC gated lymphocytes, stained with anti-CD4 PerCP and anti-CD8 APC antibodies to distinguish the four main populations; $CD4^-CD8^-$ (DN) thymocytes in the lower left quadrant, $CD4^+CD8^+$ (DP) in the upper right quadrant, SP $CD8^+$ thymocytes in the upper left quadrant and SP $CD4^+$ thymocytes in the lower right

II. Decreased CXCR4 expression in DN thymocytes

Deregulated thymic development, increased number of thymocytes due to early T-cell development changes in N3-IC Tg mice (affirmed by increased DN3 vs. DN4 thymocytes), and the role of Notch, pre-TCR and CXCR4 in T-cell differentiation - particularly the DN to DP transition, all suggested the analysis of CXCR4 expression on DN cells, the most undeveloped thymocytes (16). The importance of Notch3/pre-TCR cooperation in T-cell differentiation (DN-DP transition) has already been reported (44), and some further research has shown a peak CXCR4 expression in transitions DN2-DN3 and DN-DP (63).

Figure 15 depicts the Gaussians of CXCR4 and control fluorescence. The area between the curves varies between two mice models, confirming that there is, in fact, a strong reduction in N3-IC Tg compared to WT.

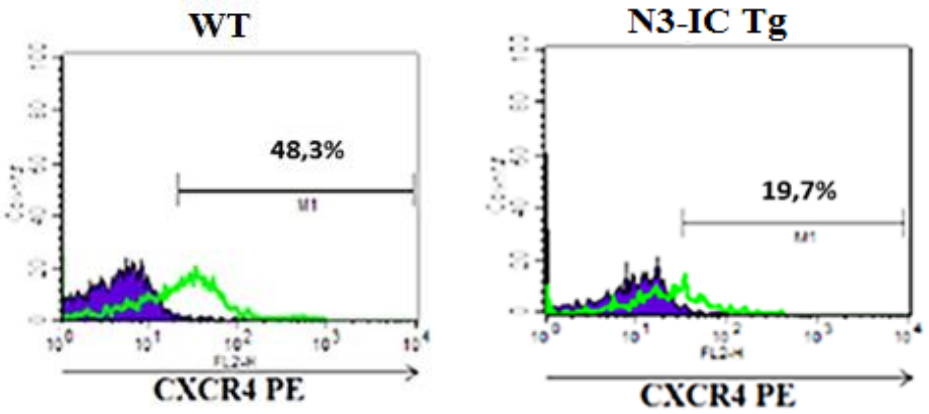

Figure 15: DN cells CXCR4 expression (green curve) compared to the control IgG2b (blue). Thymocytes ($8x10^5$) obtained from both WT and N3-IC Tg mice were stained with anti-CD4 PerCP-Cy5.5, anti-CD8 APC and anti-CXCR4 PE or IgG2b PE (control) antibodies and analyzed with FACS. The panels indicate an overlap of curves corresponding to the fluorescence of CXCR4 (green) and IgG2b (blue).

This data shows that expression of CXCR4 receptor on DN thymocyte plasma membranes is reduced in N3-IC Tg mice compared to WT. The positivity for CXCR4 receptor decreases from 48.3% to 19.7%. In conclusion, the DN thymocytes, derived from transgenic mice, show a decrease of the CXCR4 surface expression of at least 20 - 30 %.

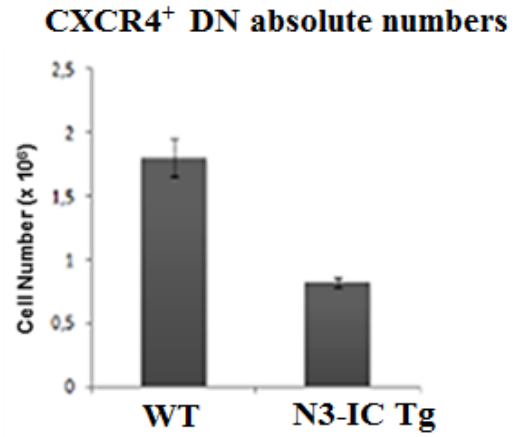

Figure 16: Absolute numbers of CXCR4 positive DN thymocytes. The numbers were calculated based on the percentage of fluorescent ($CXCR4^+$) cells and the actual number of the cells examined. The data were calculated from three independent experiments and are presented with the standard deviation.

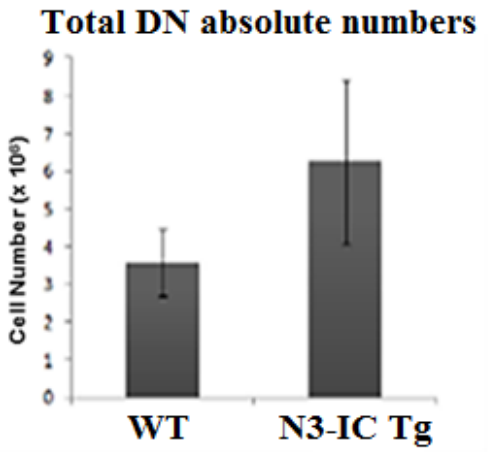

Figure 17: Absolute numbers of the total DN thymocyte population. The data were calculated from three independent experiments and are presented with the standard deviation.

After reviewing the absolute numbers of two populations; DN/$CXCR4^+$ and total DN (Figures 16 and 17), it appears that over-expression of N3-IC slightly increases the *proportion* of DN T-cells in the thymus (Fig. 14, from 4% to 6.1%), but drastically reduces the *absolute number* of DN/$CXCR4^+$ in N3-IC Tg model in respect to WT mice. This effect is not due to any reduction of the total DN thymocytes number (Fig. 17). We can therefore assume that Notch3 deregulates the CXCR4 surface expression, perhaps by modulating its transcriptional or post-translational expression. Similar results were also observed in animals, younger than six weeks old.

This data indicates that over-expression of N3-IC increases the absolute number of DN T-cells alone (stopping the thymocyte development), but it drastically reduces the number of $CXCR4^+$ DN thymocytes. This is probably correlated to a deranged T-cell development.

Natural killer cells exclusion

In the experiments with N3-IC Tg mice, the percentage of NK cells increased (16.7%) compared to WT

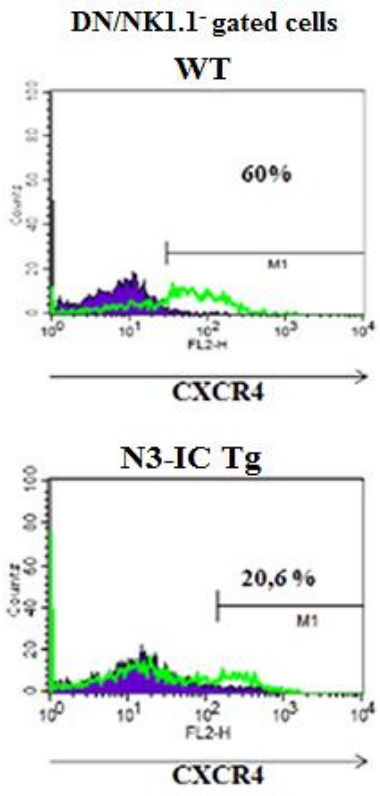

Figure 18: CXCR4 expression in DN thymocytes, excluding the NK cells (DN/NK1.1$^-$). Each sample ($8x10^5$ cells) was stained with anti-CD4, anti-CD8, anti-NK1 and then with anti-CXCR4 PE or IgG2b PE. The percentage of CXCR4$^+$ cells (green) has been normalized to its IgG2b control (blue).

NK$^+$ cells presence is also demonstrated in form of absolute number graphs, showing a drastic reduction of the number of DN/NK$^-$ T-cells, positive for CXCR4 in the thymus (Fig. 19).

Ultimately the results suggest that over-expression of N3-IC in transgenic mice deregulates the surface expression of CXCR4 in (13.5%). To exclude the possibility that the reduced CXCR4 expression, observed in N3-IC Tg mice, could depend on dilution effect due to NK cells, expressing low levels of the chemokine receptor, we analyzed the expression of CXCR4 only in DN/NK negative thymic cells (DN/NK1.1$^-$) in N3-IC Tg and WT mice models.

From Figure 18 we can see the decreased expression of CXCR4 in DN/NK1.1$^-$ cells of N3-IC Tg mice, compared to WT.

The DN/NK1.1$^-$ thymocyte population of N3-IC Tg mice shows a great decrease in CXCR4$^+$ cells compared to WT; from 60% to 20.6% respectively.

The independence of the results from

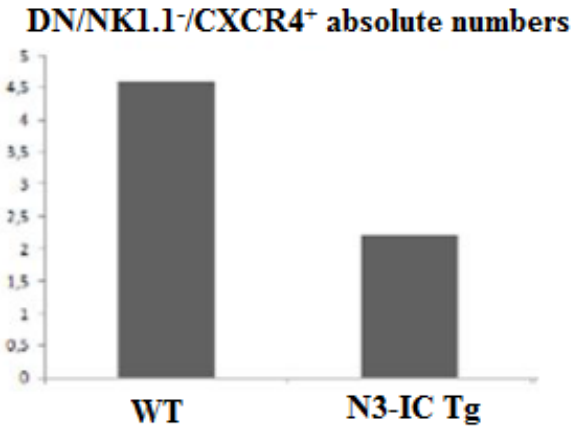

Figure 19: DN/NK1.1$^-$/CXCR4$^+$ absolute numbers. The numbers were calculated based on the percentage of fluorescent (CXCR4$^+$) cells and the actual number of the cells examined. The data were calculated from three independent experiments.

thymus in the DN stage. This receptor is involved in regulation of thymocyte proliferation and migration in between thymus areas in the transition from DN to DP stage, and for this reason, its decrease could induce alterations in the T-cells migration pattern.

III. Expression profile of CXCR4 in different DN maturation stages

Maturation of thymocytes from DN1 to DN4 is affected by their localization (within the thymus architecture; see Figure 3), as well as by the signals of receptors on T-cells' own membrane. Common lymphoid progenitor cells (CLPs) enter the thymus through blood vessels in the medulla or cortico-medullary junction, whereupon they undergo progressive differentiation from DN to DP and finally to SP thymocytes as they move through the distinct microenvironment of the thymus. The DN1 stage is situated close to the site of entry deep within the cortex. The DN2 population moves across the cortex and into the sub-capsular zone. The DN3 population stays within and accumulates in the subcapsula, where the transition to DN4 stage happens. Progression to the DP stage, through an immature CD8+ SP (ISP) cell intermediate, is accompanied by a reversal in direction of migration of the cells back across the cortex and towards the medulla. As for the surface receptors, it has been

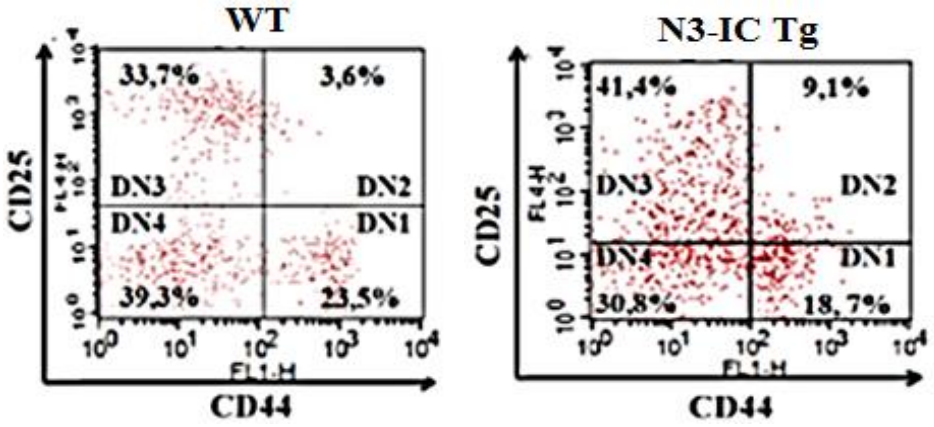

Figure 20: DN 1-4 cells distribution. Approximately $1x10^6$ thymocytes from WT and N3-IC Tg mice were treated with specific anti-CD4 and anti-CD8 antibodies, and then with anti-CD44 FITC and anti-CD25 APC. Lymphocytes and DN cells were already gated based on their FSC/SSC and CD4/CD8 expressions, respectively. DN subsets were analyzed based on their expression of markers CD44 and CD25; lower right quadrant=DN1 ($CD25^-CD44^+$), upper right quadrant= DN2 ($CD25^+CD44^+$), upper left quadrant=DN3 ($CD25^+$, $CD44^-$), lower left quadrant=DN4 ($CD25^-$, $CD44^-$).

previously reported that in physiological conditions, DN cells express different levels of CXCR4 in throughout their developmental stages.

Based on these observations, we evaluated the differences of CXCR4 expression in all four DN stages, using the anti-CD44 and anti-CD25 antibodies. The cells from both WT and N3-IC Tg mice are depicted in Figure 20, divided into subpopulations. From these results we can observe how DN2 population percentage is increased inn N3-IC Tg model compared to the WT, and DN1, on the contrary, experienced a decrease. While in WT the DN3 are percentage-wise roughly equal to DN4 (33.7% and 39.3%, respectively), in N3-IC Tg the DN3 show an increase compared to DN4 (41.4% vs. 30.8 %, respectively).
This observation, combined with the increase of DN2, suggests that over-expression of Notch3 causes a decreased progression through β-selection, followed by a blockage of thymocytes maturation at the DN3 stage.

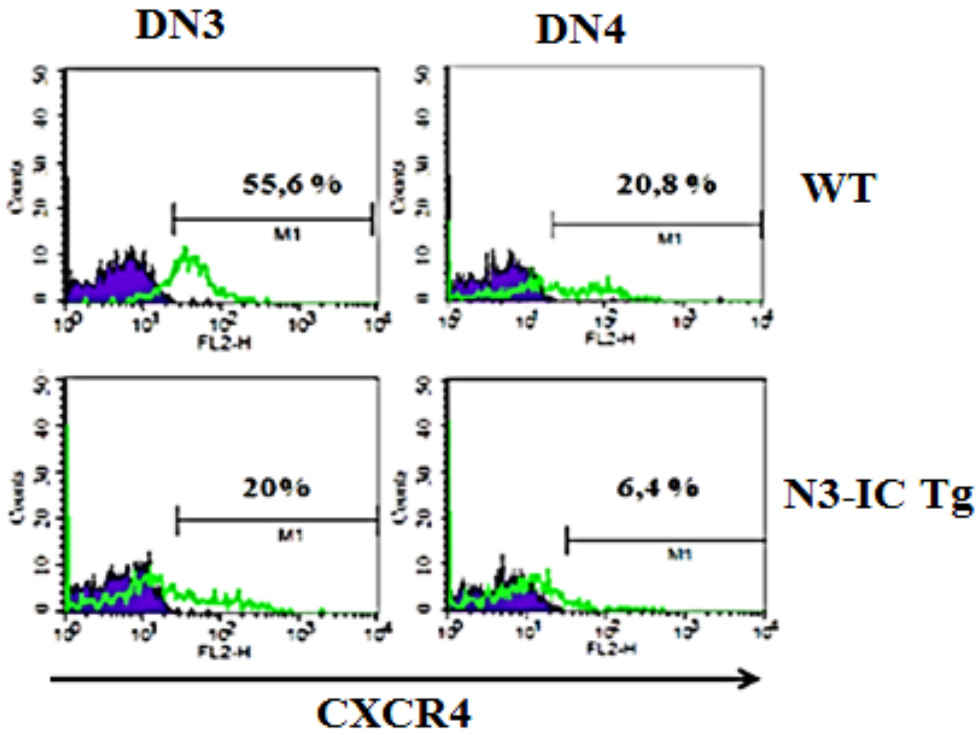

Figure 21: Expression of CXCR4 in populations of DN3-4 cells. Samples ($1x10^6$ cells) were stained with anti-CD4 and anti-CD8 antibodies for DN gating, then anti-CD44 FITC and anti-CD25 APC antibodies for determining the DN stage and last, anti-CXCR4 (PE) or its isotype control IgG2b (PE) antibodies. The overlapping curves show CXCR4 (green) and its control (blue) expressions and the positivity percentage.

All the data shown so far has demonstrated that the double negative cells of transgenic mice display a visibly decreased expression of CXCR4 in regard to wild type. For this reason, we decided to perform a more detailed analysis of CXCR4 expression; in particular, of DN3 and DN4 maturation stages in order to reveal the effects of Notch3 in DN-DP transition in N3-IC Tg mice compared to WT model.

Analysis of the absolute cell numbers (data not shown) for all four subpopulations showed that about half of DN3 cells progress to DN4 in WT (1.63 of 4.3 X 10^6) and only a third (0.7 of 2.3 X 10^6) in Tg sample, suggesting a restriction in transition from DN3 to DN4. In Figure 21 presented Cell Quest-generated analysis of N3-IC Tg models shows a slight CXCR4 expression increase in DN1 stage, a small decline in DN2 expression and then a strong decrease in DN3 and DN4 stages, compared to WT.

This drastic reduction coincides with DN3 and DN4 stages, transition under control of Notch, pre-TCR and CXCR4. These results confirm that over-expression of Notch3 causes the CXCR4 receptor down-modulation in DN cells of transgenic mice, and that such event is specific for the DN3 / DN4 stages, reaching its maximum reduction at the DN3 stage.

Overall, the data clearly indicates that there is a drastic reduction in both the number and the percentage of $CXCR4^+$ cells alone in DN3 and DN4 stages. Knowing that CXCR4 is a co-stimulator, along with pre-TCR, specifically during the step of β-selection, and having observed a small proportion of cells that pass from DN3 to DN4 in N3-IC Tg model compared to WT, it can be assumed that Notch3-IC is deregulating the expression of CXCR4 in immature DN thymocytes and therefore negatively affecting the thymocyte differentiation, and positively contributes to development of leukemia in the transgenic model.

IV. Surface expression of EpCAM molecule in DN thymocytes

So far the displayed evidence suggests that Notch3 is able to compromise the first events in maturation of pre-T cells, altering the migration through the thymus, or modulating the interactions between thymocytes and the stroma. In regard to the latter, we chose to examine the epithelial cell adhesion molecule (EpCAM) expression, which, as known from previous research, has a role directly in the interaction between thymocytes and epithelial cells and in homotypic interactions. EpCAM is a trans-membrane glycoprotein that mediates cell-cell adhesion, and is involved in cell signaling, migration, proliferation and differentiation (73,

75). Since it also promotes tumor metastasis, is considered an important marker for several cancers.

The results (Figure 22) show a differential expression of EpCAM on DN thymocyte membranes of transgenic mice compared to WT; in particular, a strong reduction in transgenic model can be detected, decreasing from 49.2% down to 9.6%. This data was reconfirmed with two independent experiments.

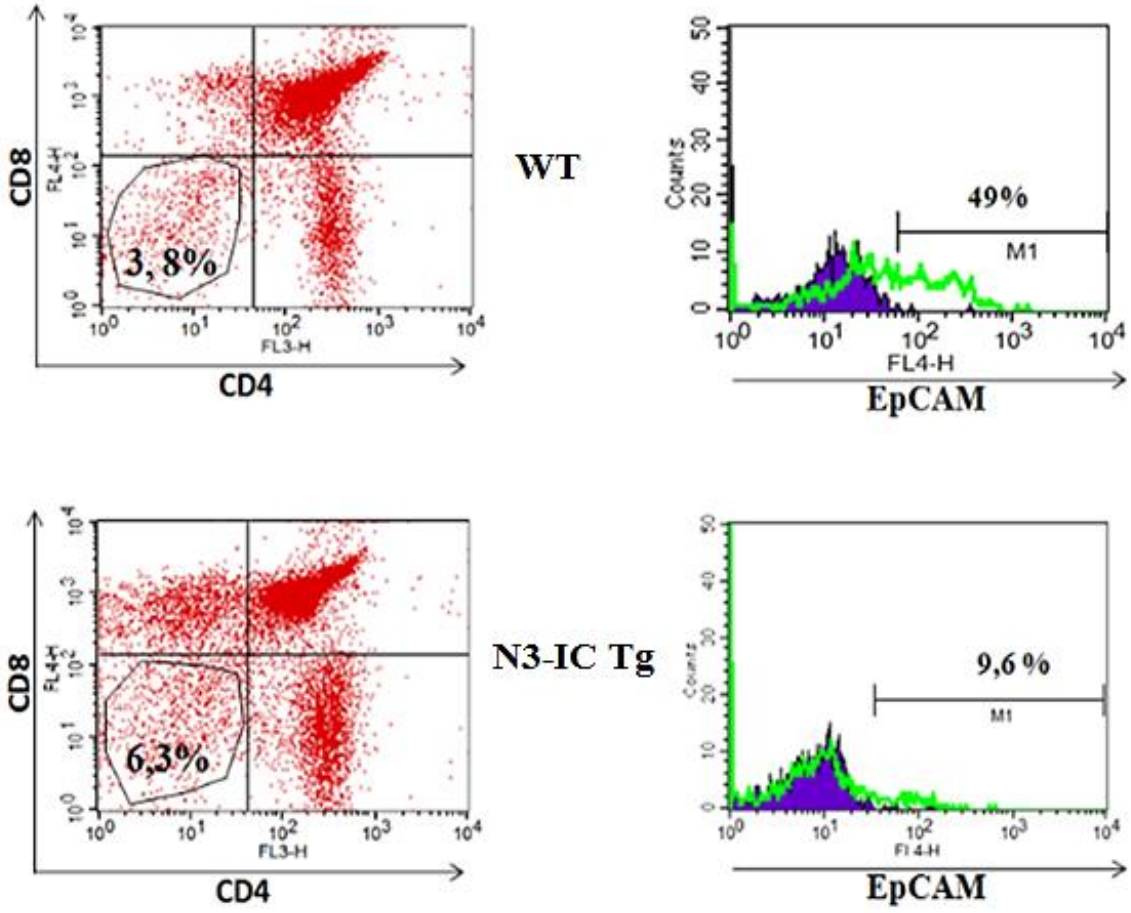

Figure 22: EpCAM expression in DN thymocytes. Approximately $1x10^6$ thymocytes of WT and N3-IC Tg mice were stained with anti-CD4 PerCP, anti-CD8 APC to enable DN cell gating (left panels) and then with anti-Ep-CAM APC or its control IgG2a APC antibodies to determine its expression (right panels). The positivity for EpCAM decreases significantly in the N3-IC Tg model.

It is likely that in Tg mice, over-expression of Notch3-IC results in accumulation of DN thymocytes more resistant to apoptosis, switching off EpCAM expression and thus deregulating the surface receptor signals that normally take part in thymocyte/stroma or

thymocyte/thymocyte interactions, necessary for progressive differentiation. In conclusion, the DN thymocytes derived from transgenic mouse models show an important decrease of surface EpCAM expression, quantified at approximately 40 percentage points. This has already been explained as how some soft tissue tumors and, in fact, all lymphomas are EpCAM negative (71).

These results suggest that over-expression of N3-IC increases the proportion of DN T-cells, but drastically reduces the number of DN thymocytes that are EpCAM positive in N3-IC Tg mice compared to WT. This, in association with a decreased CXCR4 expression, is possibly related with decreased cell-cell adhesion and a subsequent deregulation of DN differentiation.

V. Transcriptional analysis of CXCR4 and Bcl2-A1 in DN/NK$^-$ cell

The CXCR4 expression is regulated at transcriptional, post-transcriptional and post-translational level by numerous factors (58). We have observed reduced CXCR4 surface expression in DN thymocytes of transgenic mice, which could be a result of direct Notch3 effect on CXCR4 gene transcription.

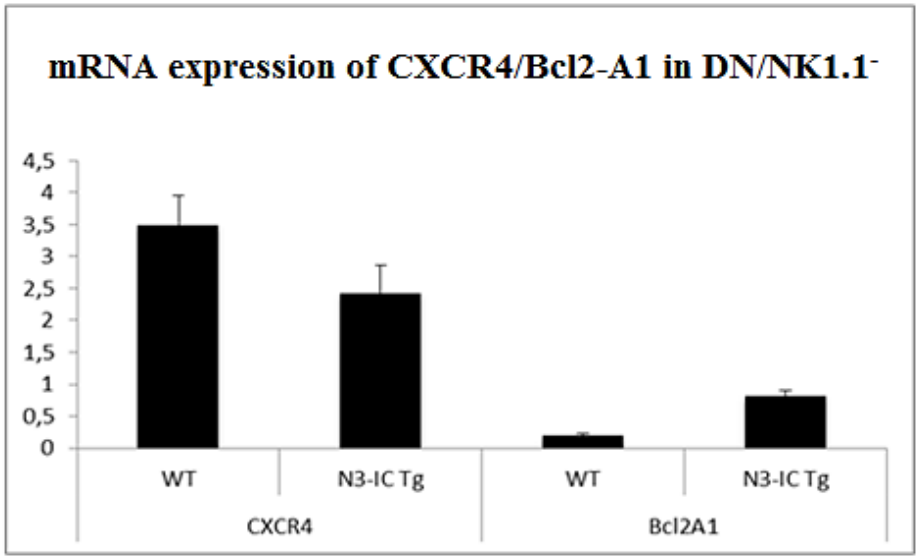

Figure 23: Histograms of densitometric analysis of CXCR/Bcl2-A1 mRNA expression in DN/NK1.1$^-$ thymocytes. The quantity of mRNA, extracted from thymuses of WT and N3-IC Tg models in this analysis was 1 μg. The densitometry was performed with 1D gel electrophoresis software Quantity One, using the average value expression obtained in several experiments, normalized on the housekeeping β-actin mRNA gene expression. The figure depicts error bars, calculated as the standard deviation, which gives significance to the observed difference.

To determine if the mechanism of reduction of CXCR4 was cell-autonomous, we analyzed mRNA expression of this receptor in DN cells. Considering the data from the previous FACS analyses, steps were taken to extract mRNA from cells $CD4^-/CD8^-/NK1.1^-$ alone.
Histograms of Figure 23 display densitometric analysis of electrophoretic gel bands (Figure 24) and show that CXCR4 mRNA expression is reduced in N3-IC Tg compared to WT model. This data indicates that Notch3 transcriptionally regulates the expression of CXCR4, which is important because it shows that reduced presence in the membrane is not governed by a mechanism of increased receptor internalization due to stimulation of SDF-1α / CXCL12.

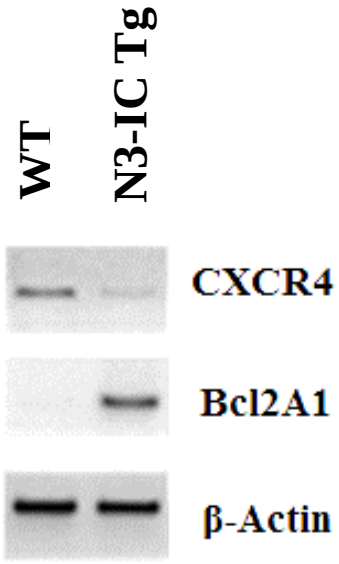

Figure 24 : mRNA expression of CXCR/Bcl2A1 in $DN/NK1.1^-$ thymocytes. RNA was extracted from thymuses of WT and N3-IC Tg models. 1 μg of the mRNA extracted was subsequently reverse transcribed into cDNA and used as a template for semi-quantitative PCR. The PCR allowed, through the use of murine-specific primers, to evaluate expressions of CXCR4 and Bcl2-A1 genes, normalized on the housekeeping β-actin mRNA gene expression. The PCR products were analyzed using 1.5% agarose gel with Midori green nucleic acid stain solution and were loaded using loading buffer.

It is possible to assume a cell-autonomous mechanism, perhaps even free of modulation by SDF-1α. Future studies will definitely be needed to clarify the role of Notch3 and how it can act on reduction of CXCR4 expression.

Aside from CXCR4 mRNA expression, we also wanted to see how Bcl2-A1 acts in these circumstances. As known from previous research, pre-TCR and Notch receptors, expressed on DN thymocytes, influence the processes of proliferation and differentiation during the β-selection (47). For this purpose, the analysis of this anti-apoptotic protein expression was carried out in two ways; as a gel analysis (Fig. 24), and a densitometric analysis (Fig. 23).
In both of these figures we can see how the expression of Bcl2-A1 increased in DN/NK1.1$^-$ population. This means that expressions of the CXCR4 receptor and anti-apoptotic protein Bcl2-A1 actually have the exactly opposite patterns of expression. This might at first seem at odds with previous research, which shows that physiologically increased expression of CXCR4 receptor goes hand in hand with an increase in mRNA levels of Bcl2-A1 factor (62). But experiments, performed on DN cells of N3-IC Tg mice, on the contrary show a different trend. From this finding we can only assume that perhaps Notch3 even further releases these immature cells from normal differentiation and developmental mechanisms. This could be due to the activation of canonical p65 / NF- κB in the transgenic mouse model. Our hypothesis is that the activity of Notch3 and pre-TCR in transgenic mice can favor the increase in levels of Bcl2-A1 expression through activation of NF- κB by Notch3, regardless of the CXCR4 receptor.
Studies on molecular mechanisms that regulate the expression of CXCR4 will need to be performed to in more detail outline the crosstalk of Notch3 and other pathways involved in lymphomagenesis of this disease model.

VI. Increased CXCR4 expression in DP thymocytes

After observing the deregulated expression of CXCR4 in DN T-cells, the next step was to analyze its expression on the surface of DP thymocytes in two mice systems (WT and N3-IC Tg) (Figure 25). Both genotypes, WT and N3-IC Tg, show a high positivity rate for CXCR4 (93.2% and 96.2%, respectively) in selected DP T-cells. For a more detailed analysis, we assessed the difference (Δ) of the Mean Fluorescence Intensity (FI) and Δ of Median FI of thymocytes from 5 WT and 6 N3-IC Tg mice. The data revealed higher levels of CXCR4 receptors **per cell** in DP thymocytes of N3-IC Tg mice compared to WT. These results were obtained from three different experiments averages, with the standard deviation, making the data statistically significant.

The increased expression of CXCR4 at the single cell level of DP cells in N3-IC Tg mice compared to WT suggests that over-expression of Notch3 deregulates CXCR4 in opposite fashion in the two cell sub-populations, perhaps by promoting CXCR4 function in DP and significantly reducing it in DN stage. In support of increased function of CXCR4, previous

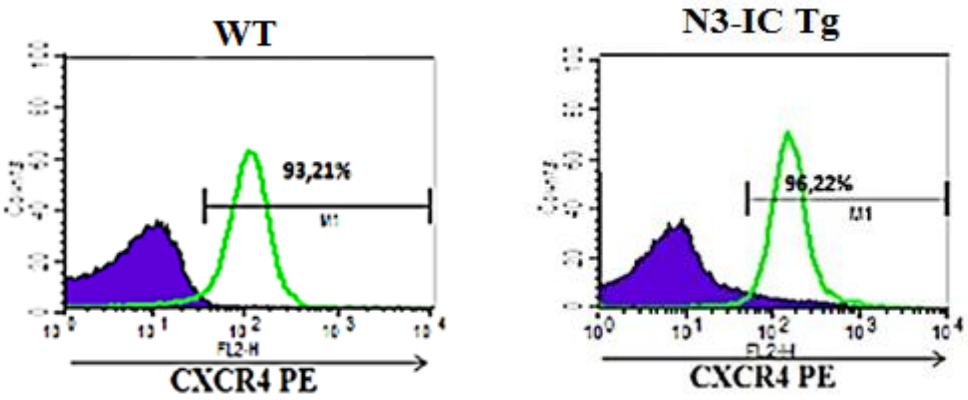

Figure 25: CXCR4 expression in DP thymocytes. Both WT and N3-IC Tg genotypes ($1x10^6$ cells) were stained with anti-CD4, anti-CD8 and anti-CXCR4 or the control, IgG2b antibodies. The curves show the evaluation of the CXCR4 positivity rate in DP population only (CD4/CD8 expression-based gating).

data have shown increased expression of phosphorylated forms of Extracellular signal-regulated Kinase (Erk) in selected DP populations, which is a known target for CXCR4 activation, correlated with thymocyte migration ability (67).

Notch3 and CXCR4 co-expression

To support the hypothesis of Notch3-mediated modulation of CXCR4 expression, we considered the possibility of co-expression of the two receptors on DP cells in the pathological mouse model, N3-IC Tg, compared to WT.

On the left side of Figure 26, we can see the classic distribution of four thymocyte subpopulations in WT and N3-IC Tg mice as function of CD4 and CD8 markers (panels A and C). From these cells, we gated the DP subset to clarify the simultaneous presence of CXCR4 and Notch3 on this population. Comparison between panels B and D shows very different patterns of positivity distribution of the two receptors. Both are clearly much more

expressed on the membrane of N3-IC Tg DP cells in respect to WT, with a real shift of co-presence of Notch3^{+}/CXCR4^{+} population towards the right.

This confirms our hypothesis and suggests close cooperation between the two receptors in DP cells of Tg mice. We believe that this co-expression might favorite DP thymocytes egress toward periphery, thus regulating leukemia-propagating potential. To corroborate this hypothesis, we will need to further test DP CXCR4^{+}/Notch3^{+} T-cells in blood, bone marrow and peripheral organs, too.

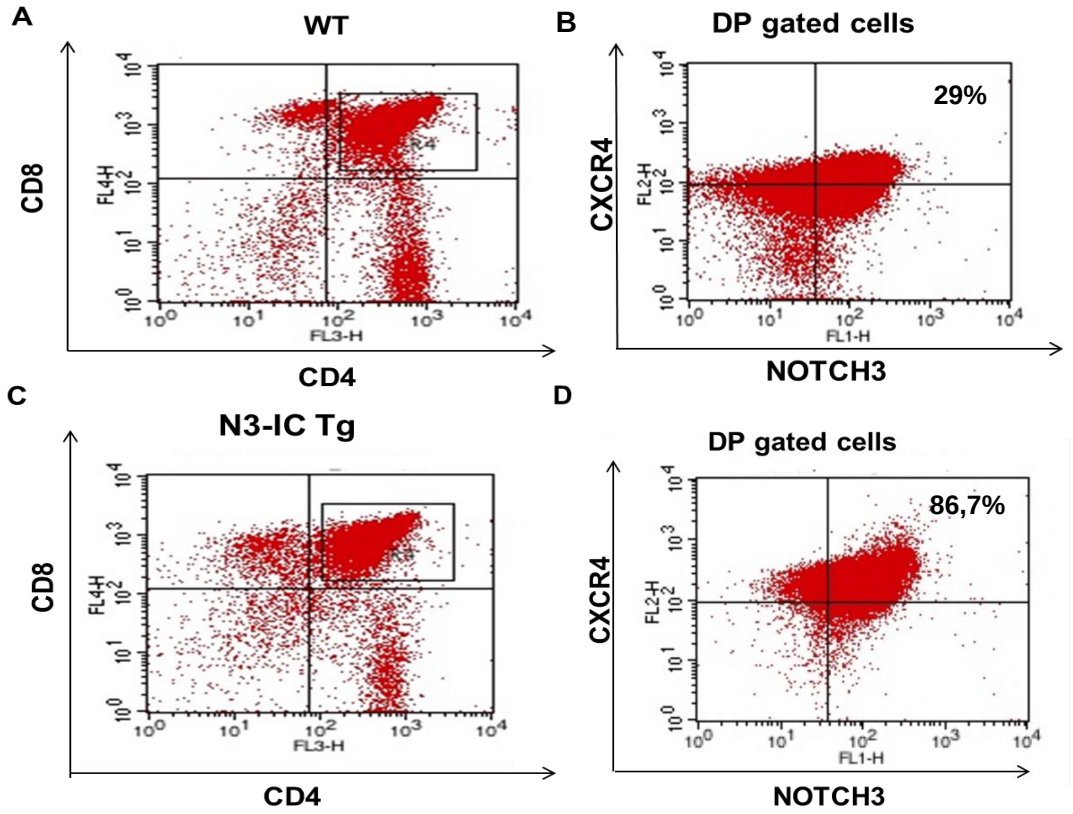

Figure 26: Analysis of CXCR4 and Notch3 co-expression in DP cells. With CellQuest software we evaluated the events (cells) from FACS analysis. DP T-cells (1x10^{6}) obtained from both models were stained using anti-CD4 PerCP Cy5.5, anti-CD8 APC, anti-CXCR4 PE and anti-Notch3 FITC antibodies. As controls, we used anti-IgG2b PE for CXCR4 and goat anti-IgG for Notch3 (together with secondary antibody donkey anti-goat FITC incubation to provide for a fluorophore).

VII. Migration of DN and DP thymocytes in response to the chemokine SDF-1α

Natural ligand for CXCR4 is SDF-1α (CXCL12), expressed in cortex and sub-capsular region of the thymus (69). Both of them are widely distributed in tissues and play an important role in embryonic development, haematopoiesis, organogenesis and vascularization. So far, the data analyses have shown that Notch3 regulates expression of CXCR4 in DN and DP cells, and can also influence the progressive development of DN thymocytes by directing their localization inside the thymus. Based on these premises, we wanted to analyze the ability of T-cell migration *in vitro*.

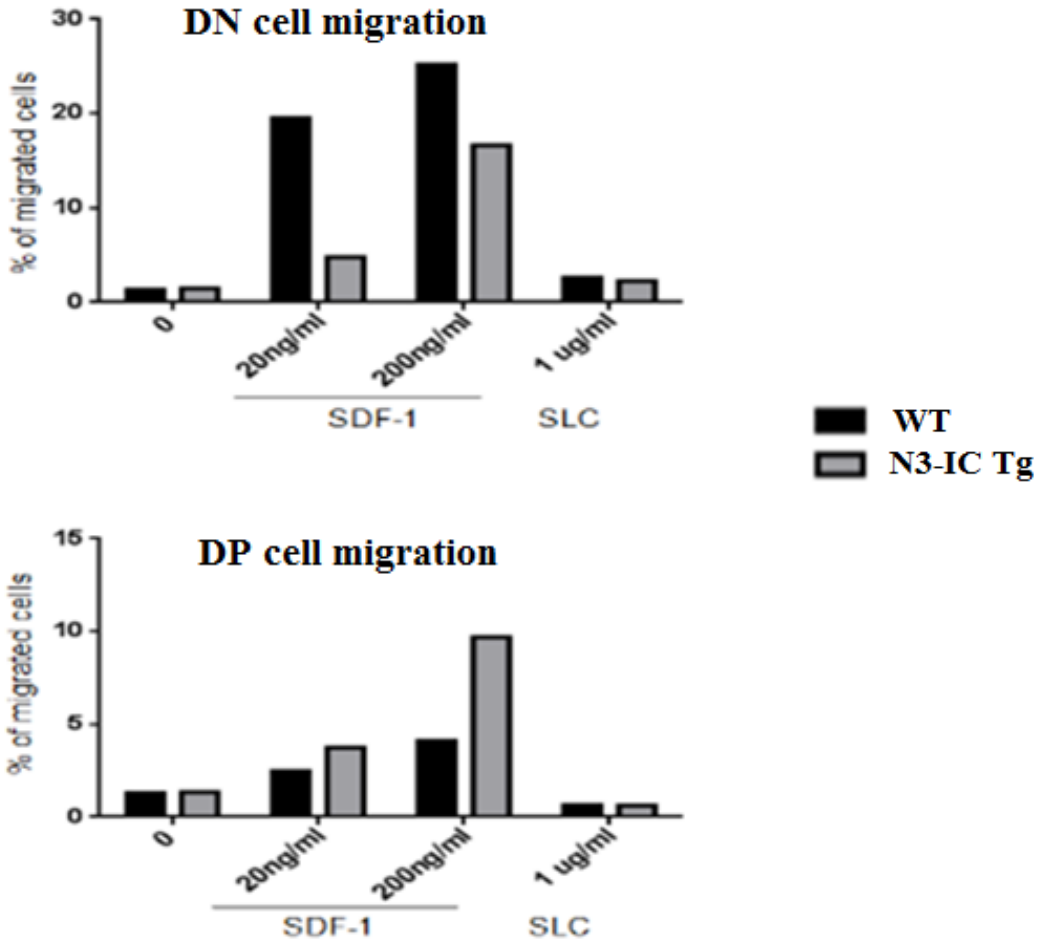

Figure 27: Histograms of DN and DP cell migration in response to chemokines SDF-1 and SLC. Migration tests using $1x10^6$ thymocytes, taken from thymuses of N3-IC Tg and WT mice, were performed using chemokine SDF-1α at two different concentrations, 20 and 200 ng/ml, and chemokine SLC at concentration of 1 μg/ml. After 90 minutes, the migrated cells (from the lower chamber of the Transwell system (see Figure 13) were stained with anti-CD4 and anti-CD8 antibodies. Cell migration was assessed by flow cytometry (FACS). The results are expressed as a percentage of migrated cells; sample acquired events/control acquired events x 100.

In the top panel of Figure 27, a histogram shows the percentage of cells that have migrated in response to SDF1. At minimal concentrations of SDF-1, the percentage of DN that passed into the chamber below (towards the chemokine), dropped dramatically in N3-IC Tg compared to WT. At higher doses, this reduction is less evident, which coincides with decreased CXCR4 surface expression, observed in DN cells of N3-IC Tg. It is evident that migration ability of DN thymocytes is greatly reduced in N3-IC Tg compared to WT, suggesting that Notch3 can alter this capacity together with that of localization in the thymus by interfering with normal differentiation processes, supported by previous data that demonstrate a disorganization of the thymus (16).

Regarding the DP cells of N3-IC Tg, concurring with the increased expression of CXCR4 per cell, observed earlier, their ability to migrate is already increased in at low concentrations of SDF-1, compared to WT. Moreover, contrary to what we observed with DN cells, a higher dosage of chemokine raised the DP percentage in the migration chamber even more. Finally, no difference between WT and N3-IC Tg models was observed in response to SLC ligand (more specific for CCR7), confirming the specificity of the test.

In conclusion, the results of all thymocyte analyses demonstrate that continuous activation of Notch3 is able to compromise the early events in progressive maturation of immature T-cells, probably affecting the migration and localization within the thymus. CXCR4 is also involved in the mechanisms of mature cells migration outside the thymus, suggesting the possibility that a greater amount of DP cells leave the thymus. This would agree with different percentages of DN and DP observed in WT and N3-IC Tg along the disease progression.

VIII. CXCR4 expression in abnormal DP splenocytes

The transgenic mouse model after 8 weeks of age developed a Notch3-induced T-cell leukemia/lymphoma, also impairing peripheral lymphoid organs (Fig. 28, splenomegaly). Just like thymus, spleen also shows altered distribution of splenic T-cell subpopulations; indeed, anomalous DP T-cells were represented in spleens of N3-IC Tg mice. Given this evidence, associated to increased CXCR4 expression in DP thymocytes, we analyzed the distribution profile and chemokine receptor expression in T-cells of the spleen.

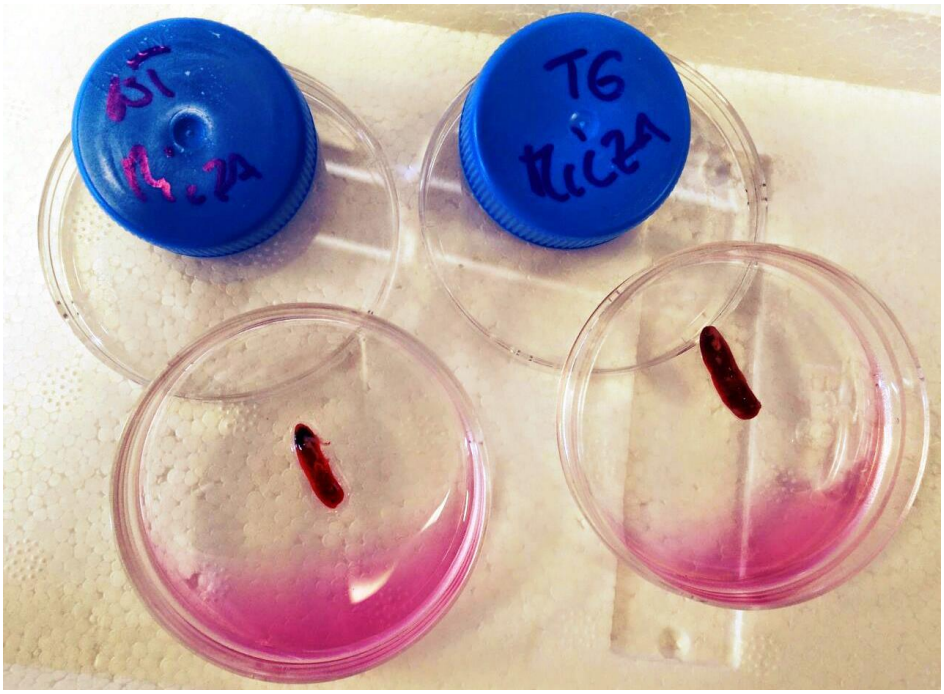

Figure 28: Comparison between WT (left) and N3-IC Tg (right) spleens. Transgenic spleen shows splenomegaly, typical for T-ALL.

Results of characterization (Fig. 29) show that in WT mice, population of splenocytes consists of 67% DN cells, while the SP $CD4^+$ and SP $CD8^+$ represent 10% and 22%, respectively. In these WT samples, DP cells are almost or completely absent, with a maximum frequency to below 1%. Distribution of splenocytes is severely altered in transgenic mice. More precisely, there is a decrease of both $CD8^+$ and $CD4^+$ SP thymocytes, in addition to an anomalous presence of DP T-cells (13.5%) compared to WT. This prompted us to study the surface expression of CXCR4 alone or in combination with Notch3, considering the already observed co-expression in DP thymocytes.

We only further analyzed the DP cells of N3-IC Tg model, because they represent the anomalous population; possibly circulating leukemic cells. These DP cells show an increased percentage of positivity for the CXCR4 specific antibody; meaning there is a high expression of CXCR4 receptor (67.6%) on the membranes of splenocytes of DP transgenic mice (Figure 30).

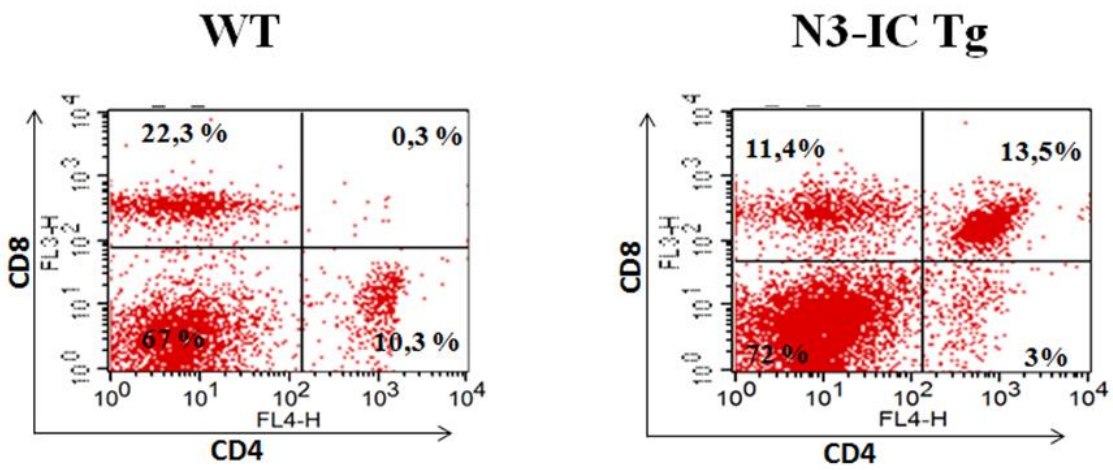

Figure 29: Distribution of the four T-cell subpopulations in spleens of WT and N3-IC Tg mice. Spleen cells were harvested from the same animals we used for the study of thymocytes. The immuno-phenotypic characterization with FACS was carried out with $1x10^6$ cells, stained with specific anti-CD4 APC, anti-CD8 PerCP and anti-FITC Thy1 antibodies. Incubation with anti-Thy1, antibody specific for T-cells, was necessary to ensure that solely T-cells from the spleen were processed and isolated. The results were analyzed with the use of CellQuest software.

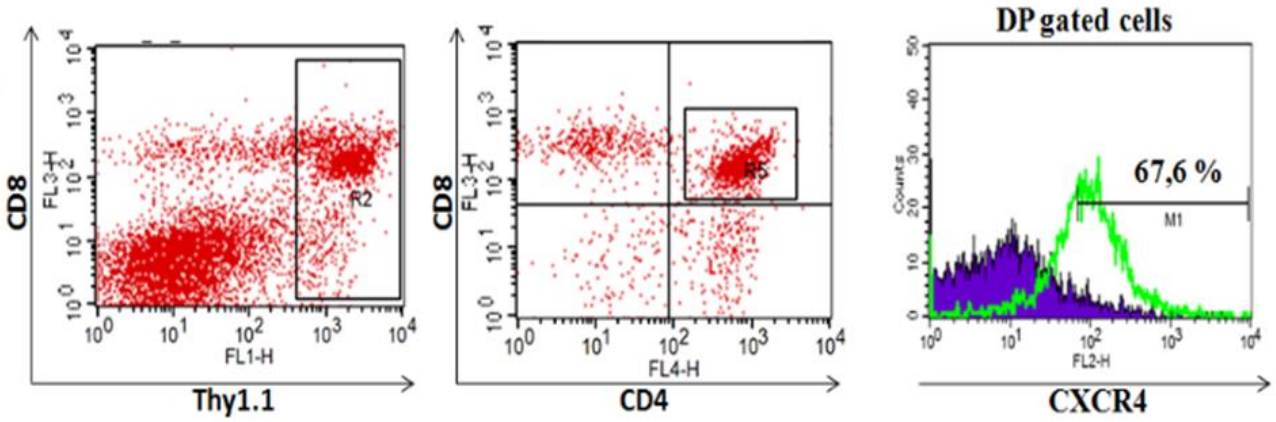

Figure 30: Increased expression of CXCR4 in anomalous present DP T-cells of the spleen. Each sample ($1x10^6$ cells) of N3-IC Tg mice was stained with specific anti-CD4 PerCP, anti-CD8 APC and anti-Thy FITC antibodies. The DP T-cells were separated with the use of anti-Thy1.1 antibody and later with CD4/CD8 gating (left and middle panels). The right panel shows the overlapping curves of CXCR4 (green) and isotype control (blue) expressions and a positivity percentage for CXCR4.

Next step was to analyze the cell-surface co-expression of CXCR4 and Notch3in N3-IC Tg samples. The left graph of Figure 31 shows the distribution of T-cell subpopulations in the spleen of N3-IC Tg mice. The right panel of Figure 31 shows a high positivity (71.45%) for presence of both CXCR4 and Notch3 on DP splenocytes surface, with a pattern extremely similar to that of DP thymocytes of N3-IC Tg mice (Figure 26).

In conclusion, this data indicates that Notch3 (also) deregulates expression of CXCR4 receptor in peripheral organs, such as the spleen, and allows us to hypothesize that the increased *in vitro* migration of DP T-cells and the increased co-expressions of CXCR4 and Notch3 receptors on splenocyte membranes in *ex vivo* experiments may be a result of increased release of DP thymocytes from the thymus.

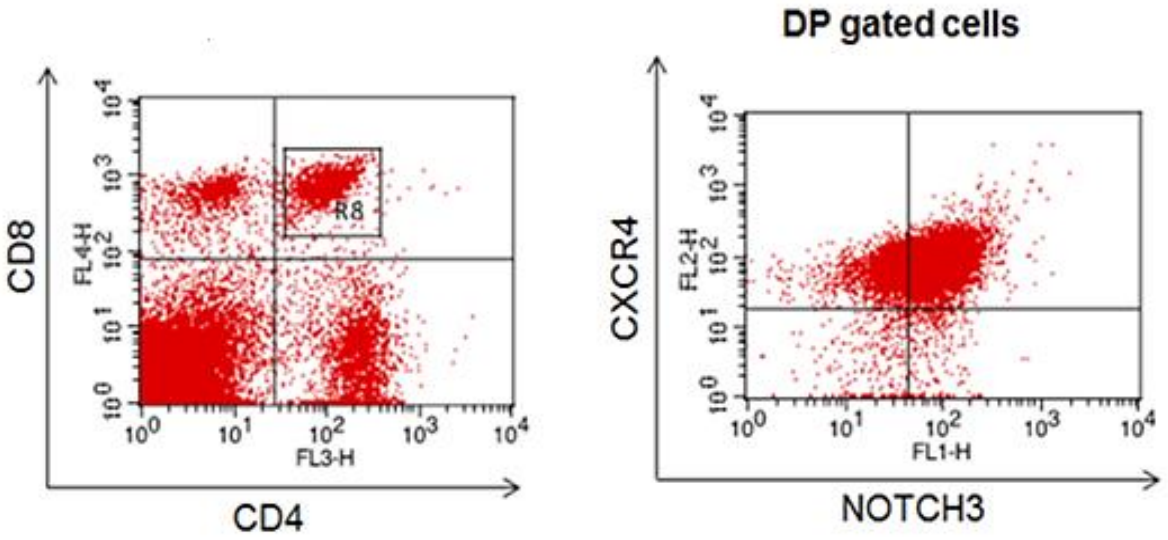

Figure 31: Co-expression of CXCR4 and Notch3 in DP cells of the spleen. Splenocytes ($1x10^6$) of N3-IC Tg model were stained with antibodies specific for surface markers CD4 and CD8, following by antibodies specific for CXCR4 and Notch3. As a control, we used anti-IgG2b PE for CXCR4 and goat anti-IgG for Notch3 (secondary incubation of the latter with secondary fluorescent antibody donkey anti-goat FITC). FACS assessment results were analyzed with CellQuest software. The use of markers CD4 and CD8 allowed for specific selection of DP spleen cell population (left panel, gate R8) for further analysis of combined cell-surface CXCR4 and Notch3 presence.

5. CONCLUSIONS

Notch3 over-expression in transgenic mice has already been proven to have an important oncogenic effect to inducing the onset of acute T-cell lymphoblastic leukemia (T-ALL) (16). However capable, Notch3 signaling alone is not a sufficient factor to make such β-selection-associated metabolism alterations, where it is known to cooperate with both pre-TCR and CXCR4 receptors. Previous reports show that deletion of CXCR4 causes a reduction of DP T-cells and increases the number of DN thymocytes. This and total thymic architecture subversion (see Figure 8) with reduction of subpopulations from DN2 to DP suggested an important role in these phases. In fact, DN3 cells for their development essentially require CXCR4 in addition to pre-TCR and Notch signals; for progression in the β-selection (47). This cooperation suggests that CXCR4 receptor could play an *in vivo* role in T-ALL, where these pathways are repeatedly altered.

On this basis, we used transgenic N3-IC mice models and compared it to WT to examine possible mechanisms of interference between the paths of Notch3 and CXCR4. In addition to finding a crosstalk between Notch3 and CXCR4, we also wanted to see if this Notch3-induced CXCR4 deregulation influences onset and progression of T-ALL.

We have arrived to following main conclusions:

A. CXCR4 expression is decreased in double negative thymocyte population

FACS analyses demonstrated an exceptional decrease of surface CXCR4 expression, mostly in immature thymocytes. This was not due to decrease of a DN cell total number; on the contrary, detailed analyses excluding the NK cells evidently showed a drastic reduction in both the number and the percentage of $CXCR4^+$ cells alone, specifically at DN3 and DN4 stages. These observations suggest that over-expression of Notch3 by impairing the CXCR4 gene causes a reduced progression through β-selection and then a block of thymocytes at the DN3 stage.

Notch3, especially in advanced stages of the disease, therefore, in addition to subversion of the thymic architecture, is also capable of cancelling alternative early maturational processes. Confirming this premise, we detected a complete opposite pattern of CXCR4 receptor and anti-apoptotic protein Bcl2A1 mRNA expressions; showing an increase of Bcl2A1 in DN cells, which could mean that Notch3 even further releases these cells from

normal differentiation. In the latest examinations of the DN cell membrane, decrease of EpCAM expression in Tg mice demonstrated that Notch3 might deregulate not only differentiation, but also cell-cell and cell-stroma interactions, fundamental in thymocyte maturation. The EpCAM turning off could therefore disrupt the interactions between thymocytes and epithelial cells of the stroma (producing CXCL12); interfere with the CXCL12/CXCR4 axis and free thymocytes of pro-proliferative signals. This means that Notch3, through CXCR4 deregulation, definitely negatively affects the thymocyte differentiation and the thymic microenvironment, possibly affecting the cell interactions and in summary critically contributes to development of leukemia in N3-IC Tg model.

B. CXCR4 expression is increased in double positive population

DP cells showed increased CXCR4 receptor presence on the cell surface. Since CXCR4 is also involved in the mechanisms of mature cells migration outside the thymus, this alteration implies an increased migration capacity of DP cells, as confirmed with migration tests. The high percentage of DP cells not only in the thymus, but the spleen of Tg mice as well, suggests that Notch3 over-expression may result in greater emigration of DP thymocytes from the thymus. Particularly the mechanism involving a mutual surface presence of CXCR4 and Notch3 seems to be favoring the migration of DP cells to periphery and hence increasing the T-ALL aggressiveness. The results show that more than half of DP cells found in the spleen are co-expressing CXCR4 and Notch3. This supports our hypothesis and suggests close cooperation between the two receptors in DP cells of Tg mice.

We also think that extensive future studies are necessary to analyze the possible interaction between CXCR4 and Notch3 pathways as a mechanism that regulates CXCR4 signaling and the consequent migration of DP cells from the thymus to the periphery. According to some research, the positive selection is accompanied by down-regulation of CXCR4 and up-regulation of CCR7, and that migration of naïve cells to periphery is guided by gradients of CCL19 and CCL21, both CCR7 receptor ligands (64, 76). CCR7 is allegedly co-expressed on the surface with CXCR4 on T-cells in the blood, spleen and lymph nodes. The persistence of high levels of CXCR4 in the analyzed DP cells suggests a closer look into CCR7 expression in the disease model and its possible involvement in T-ALL (58, 77).

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