NF-κB-dependent RANKL expression in a mouse model of immature T-cell leukemia

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ABSTRACT

Activation of the receptor activator of nuclear factor-κB (RANK) by its ligand (RANKL) is involved in both solid and hematological malignancies, including multiple myeloma, acute myeloid leukemia and B-cell leukemia. Although RANKL expression has been described in normal T cells, a potential role in T-cell leukemia remains undefined. Here, we used a model of immature T-cell leukemia/lymphoma, the TEL-JAK2 transgenic mice, to assess RANKL expression in leukemic cells and its regulatory mechanisms. We found that *Rankl* mRNA was significantly overexpressed in leukemic T cells when compared to wild-type thymocytes, their nonmalignant counterparts. Moreover, *Rankl* mRNA and RANKL surface expression in leukemic cells was induced by T-cell receptor (TCR) signaling activation, dependently on the NF-κB signaling pathway. These results indicate that TCR-activated leukemic T cells express high levels of RANKL and are potential inducers of RANK signaling in microenvironmental cells.

Keywords: Leukemia; Lymphoma; NF-κB; RANKL; T cell; TNF family

Abbreviations: ATL, adult T-cell leukemia; LGR4, leucine rich repeat containing G protein-coupled receptor 4; NF-κB, nuclear factor kappa B; OPG, osteoprotegerin; PMA, phorbol 12-myristate 13-acetate; RANK, receptor activator of NF-κB; RANKL, receptor activator of NF-κB ligand; T-ALL, T-cell acute lymphoblastic leukemia; TCR, T-cell receptor; TJ2-Tg, TEL-JAK2 transgene.

1. Introduction

The receptor activator of NF-κB (RANK) and its only known ligand, RANK ligand (RANKL), are members of the tumor necrosis factor (TNF) superfamily, critical for several physiological processes, including bone remodeling, lymphoid organ organogenesis, adaptive immunity, mammary gland development and thermoregulation [1]. RANKL (encoded by the *Tnfsf11* gene) is a type II transmembrane protein that may also exist in a soluble form, generated by either proteolytic processing [2] or alternative mRNA splicing [3]. RANKL binds not only RANK (encoded by the *Tnfrsf11a* gene) but also two other proteins, osteoprotegerin (OPG) [4,5], a secreted decoy receptor encoded by the *Tnfrsf11b* gene, and LGR4, a G-protein-coupled receptor of the LGR family [6]. By sequestering RANKL, these proteins negatively regulate RANK signaling.

RANK is mainly expressed in mature dendritic cells (DCs) [7], osteoclasts [4], mammary epithelial cells [8], and thymic medullary epithelial cells [9]. In turn, RANKL is expressed in pro-B cells [10], T cells, mature DCs [7], osteocytes [11], osteoblasts and bone marrow stromal cells [5]. RANKL expression was reported during thymocyte development, transiently in CD4/CD8 double-negative (DN) thymocytes, and then in positively selected mature thymocytes, mainly in CD4 single-positive (CD4SP) thymocytes [7,9,10,12]. RANKL is weakly expressed on human or mouse naive T cells, but is highly induced at the membrane upon T-cell receptor (TCR)-mediated activation [3,7,13,14]. This induction by TCR stimulation was shown to be controlled by calcineurin-regulated transcription factors [3,12–15].

Interestingly, deregulated RANKL-to-RANK signaling is critically involved in both solid and hematological cancers, turning this axis into a therapeutic target [16]. RANKL was shown to be expressed at high levels and to favor the development of hematological cancers such as acute myeloid leukemia (AML) [17], multiple myeloma and B-cell chronic lymphocytic leukemia [18]. In addition, RANKL was shown to be overexpressed in adult T-cell leukemia (ATL), in association with the frequently observed hypercalcemia [19]. The RANKL/RANK axis was also

implicated in the crosstalk of tumor cells with the immune system, having an important role in suppressing antitumor immunity [16].

Although RANKL is expressed in particular thymocyte subsets, its role in immature T cell leukemia has so far not been reported. Herein, we show that RANKL mRNA and protein is highly expressed in leukemic T cells from the TEL-JAK2 transgenic mouse model of acute T-cell leukemia. In addition, we found that RANKL protein surface expression in leukemic cells is increased by TCR signaling in an NF-κB-dependent manner.

2. Materials and methods

2.1. Mice

EμSRα-TEL-JAK2 (B6.Cg-Tg(Emu-ETV6/JAK2)71Ghy) transgenic (TJ2-Tg) mice, previously provided by Dr. Jacques Ghysdael (Institut Curie, France), were described before [20,21]. C57BL/6J mice were purchased from Harlan Laboratories (C57BL/6JRccHsd substrain) and bred locally. Mice were maintained in the CBMR/UAlg barrier animal facility (HEPA filtration of incoming air, differential pressure and disinfection or sterilization of room equipment and supplies), under 12 h light/dark cycles, controlled room temperature (22ºC), in type II polycarbonate cages (Tecniplast, Italy) and with autoclaved food (4RF25 diet; Mucedola, Settimo Milanese, Italy) and water *ad libitum*. Microorganism screening (Charles River, L'Arbresle, France and Idexx Bioresearch, Ludwigsburg, Germany) detected opportunistic pathogens (*Helicobacter* spp., *Pasteurella pneumotropica* and murine norovirus) in the CBMR/UAlg housing room. All experimental procedures followed recommendations for the care and use of laboratory animals from the European Commission (Directive 2010/63/UE) and Portuguese authorities (Decreto- Lei nº113/2013). TJ2-Tg mice were generated by backcrossing with C57BL/6J females, thus keeping the transgene in heterozygosity, were monitored for signs of spontaneous disease (i.e. dyspnea, lethargy, enlarged lymph nodes or enlarged abdomen) and killed by CO_2 inhalation when reaching predefined experimental endpoints. Both female and male mice were used for experiments.

2.2. Cell culture

The mouse thymic lymphoma EL4.2 cell line was provided by Dr. José M. Almendral (Madrid, Spain) and shown to express at the surface CD90.2 and CD44, but not CD3ε, CD4 and CD8 (data not shown). EL4.2 cells were cultured in complete RPMI medium [RPMI1640 medium (Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated foetal bovine serum (PAA Laboratories, Linz, Austria), 2 mmol/l L-glutamine, 100 U/ml of penicillin and 100 μg/ml of

streptomycin (Lonza)] and maintained at 37°C under a humidified atmosphere with 5% CO_2. Primary TJ2-Tg leukemic cells were obtained as single cell suspensions from the thymus or lymph nodes, after gentle tissue dissociation and filtration through 70 µm nylon cell strainers. Thymocyte single-cell suspensions were obtained from wild-type C57BL/6J mice, following the same procedure. For cell treatments, EL4.2 or mouse primary cells were cultured in complete RPMI medium at 2×10^6 cells/ml and treated with either DMSO (control), 50 µg/ml cycloheximide (CHX), 10 ng/ml phorbol 12-myristate 13-acetate (PMA) and 250 ng/ ml ionomycin (PMA+Iono) (all from Sigma-Aldrich), 10 µg/ml plate-bound CD3ε antibody (BioLegend, San Diego, CA USA), 10 µmol/l tetracyclic pyridone 6 (InSolution Jak Inhibitor I, JAKi) or 10 µmol/l BMS-345541 (IKKi) (both from Calbiochem), as indicated.

2.3. Reverse transcription quantitative PCR (RT-qPCR)

Total RNA from mouse cells and cell lines was prepared using Trizol reagent (Life Technologies) and Direct-zol RNA miniprep (Zymo Research, Irvine, CA, USA), following the manufacturers' instructions. RNA samples were treated with DNase I (Thermo Fisher Scientific, Waltham, MA, USA) to remove remaining genomic DNA. Then, total RNA (1 µg) was reverse-transcribed using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) and oligo(dT)18 primers. Quantitative PCR reactions, containing 2 µl of 1:20 diluted cDNA in 20 µl, SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) and 300 nmol/l of murine *Rankl* primers (5'-CGC CTC CCG CTC CAT GTT CC-3'; 5'-GTT CCT TCT GCA CGG CCC CC-3') were performed on a C1000 Thermal Cycler coupled to a CFX 96 Real-time PCR detection system (Bio-Rad). PCR results were analyzed by Bio-Rad CFX Manager software, version 3.0, treated using the comparative C_T method ($2^{-\Delta\Delta C(T)}$ method), and the mean fold change in expression of the target gene was calculated in relation to *Gapdh* reference gene expression (5'-GGT GAA GGT CGG TGT GAA CG-3'; 5'-ACC ATG TAG TTG AGG TCA ATG AAG G-3').

2.4. Flow cytometry and cell sorting

Single-cell suspensions were stained with fluorochrome-labeled antibodies as previously described [21] and detected in a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA, USA) or sorted using a FACSAria I (BD Biosciences). Fluorescein isothiocyanate (FITC)-, R-phycoerythrin (PE)-, or PE-cyanin 5 (PE-Cy5)-conjugated antibodies specific for RANKL (IK22/5), CD69 (H1.2F3), CD4 (GK1.5), and CD8 (53-6.7) (BioLegend, San Diego, CA USA) were used. For RANKL intracellular staining, cells were fixed with 4% paraformaldehyde for 20 minutes at 4°C, rinsed twice in PBS containing 0.5% BSA and 0.1% saponin, and incubated for 1 hour on ice with PE-conjugated anti-RANKL diluted in the latter solution. Nonviable cells were excluded from analyses by propidium iodide (Sigma-Aldrich) or 7-AAD (BioLegend) staining and appropriate gating. The data were analysed on CellQuest (BD Biosciences) and FlowJo (FlowJo LLC, Ashland, OR) software.

2.5. Statistics

Statistical analyses were performed using Prism 5 (GraphPad Software, La Jolla CA, USA). Statistical tests were used as indicated in figure legends. Two-tailed *t* tests were used to determine how significant were the differences between the means of two independent samples. Welch's correction was applied when variances were unequal, as determined by the F-test. A *P* value below 0.05 was considered statistically significant.

3. Results

3.1. RANKL expression in mouse leukemic T cells

To assess RANKL expression in immature T-cell leukemia, we assessed *Rankl* mRNA levels in primary leukemic T cells from TEL-JAK2 transgenic (TJ2-Tg) mice. Analysis of publicly available microarray datasets [22] revealed that *Rankl* (but not the genes encoding the RANK and OPG receptors) was upregulated in TJ2-Tg leukemic samples as compared to control thymocyte samples (Supplementary Fig. 1). RT-qPCR analyses confirmed that *Rankl* expression, albeit heterogeneous, was significantly higher in TJ2-Tg leukemic cells that in control wild-type thymocytes (Fig. 1A). Since in the thymus, *Rankl* is mostly expressed in mature CD4SP thymocytes [7,9], we compared *Rankl* expression in TJ2-Tg leukemic cells with that in sorted CD4SP cells. Although TJ2-Tg leukemic cells were previously shown to be immunophenotypically and transcriptomically more related to immature CD4/CD8 double-positive (DP) than mature thymocytes [20,22], the *Rankl* expression levels in leukemic cells were comparable to those in CD4SPs and notably higher than those in DPs (Fig. 1B). Transcriptomic analysis of another mouse model, the LN3 murine model, which is driven by inherited prerearranged TCRαβ genes [23], showed high *Rankl* expression in thymic lymphomas, as compared to expression in normal thymocyte subpopulations (Supplementary Fig. 2). Together, these data show that the *Rankl* gene is upregulated in mouse models of acute T-cell leukemia/lymphoma.

In contrast to the gene expression results, the RANKL protein was barely detectable at the surface of TJ2-Tg leukemic cells (Fig. 1C). However, fixation and permeabilization of TJ2-Tg leukemic cells revealed that RANKL protein was consistently expressed in intracellular compartments at similar or higher levels than in wild-type thymocytes (Fig. 1D). These data thus indicate that TJ2-Tg leukemic T cells express high levels of RANKL mRNA and protein, as compared to normal thymocytes.

3.2. RANKL surface expression is induced upon leukemic T-cell activation

Since TCR stimulation of T cells or activation of signaling pathways downstream of TCR result in RANKL induction [7,9,12,13,15], we assessed the impact of compounds mimicking TCR signaling on *Rankl* expression in leukemic cells. It was previously shown that PMA/ionomycin treatment of TJ2-Tg leukemic cells induces their proliferation and expression of the CD69 activation marker and lymphotoxin proteins [20,22]. Although leukemic cell culture alone led to increased *Rankl* gene expression (Fig. 2A), *Rankl* was further upregulated upon PMA/ionomycin stimulation (Fig. 2B). PMA/ionomycin stimulation led to increased size (Fig. 2C) and surface CD69 upregulation (Fig. 2D) of both normal thymocytes and TJ2-Tg leukemic cells. However, under the same conditions leukemic cells displayed much higher surface RANKL upregulation than normal thymocytes (Fig. 2D). In addition, direct stimulation of the TCR/CD3 complex by an agonist antibody also led to strong RANKL induction in leukemic cells (Fig. 2E).

Although PMA alone was able to induce TJ2-Tg leukemic cell proliferation [22], neither PMA nor ionomycin alone induced RANKL surface expression in TJ2-Tg leukemic cells (data not shown). In addition, cell permeabilization showed that PMA/ionomycin-mediated increase in cell surface RANKL corresponded to an increase in total protein levels (Fig. 3A). Indeed, inhibition of protein synthesis by cycloheximide treatment prevented PMA/ionomycin-induced RANKL surface expression but not growth of leukemic cells (Fig. 3B and C). Together, these results indicate that stimulated TJ2-Tg leukemic cells increase RANKL synthesis and surface expression.

3.3. RANKL expression in TEL-JAK2 leukemic cells depends on NF-κB activity

TJ2-Tg leukemic cells are characterized by constitutive activation of the JAK/STAT and IKK/NF-κB signaling pathways [24,25]. To understand if these pathways regulate *Rankl* expression in TJ2-Tg leukemic cells, these cells were treated *in vitro* with specific pharmacological inhibitors. While a pan-JAK inhibitor had no effect on *Rankl* expression (Fig. 4A), this gene was

significantly downregulated in TJ2-Tg leukemic cells treated with BMS-345541 (Fig. 4A), an IKK inhibitor previously shown to decrease the expression of NF-κB target genes in TJ2-Tg leukemic cells [20]. Further supporting a role for NF-κB activity in controlling *Rankl* expression in leukemic cells, the IKK inhibitor strongly impaired RANKL protein upregulation by PMA/ionomycin treatment (Fig. 4B). Leukemic cell growth induced by PMA/ionomycin was also blocked by BMS-345541 (Fig. 4C), indicating that the NF-κB pathway is an important mediator of PMA/ionomycin activity in these cells. In all, these results indicate that leukemic cell surface RANKL upregulation upon PMA/ionomycin treatment is chiefly due to NF-κB-dependent *Rankl* gene transcription and translation.

4. Discussion

RANKL is expressed at different stages of T-cell development and activation states, but the potential role of RANKL in T-cell malignancies has been less well characterized. In the present study, we found that RANKL mRNA and protein is highly expressed in mouse immature T cell leukemia/lymphoma, as compared to normal thymocytes. Within thymocyte subsets, the highest expression of RANKL mRNA and protein is found in CD4SP thymocytes that recently underwent TCR-mediated positive selection [9,26]. Although expressing surface TCR, TJ2-Tg leukemic cells do not require this receptor for their development and appear to arise from pre-selection thymocytes [22]. We also observed constitutive RANKL surface expression on a TCR/CD3-negative mouse thymic lymphoma cell line (Supplementary Fig. 3). Thus, in contrast to normal CD4SP thymocytes, leukemic cells do not upregulate RANKL in response to positive selection signals, but likely in response to other signaling mechanisms.

Although RANKL expression at the surface of freshly collected TJ2-Tg leukemic cells was barely detectable, it was abundant in intracellular compartments. RANKL surface expression in normal thymocytes was shown to be detectable only when briefly cultured *ex vivo* [9,26]. We observed a slight increase in RANKL surface expression in cultured leukemic cells (Fig. 3B), further confirming that *in vivo* surface RANKL expression is transient. Reduced RANKL surface expression on leukemic cells may result from protein cleavage and shedding, as demonstrated in cell lines [2], and/or RANKL intracellular storage in secretory lysosomes and translocation to the cell surface only upon RANK binding, like previously shown for osteoblasts and osteocytes [27,28].

Like in human and mouse T cells [7,13,14], both *Rankl* mRNA and RANKL cell surface expression were induced in TJ2-Tg leukemic cells following anti-CD3ε or PMA/ionomycin stimulation. In addition, we found that treatment with an IKK inhibitor could reduce RANKL mRNA and protein expression in cultured cells treated or not with PMA/ionomycin. Leukemic cells from TJ2-Tg mice present constitutive NF-κB activation [25], and its inhibition was shown

to downregulate expression of target genes, including the TNFSF members lymphotoxin-α and -β genes [20]. The *Rankl* promoter was shown to carry NF-κB consensus sites and to bind these factors in PMA/ionomycin-stimulated T cells [13]. Together, these experiments define IKK kinases and NF-κB transcription factors as key regulators of RANKL expression in TJ2-Tg leukemic cells.

Previous reports uncovered overexpression of RANKL in human ATL, a highly aggressive neoplastic disease of peripheral T lymphocytes [19,29]. Although no report has addressed RANKL expression in T-cell acute lymphoblastic leukemia (T-ALL), a T-cell precursor malignancy, this gene was found to be expressed in the Jurkat T-ALL cell line after anti-CD3/CD28 or PMA/ionomycin stimulation [3,30]. This notion raises the possibility that TCR/CD3 signaling in patient T-ALL cells may induce RANKL expression.

Although several cell types express RANK and are potential targets of RANKL-expressing leukemic cells, RANKL expression in hematological malignancies has been mainly linked to increased osteoclastogenesis and bone resorption [18,19,30–32]. RANKL in leukemic T cells may activate and modulate specific RANK-expressing microenvironmental cells, such as osteoclasts in the bone marrow microenvironment. TJ2-Tg leukemia arises in the thymus, leading to the development of lymphomas characterized by progressive alterations in epithelial cell composition [21]. Since medullary thymic epithelial cells (mTECs) express RANK and differentiate in response to RANKL [9,33], it is possible that RANKL expression by leukemic cells influences mTEC function. RANKL-RANK signaling was also shown to mediate interactions between T cells and DCs, thus favoring T-cell proliferation and DC function and survival [10]. DCs are present in thymic lymphomas from TJ2-Tg mice [21], and are therefore potential targets of RANKL-expressing leukemic cells. Future studies should address the potential role for RANKL-RANK signaling in T-cell leukemia.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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Legends to the Figures

Fig. 1. High RANKL mRNA and protein expression in TEL-JAK2 mouse leukemic cells. (A) RT-qPCR quantification of *Rankl* in thymocyte-enriched fractions collected from wild-type thymi (WT; n=4) and leukemic cell-enriched fractions collected from TJ2-Tg thymic lymphomas (n=11). Results are expressed as mean ± SEM. ***, $P < 0.001$, two-tailed, unpaired *t*-test with Welch's correction. B) *Rankl* RT-qPCR analysis in WT thymocytes (n=2), TJ2-Tg leukemic cells (n=4), and sorted CD4/CD8 double-positive (DP; n=2) and CD4 single-positive (CD4SP; n=2) thymocytes. C,D) Flow cytometry RANKL immunostaining of either viable (C) or fixed and permeabilized (D) WT thymocytes and TJ2-Tg thymic (Thy) or lymph node (LN) leukemic cells, collected from the indicated biological replicates. TJ2-Tg nº133, 134, 138, 251, 367, 544 and WT nº1 and 3 were female, while TJ2-Tg nº139 and WT nº2 and 4 were male

Fig. 2. RANKL expression in leukemic T cells is induced by signals mimicking TCR stimulation. A) RT-qPCR analysis of *Rankl* mRNA levels in TJ2-Tg leukemic cells freshly collected (0 h) or after *ex vivo* culture for 10 h (n=6). Results for each sample were normalized to *Gapdh* mRNA expression and are represented as the mean of triplicates. *, $P < 0.05$, two-tailed, paired t-test. B) RT-qPCR quantification of *Rankl* mRNA in two independent TJ2-Tg leukemic cell samples treated *ex vivo* for 10 h with either DMSO (vehicle) or PMA/ionomycin (PMA+Iono). C) Determination of wild-type (WT) thymocyte or TJ2-Tg leukemic cell size by flow cytometry, assessed by forward scatter (FSC-H) signal in gated viable cells, upon 22 h treatment with PMA+Iono or DMSO. One representative of three biological replicates (2 female and 1 male) is shown. D) CD69 and RANKL upregulation detected in cells treated as in (C). E) CD69 and RANKL surface detection in leukemic cells treated with PMA+Iono, plate-bound anti-CD3ε antibody, or DMSO.

Fig. 3. RANKL surface expression in TJ2-Tg leukemic cells depends on *de novo* protein synthesis. A) Detection of total RANKL in fixed and permeabilized normal thymocytes and TJ2-Tg leukemic cells after 18 h stimulation with PMA/ionomycin (PMA+Iono) or DMSO (vehicle). One representative (nº251) of two biological replicates (both female) is shown. B) Flow cytometry immunostaining of RANKL (solid line) or isotype control (gray shading) of TJ2-Tg (male nº199) leukemic cells freshly collected (ex vivo), or cultured for 6 h with DMSO, PMA/ionomycin (P+I), cycloheximide (CHX), or these two conditions combined (CHX + P+I). C) Cell size assessment by flow cytometry FSC-H signal of cells treated as in B).

Fig. 4. Rankl mRNA expression in TEL-JAK2 mouse leukemic cells is mediated by IKK/NF-κB signaling. A) RT-qPCR quantification of *Rankl* mRNA in three independent TJ2-Tg leukemic cell samples treated *ex vivo* for 10 h with either DMSO (vehicle) or a pan-JAK inhibitor (JAKi) (left) or an IKK inhibitor (IKKi) (right). ns, non significant; ***, $P < 0.001$, two-tailed, paired t-test. B) Flow cytometry immunostaining of total RANKL protein in fixed and permeabilized TJ2-Tg leukemic cells (female nº389) cultured for 18 h with the indicated compounds. C) Cell size assessment by flow cytometry FSC-H signal of cells treated as in B).