Notch3-regulated microRNAs impair CXCR4-dependent maturation of thymocytes allowing maintenance and progression of T-ALL

Ilaria Sergio[1]*, Martina Del Gaizo[2]*, Sandesh Kumar Patel[3]*, Claudia Varricchio[3], Eleonora Russo[3], Andrea Orlando[4], Giovanna Peruzzi[5], Francesca Ferrandino[6], Georgia Tsaouli[7], Daniele Peluso[8], Zein Mersini Besharat[1], Federica Campolo[1], Mary Anna Venneri[1], Donatella Del Bufalo[9], Giovanni Bernardini[3], Isabella Screpanti[3], Antonio Francesco Campese[3#], Maria Pia Felli[1#"].

Supplementary Information:

Supplementary Materials and Methods

Supplementary Figures 1-7

Supplementary References: 4

Supplementary Materials and Methods

Mice

Animal experiments performed with at least three animals of each genotype. The number of used mice is reported in each Figure legend. Experimental mice groups were based on age and genotype. No mice were excluded during the experiments. All animal experiments were performed according to Italian D. Lgs. n.26/2014 and European Directive 2010/63/ UE, approved by the Italian Ministry Committee and Sapienza University (Authorization number 1368.17).

Cell Culture

Murine N3-232T cells are a DN immature phenotype derived from the thymocytes of a 6-week-old N3-ICTg thymus. Human TALL1 cells are a DP immature phenotype with N3 over-expression. All the cells were tested and are mycoplasma-free.

Flow cytometry

T-cells from the thymus and BM were stained with the following antibodies: anti-CD4-PerCP-Cy5.5, anti-CD8-APC, IgG2bPE (12-4031) (BD-Biosciences); anti-CXCR4-PECy-7 and anti-CXCR4-PE (eBioscience); anti-CD25 (BD-Biosciences); and anti-CD3 (ε chain: 145-2C11) (eBioscience); anti-NK1.1 PE, anti MAC1 PE, anti-Gr1 PE (553128 BD Biosciences); CD8-APC-H7(560182). To test vitality and proliferation anti-AnnexinV-APC (550474) (BD-Biosciences) was used with anti-KI67-BV510 clone B56 (BD-Biosciences). Samples analysis was carried out either on FACSCalibur (1) using CellQuest-software (BD-Biosciences), but all the multiparametric on FACS Canto II (Becton-Dickinson) analysis with DivaVersion6.1.3 or FlowJoVersion8.5.2 software. Blinded-conducted analysis performed.

Isolation of DN T-cell subsets by fluorescent-activated cell sorting

Thymocytes of 12-14-week-old N3-ICtg mice were stained with antibodies abovementioned in flow cytometry. Specific DN T-cell subsets were prepared for sorting upon pre-enrichment of DN/$CD4^+$ by using magnetic CD8a (Ly-2) microbeads mouse (cod 130-117-044, Miltenybiotec). Cells were sorted using a FACSAriaIII (Becton Dickinson, BD Biosciences) equipped among others with 488nm, 561nm and 633nm laser and FACSDiva software (BD Biosciences version 6.1.3). Data were analyzed using a FlowJo software (Tree Star, version 9.3.2). Briefly, cells were first gated based on morphology using forward versus side scatter area parameter (FSC-A versus SSC-A) strategy followed by doublets exclusion with morphology parameter area versus width (A versus W).
According to the experiments DN T-cell subsets or CXCR4 positive and negative cells starting from DNCD3ε^+ positive cells were sorted. To reduce stress, cells were isolated in gentle conditions using a ceramic nozzle of size 100µm, a low sheath pressure of 19.84 pound-force per square inch (psi) that maintain the sample pressure at 18.96 psi. Following isolation, an aliquot of each tube of the sorted cells was evaluated for purity at the same instrument resulting in an enrichment >95% for each sample.

Transfections of N3-232T and TALL1 cell lines

N3-232T cells were transfected with 100nM mimic miR-139-5p (mmu-miR-139, ID: MC12466, Invitrogen), antagomir miR-139-5p (mmu-miR-139, ID: MH12466, Invitrogen), mimic miR-150-5p (hsa-miR-150, ID: MC10070, Invitrogen), and their corresponding miRNA mimic 2.0 control negative (Negative Control #1 Invitrogen). Human TALL1 cells were transfected with 100 nM and 200 nM miRNA mimic miR-150-5p (has-miR-150, ID: MC10070, Invitrogen) and their corresponding miRNA mimic 2.0 Control Negative (Negative Control #1 Invitrogen). Neon Transfection System (Invitrogen)

was used for both cell lines. 72h post-transfection, mRNA extraction (see RNA extraction and RT-qPCR) and staining with anti-CXCR4 PE (murine CD184, clone 2B11, 12911-82, eBioscience) and anti-CXCR4 APC (human CD184, BD-Biosciences) were performed for both cell lines. Data analyzed on FacsCalibur with Cell Quest software (BD-Biosciences).

RNA extraction and RT-qPCR

Total RNA was extracted with Trizol reagent (Invitrogen/ThermoFisher, Waltham, MA, USA) and reverse-transcription was performed with High-Capacity cDNA Reverse transcription Kit (ThermoFisher, Waltham, MA, USA). CXCR4 (Mm01996749_s1), β-arrestin1 (Mm00617540_m1), Notch3 (Hs00166432_m1) and β-actin (Mm02619580_g1) mRNA levels were determined by TaqMan quantitative real-time RT-PCR (RT-qPCR) performed on cDNA following manufacturer's instructions (Applied-Biosystems). Data analysis by ΔΔCt method (β-actin use for normalization of mRNA levels); murine β-actin was use as reference. miR-150-5p (has-miR-150, 000473), miR-139-5p (has-miR-139 002289), miR-9-5p (has-miR-9 000583), and miR-223-5p levels were monitored comparatively to U6 (snRNA 001973) as a reference.

Droplet digital PCR (ddPCR)

Amplification was carried out on using the following TaqMan probes: U6 (snRNA 001973), miR-150-5p (has-miR-150, 000473), miR-139-5p (has-miR-139 002289), miR-9-5p (has-miR-9 000583) (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) and ddPCR supermix for probe no dUTP (Bio-Rad Laboratories, Hercules, CA, USA). The PCR mixture was loaded in a disposable droplet generator to create oil-emulsion droplets and transferred to a 96-well PCR plate, sealed, and quantified at the endpoint in a QX200 Droplet Reader System using QuantaSoft software (Bio-Rad Laboratories, Hercules, CA, USA). The fraction of positive droplets was quantified assuming a Poisson distribution. Samples were run in triplicate, and concentration results were expressed as the number of copies of each transcript/μL sample.

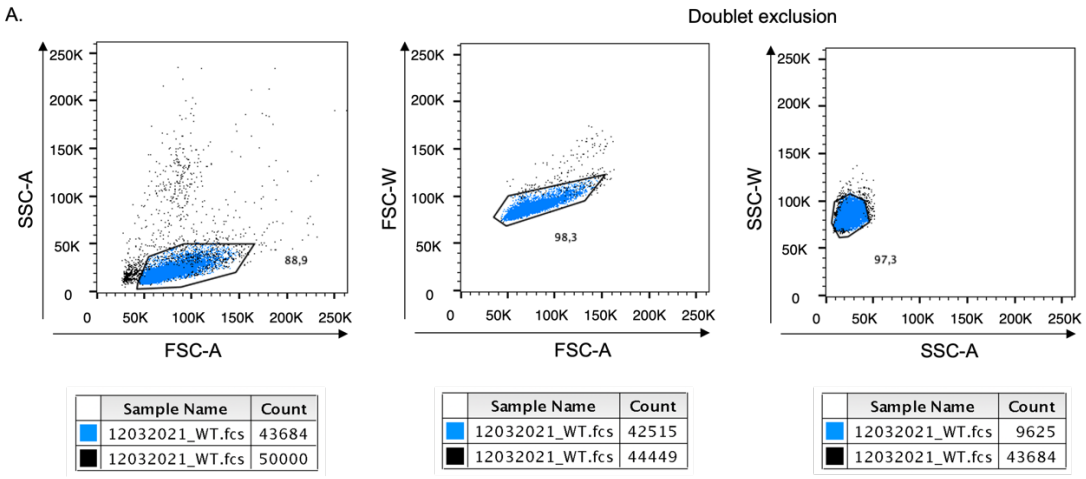
In vivo intravenous CD45 Labeling and analysis of thymic DN T-cells.

In vivo labeling and analysis of DN CD3ε$^+$ thymocytes in the thymic perivascular spaces was performed according to a modification of (2, 3). Briefly, 1ug of anti-CD45.2 PE clone 104 antibody (0.2 mg/ml) in 300mL of PBS was injected intravenously in 12-14-week-old WT and N3-ICtg mice. After 3 minutes, mice were euthanized. Cells from the thymus, the BM and the blood were isolated and stained with the antibodies indicated in the legend of figure 8 and analyzed by flow cytometry. The DN T-cells positive for CD45.2 PE are referred as vasculature-associated circulating cells, whereas CD45.2 PE negative as those residing in the thymic parenchyma at the time of analysis (2, 4). FACS Canto II (Becton-Dickinson) analysis with FlowJoVersion8.5.2 software was done.

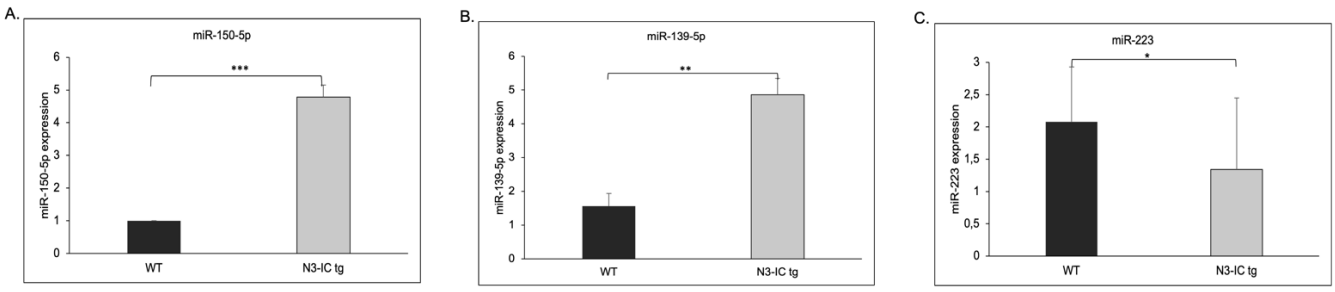
Statistical Analysis

For comparison of two independent groups unpaired *t*-test was done. Comparison of more groups using one-way ANOVA (Tukey's post-test) was performed. The index Pearson r expresses the linear relation between paired samples, and p-values were calculated using Student's t-test. A p-value of <0.05 was considered statistically significant (*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$). At least three independent experiments are reported as mean ± SD (the repeat number was increased according to the effect size or sample variation). Estimation of sample's size considering variation and mean of samples was performed. No animals/samples were excluded from analysis. All analyses and data visualization were carried out with GraphPad Prism 8 software.

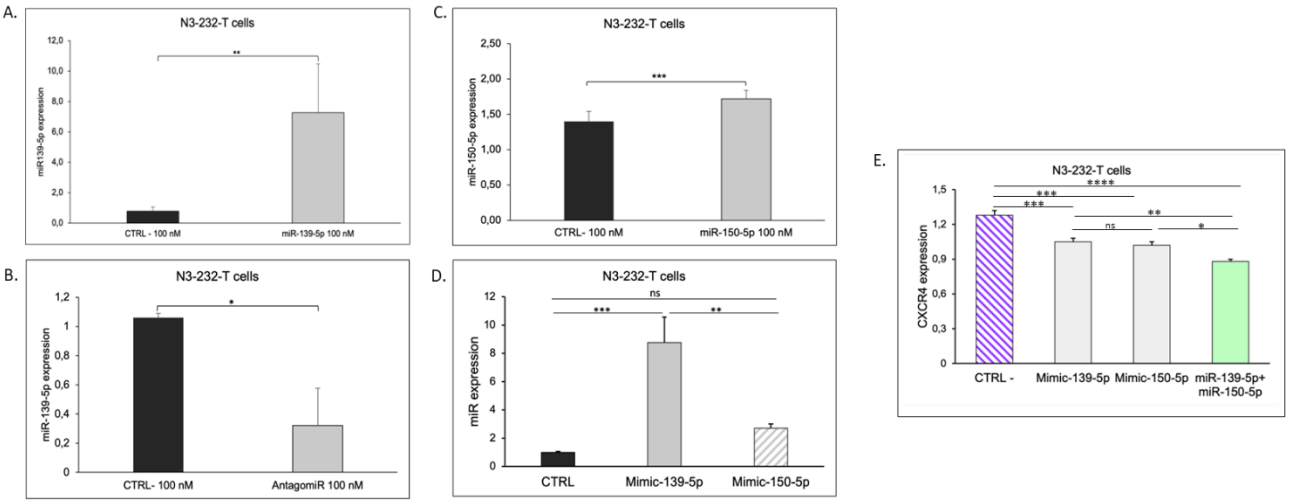
Supplementary Figures



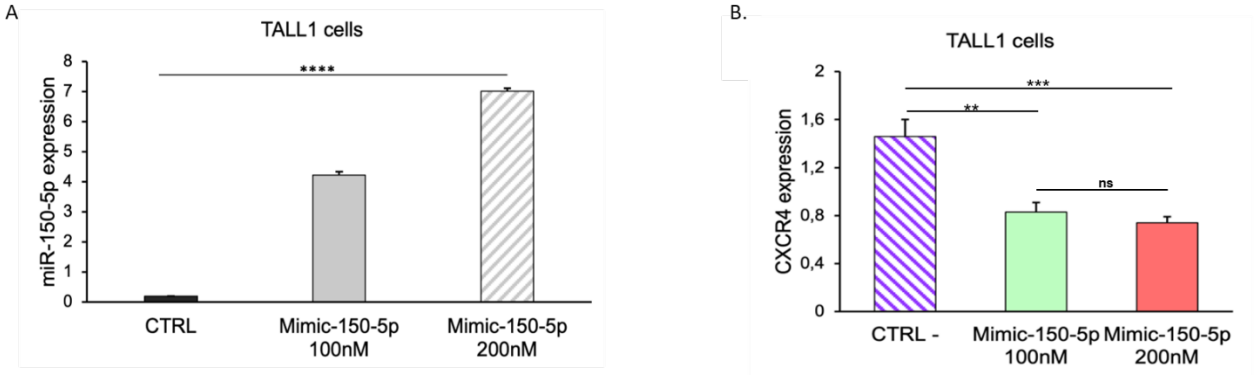
Supplementary Figure 1. Selection of CD4⁻CD8⁻ Thymocytes. CD4⁻CD8⁻ (DN) gating strategy to isolate DN subsets from WT. The same procedure was used with the N3-IC tg Thymocytes. The check purity of the selected DN T-cells is shown in figure 5.A (right panel).



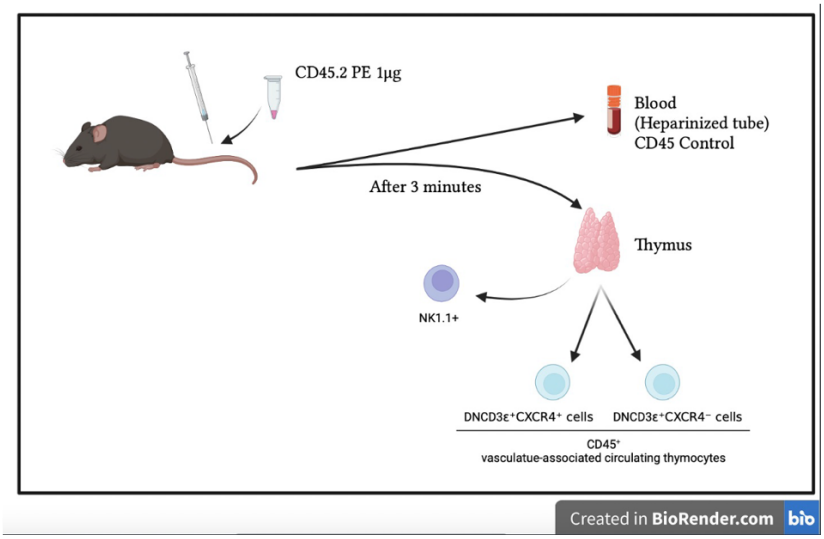
Supplementary Figure 2. miRNA expression in Selected DN T-cells of 12-14-week-old WT and N3-ICtg mice. miR150-5p (A), miR139-5p (B), and miR223 (C) evaluated by RT-qPCR in selected DN thymocytes (NK1.1⁻Mac1⁻Gr1⁻ sorted cells) of 12-14-week-old WT and N3-IC-Tg mice. Student's t-test (*p< 0.05; **p<0.01; ***p<0.001).



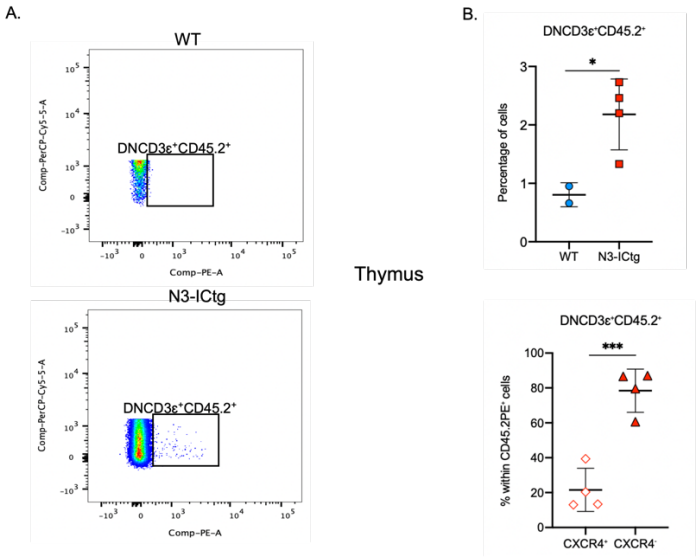
Supplementary Figure 3. CXCR4 and miRNA expression in transfected murine N3-232T cells.
Evaluation of miRNA expression 72h after their transfection in murine N3-232T cells, a Notch3 transgenic model, as compared to the negative control (CTRL): A) mimic miR139-5p and B)Antagomir 139-5p; C) mimic miR150-5p and D) the combined transfection of miR-139-5p and miR-150-5p; E) The synergic action of miR-150-5p and miR-139-5p on CXCR4 expression as compared to single miRNA and CTRL- in N3-232-T cells. One way ANOVA, Tukey's post-test and Student's t-test (ns, not significant, *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001).



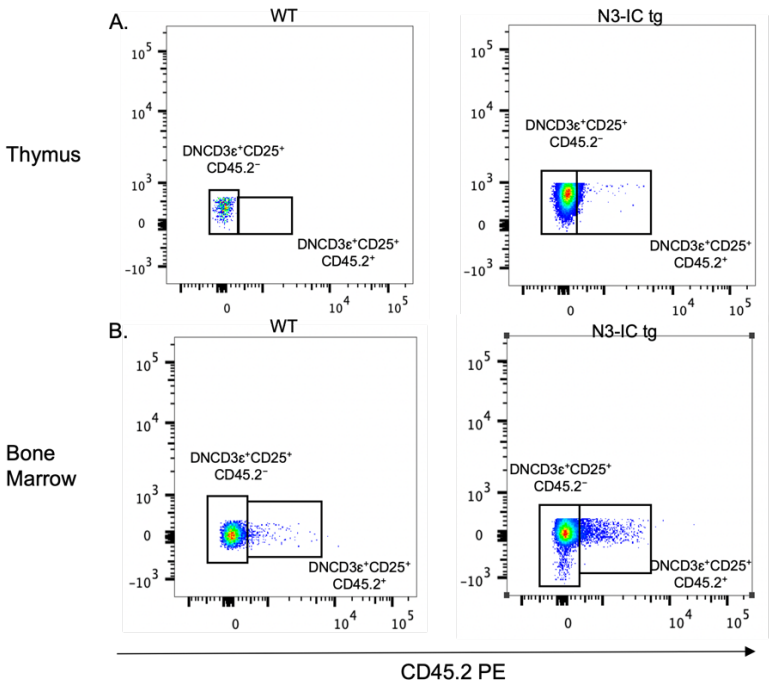
Supplementary Figure 4. CXCR4 mRNA expression in miRNA transfected human TALL1 cells.
A) Increasing mimic miR150-5p (100-200nM) in human TALL1 cell line; B) CXCR4 expression in human TALL1 cells transfected with increasing amount of mimic miR-150-5p (100-200mM); One way ANOVA, Tukey's post-test and Student's t-test (**p<0.01; ***p<0.001, ****p<0.0001; ns, not statistically significant).



Supplementary Figure 5. A visual flow chart of in vivo CD45 labeling in 12-14-week-old WT and N3-IC-Tg mice. Intravenous in vivo CD45.2 labeling and analysis of thymocytes in the perivascular space of 12-14-week-old WT and N3-IC-Tg mice. Schematic representation of the experimental procedures before flow cytometry analysis excluding NK1.1^{+} cells. CD45^{+} are referred as vasculature-associated circulating cells distinguished from those CD45^{-} residing within the tissues at the time of analysis.



Supplementary Figure 6. In vivo CD45.2 PE labeling of thymocytes in WT and N3-ICtg mice.
A) Gating strategy to identify DNCD3^{+}CD45.2 PE positive vascular-associated circulating thymocytes from those residing within tissues at the time of analysis and CD45.2 PE negative thymocytes associated to parenchyma in WT and N3-ICTg mice; B) analysis of DNCD3e^{+}CD45.2^{+} thymocytes in WT (n=2) and N3-ICTg (n=4) mice (upper panel) and percentage of CXCR4 positive and negative thymocytes within the CD45.2PE+ subset (lower panel). Student's t-test (*p< 0.05 ; ***p<0.001).



Supplementary Figure 7. In vivo CD45.2 labeling: thymus and bone marrow analysis of WT and N3-ICtg mice. Analysis of DNCD3ε⁺CD25⁺ cells within the thymus (A) and Bone Marrow (B) of WT and N3-Ictg mice. Gating strategy to identify CD45.2 PE positive vascular-associated circulating thymocytes from those residing within tissues at the time of analysis and CD45.2 PE negative thymocytes associated to parenchyma.

Supplementary references: 4

1. Massimi I, Ciuffetta A, Temperilli F, Ferrandino F, Zicari A, Pulcinelli FM, et al. Multidrug Resistance Protein-4 Influences Aspirin Toxicity in Human Cell Line. Mediators Inflamm. 2015;2015:607957. https://doi.org/10.1155/2015/607957.
2. Ruscher R, Hogquist KA. Intravenous Labeling and Analysis of the Content of Thymic Perivascular Spaces. Bio Protoc. 2018;8(5). https://doi.org/10.21769/bioprotoc.2757.
3. Sciume G, De Angelis G, Benigni G, Ponzetta A, Morrone S, Santoni A, et al. CX3CR1 expression defines 2 KLRG1+ mouse NK-cell subsets with distinct functional properties and positioning in the bone marrow. Blood. 2011;117(17):4467-75. https://doi.org/10.1182/blood-2010-07-297101.
4. Anderson KG, Mayer-Barber K, Sung H, Beura L, James BR, Taylor JJ, et al. Intravascular staining for discrimination of vascular and tissue leukocytes. Nat Protoc. 2014;9(1):209-22. https://doi.org/10.1038/nprot.2014.005.