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Immunoporosis: A New Role for Invariant Natural Killer T (NKT) Cells Through Overexpression of Nuclear Factor-κB Ligand (RANKL)

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Background: Osteoporosis affects millions of postmenopausal women worldwide. Invariant natural killer T cells (iNKT) are important cells for bone homeostasis. The sim of this study was to investigate the contribution of invariant natural killer T cells (iNKT) in the increased receptor activator of the nuclear factor-κB ligand (RANKL) pool and bone resorption, a characteristic of patients with osteoporosis.

Material/Methods: Whole blood was collected from 79 female patients. The dual energy x-absorptiometry scan was performed in all patients, and the T-score was calculated in order to classify our patients according to the World Human Organization (WHO) criteria for diagnosis and classification of osteoporosis. Eleven patients had a T-score <–1.0 and were encompassed in our normal donors (ND) group, 46 patients had a T-score between –1 and –2.5 and were included in the osteopenia group, while 22 patients had a T-score > –2.5 and were included in the osteoporosis group. We performed α-galactosylceramide activation of iNKT cells *in vitro*. Surface RANKL expression was detected by multicolor flow cytometry in naive and activated lymphocytes. Beta-Crosslaps (β-CTx) levels were measured in whole blood plasma by ELISA (enzyme-linked immunosorbent assay).

Results: Although iNKT cells were not clonally expanded in patients with osteoporosis, iNKT cells from osteoporotic patients overexpressed RANKL compared to ND and osteopenic patients. This is a distinctive feature of iNKT cells and is not seen in conventional T-lymphocytes. RANKL expression in iNKT cells was not related to β-CTx levels in the blood. Finally, iNKT cell activation by the prototypal glycolipid ligand α-galactosylceramide increased by 8 times their RANKL expression.

Conclusions: In patients with osteoporosis, iNKT cells specifically overexpress RANKL, a cytokine that regulates osteoclast activity. It seems that iNKT cells have a long-standing effect of on the bone physiology, which plays an important role in the bone loss of patients with osteoporosis.

MeSH Keywords: Glycolipids • Natural Killer T-Cells • Osteoporosis • RANK Ligand

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Background

Osteoporosis is the most prevalent bone disease worldwide, affecting mainly women of postmenopausal age [1]. Estrogen loss orientates the immunological bone niche in a way that enhances bone resorption [2,3]. Osteoclasts, osteoblasts, and probably the osteomacs are the cellular regulators of bone homeostasis [4]. Osteoporosis development is linked to increased osteoclast function [5].

The dominant regulator of osteoclastogenesis and bone resorption is the pathway of the receptor activator of the nuclear factor-κB ligand (RANKL). The imbalance in the RANKL/RANK/OPG pathway is linked to inherited and acquired bone diseases like cancer bone disease, immune arthritis, and osteoporosis [6]. Osteocytes, osteoblasts, the immune cells, and the mesenchymal cells are the richest sources of RANKL in a bone marrow microenvironment [7].

In the context of osteoporosis, the imbalance between Th1/Th2 and Th17/Tregs development causes a deregulation in the immunological bone niche that finally favors bone loss [2,8]. In parallel, the over-secretion of tumor nuclear factor (TNF)α, interferon gamma (IFNγ), interleukin (IL)-1, and IL-6 by the immune cells, contributes to osteoporosis development indirectly by increasing RANKL overexpression on bone marrow mesenchymal cells [2,3,5,6].

The interplay between immune cells and osteoclasts is more complicated and osteoclasts can expand the CD8+ Tregs (Foxp3+Cd25+) cells in a way that decreases bone resorption, creating thus a paradoxical autoregulatory loop [9]. Furthermore, pulsed administration of low doses of RANKL in ovariectomized mice through induction of Tregs development can protect against bone loss [10]. Thus, the inhibitory role of Tregs in bone resorption is well established.

Invariant natural killer T cells (iNKT) cells are a rare but powerful subset of regulatory T cells with invariant TCR-receptor (Va24-Ja18/Vb11 in humans) that recognizes lipid antigens presented by CD1d, a non-classic MHC-I-like antigen presenting molecule [11]. These iNKT cells can either suppress or enhance a variety of immune responses by robust secretion of immune cytokines or through direct cell to cell contact [12]. In a variety of immunological driven diseases like rheumatoid arthritis, aplastic anemia, and systematic lupus erythematosus, the percentage of iNKT cells in the peripheral blood is diminished [13–15]. In immune arthritis, iNKT cells play a contradictory role in determining the Th17/Tregs balance and affecting the Th1/Th2 fate of CD4+ T-lymphocytes [12,16,17]. Furthermore, iNKT deficient mice have a mild osteopetrotic phenotype supporting the role of iNKT, not only in bone diseases but also in bone homeostasis under physiological conditions.

In this model, iNKT activation through the prototypical glycolipid, α-galactosylceramide (agal), enhances osteoclastogenesis in an iNKT dependent manner [18]. Our group recently showed that in patients with multiple myeloma, a type of cancer that is characterized by osteolytic bone lesions, iNKT cells overexpress RANKL during disease progression from the benign MGUS (monoclonal gammopathy of uncertain significance) to overt myeloma [19]. We further showed that glycolipids – and especially GM3 – a trigger osteoclast formation in myeloma patients through activation of the RANKL pathway, by altering the cell signaling cascade, starting from the lipid rafts of their membrane [20]. On top of this evidence, our aim was to examine the contribution of iNKT cells in the increased RANKL pool, in patients with osteoporosis.

Material and Methods

Whole blood was collected, after informed consent was signed by patients who were referred to the Osteoporosis Unit of our Institution for diagnosis and potential treatment. (Demographics of patients included in this study are shown in Table 1). Female patients with autoimmune diseases, rheumatic diseases, endocrinopathies except controlled hypothyroidism, history of immunosuppression therapy or history of neoplasmatic diseases and male patients (where the osteoporosis is usually secondary to other diseases or pharmacological agents) were excluded from our study. Blood tests (including full blood count, C-reactive protein, erythrocyte sedimentation rate, etc.) were used to exclude the coexistence of conditions connected to a high expression of RANKL like metastatic cancer, bone malignancy and immune arthritis. At the end, 79 female patients were eligible to participate to our study. The dual energy x-absorptiometry scan (DEXA-scan) was performed in all patients with the WINMOC 8.0.1.2 device (LEN Laboratory Elettronici), and the T-score was calculated in order to classify our patients according to the World Human Organization (WHO) criteria, for diagnosis and classification of osteoporosis [21]. Eleven patients had T-score <–1.0 and were included in the normal donor (ND) group, 46 had T-score between –1 and –2.5 and were included in the osteopenia group, while 22 patients with T-score >–2.5 were assigned in the osteoporosis group. The study was performed according to the principles of the Declaration of Helsinki.

Surface RANKL expression was detected by multicolor flow cytometry. Initially, lymphomonocytic cells were separated by Ficoll gradient centrifugation. One million lymphomonocytes were incubated with 5 μg/mL of unconjugated anti-RANKL monoclonal antibody (MAB 6261, clone 70513, provided from R&D Systems) after being subjected to FcRγ blocking. Then, a secondary polyclonal goat-anti-mouse antibody conjugated with APC (BD-Bioscience) was used for RANKL detection. After proper

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Table 1. Demographics of patients.

T score	Total	Age	BMI	Menstruation start	Menstruation end	Pregnancies	Children	Alcohol intake (glasses/day)	Caffeine intake (cups of coffee/day)	Smoking (cigarettes/day)	Low calcium intake
Normal donors (>–1.0)	11	63.8 (43–89)	27.81 (19.92–35.29)	13.1 (11–16)	49.7 (48–55)	3.6 (1–6)	3.1 (1–5)	0	1.7 (0–3) 72.7% drinking coffee 27.3% not drinking coffee	1.1 (0–10) 72.7% non-smokers 27.3% smokers	Yes 63.63% No 36.37%
Osteopenic donors (–2.5 to –1.0)	46	62.43 (48–83)	27.59 (17.04–36.03)	13.52 (9–17)	47.44 (36–58)	3.02 (1–7)	2.64 (1–6)	0.13 (0–2) (6/46 patients ETOH – 13.04%)	1.46 (0–4) 72.73% drinking coffee 27.27% not drinking coffee	0.67 (0–16) 76.37% non-smokers 23.63% smokers	Yes 85.45% No 14.55%
Osteoporotic donors (<–2.5)	22	61.72 (45–82)	27.11 (19.92–38.27)	13.81 (9–17)	48.31 (35–57)	2.54 (0–5)	2.32 (0–5)	0.045 (1/22 patient ETOH – 4.55%)	1.82 (0-3) 90.9% drinking coffee 9.1% not drinking coffee	0.09 (0–1) 90.9% non-smokers 9.1% smokers	Yes 72.72% No 27.28%

washing, cells were incubated with a cocktail of conjugated mAbs (Anti-iNKT-FITC, Coulter and CD3-PERCP, BD-Bioscience). Isotype control was performed by using a mock mAb instead of the RANKL mAb. Finally, cells were suspended in phosphate buffered saline (PBS) and acquired through a multicolor Flow Cytometer, FACS Calibur (BD Bioscience). CellQuest & FlowJo analysis software were used to analyze the data. After proper gating strategies, RANKL was calculated on the surface of the iNKT cells, defined as CD3/iNKT double positive cells. The same procedure was performed on the rest of the T cells. The mean fluorescence intensity (MFI) of the RANKL expression was divided by the MFI of the GAM-mAb on the same cell subset (iNKT cells and T cells). Results are expressed as ratio of MFI RANKL/MFI mock (sample/isotype, an example of analysis is shown in Figure 1A).

β-CTx is a direct marker of osteoclastic activity. β-CTx levels in whole blood plasma were measured by ELISA (enzyme-linked immunosorbent assay) (β-CrossLaps/serum kit, Roche Diagnostics). Data was expressed in ng/mL. β-CTx levels were correlated with RANKL expression on iNKT cells.

Whole blood was collected from 8 normal donors and subjected to Ficoll gradient centrifugation. One million separated lymphomonocytes were incubated in RPMI medium + 5% human serum with 100 μg/mL α-galactosylceramide (agal). IL-2 was added at a concentration of 2 IU/mL. Half volume medium exchange was performed twice weekly, and reactivation with agal was performed on day 7. At day 12 cells were harvested and the percentage of iNKT cells and the RANKL expression were detected by multicolor flow cytometry.

Data represents mean values ± standard deviation. The parametric ANOVA and *t*-test were used to compare groups; *P* value <0.05 was considered as a statistical significance. The Pearson correlation test was used to detect correlations between values. Statistical analysis was performed by the SPSS-13 statistical pack.

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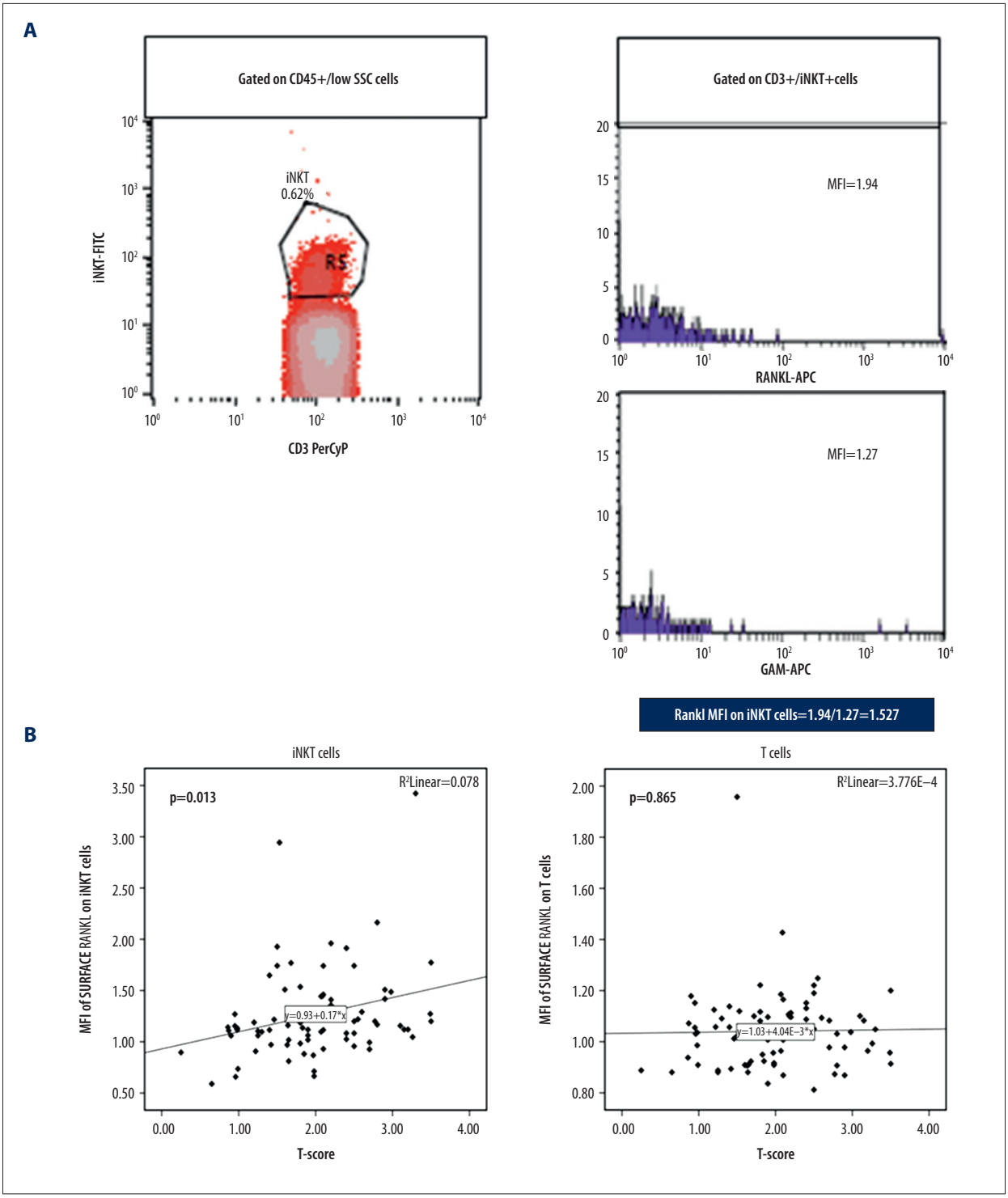

Figure 1. RANKL expression on iNKT cells as assessed by multicolor flow cytometry. (**A**) iNKT cells were identified as iNKT-FITC/CD3-PeRCyP double positive cells. RANKL-APC MFI on iNKT cells was calculated by histogram plot. GAM-APC MFI was calculated on the iNKT-FITC/CD3-PeRCyP double positive cells by histogram plot. The RANKL MFI/GAM MFI ratio was considered as RANKL-MFI expression in iNKT cells. The same strategy was followed for conventional T cells. (**B**) Correlation of the T-score to the RANKL MFI expression on iNKT cells shows a strong correlation between those two parameters, *P*=0.013. On the contrary, no correlation exists between RANKL MFI expression on T-cells and the T-score, *P*=0.865. RANKL – receptor activator of the nuclear factor-κB ligand; iNKT – invariant natural killer T cells; MFI – mean fluorescence intensity.

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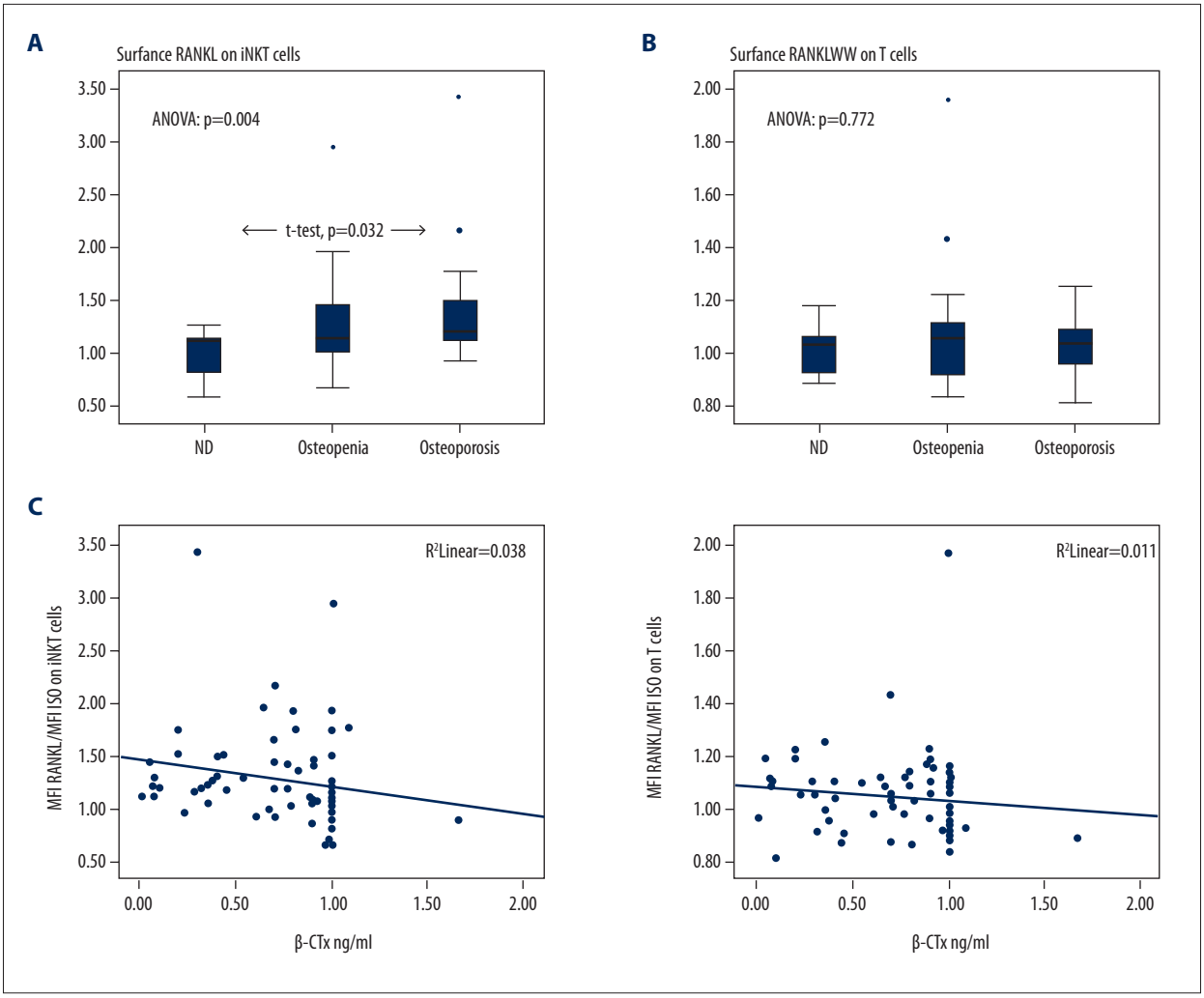

Figure 2. RANKL is specifically over-expressed on the surface of iNKT cells in patients with established osteoporosis. (**A**) RANKL MFI in iNKT cells in normal donors (n=11), patients with osteopenia (n=46) and osteoporotic patients (n=22). Patients with osteoporosis over-expressed RANKL compared to normal donors, *P*=0.032. (**B**) RANKL MFI on conventional T cells in normal donors (n=11), patients with osteopenia (n=46) and osteoporotic patients (n=22). RANKL is equally expressed in T cells in all subgroups of our study, *P*=0.77. (**C**) RANKL expression on iNKT and on T cells is not correlated to β-CTx levels (ELISA) in the blood. RANKL – receptor activator of the nuclear factor-κB ligand; iNKT – invariant natural killer T cells; MFI – mean fluorescence intensity; ELISA – enzyme-linked immunosorbent assay.

Results

We first examined the percentage of iNKT cells in the peripheral blood from all patients, which were found to be similar between the 3 subgroups included in our study. In normal donor patient group, the iNKT represented 0.23±0.07% of the total T cells. In the osteopenic patient group, the iNKT cells represented 0.21±0.04% of total T cells. In the osteoporosis patient group, the percentage was 0.24±0.04% (ANOVA test, *P*=0.8, data not shown).

The RANKL MFI ratio (sample/isotype) was calculated in double CD3/iNKT positive cells as previously described (Figure 1A). RANKL expression on iNKT cells was positively correlated to the T-score (Pearson correlation coefficient=0.279 and *P*=0.013, (Figure 1B). Interestingly, the RANKL MFI ratio on iNKT cells was found to be 0.99 ± 0.07 in the ND group, 1.27±0.06 in the osteopenia group, and 1.39±0.11 in the osteoporosis group (ANOVA test, *P*=0.004), showing a tendency for increased RANKL expression in groups with gradually increased bone loss. Performing the comparison between ND and the osteoporosis patients it is evident that osteoporosis patients express more surface RANKL in their iNKT cells compared to ND (*t*-test, *P*=0.032, Figure 2A).

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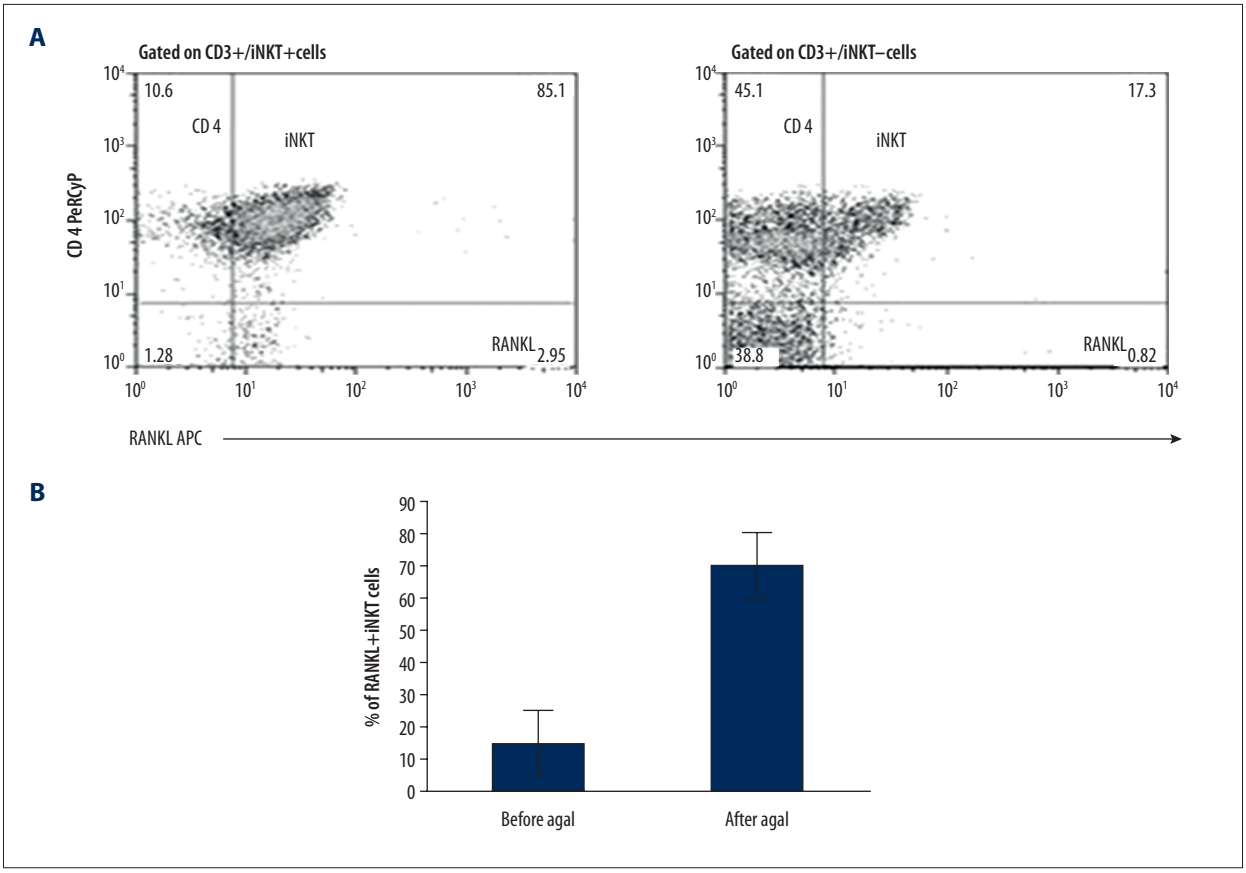

Figure 3. *Ex-vivo* activation of iNKT cells with α-galactosylceramide (agal) increased RANKL expression in their surface (**A**) plots of RANKL expression on CD4+ and CD4– iNKT cells and in CD4+ and CD4– T cells. (**B**) The percentage% of RANKL positive iNKT cells after activation with agal is increased by more than 6 times compared to pre-activation levels. iNKT – invariant natural killer T cells; RANKL – receptor activator of the nuclear factor-κB ligand.

The RANKL MFI ratio in T cells in the ND group was found to be 1.01±0.03, in the osteopenia group 1.05±0.03 and in the osteoporosis group 1.03±0.02 (ANOVA test, *P*=0.772, Figure 2B). In patients with osteoporosis, the paired t-test reveals a significant difference in RANKL expression between iNKT cells and the rest of the T cells (*t*-test, *P*=0.025). We conclude that RANKL overexpression is a distinctive feature of iNKT cells in patients with osteoporosis.

β-CTx is a direct marker of osteoclast bone resorptive activity. RANKL MFI ratio in iNKT cells is not related to β-CTx levels in the blood but neither to RANKL expression by T cells (Pearson correlation coefficient=–0.195 and *P*=0.1 for iNKT cells and *P*=0.17 for T cells, Figure 2C). We suggest that RANKL on iNKT cells has a prolonged effect on bone resorption that cannot be reflected by β-CTx levels in the blood.

The α-galactosylceramide (agal) derived from a marine sponge, is the prototypical glycolipid that activates iNKT cells, but its effect on RANKL expression is unknown. Therefore, we tested the *in vitro* agal effect in iNKT cells. After agal stimulation, the iNKT cells were expanded from a mean percentage of 0.22±0.23% to 8.48±3.66% of the total T-lymphocytes(Figure 3A). The percentage (%) of RANKL positive iNKT cells was increased from 14.5±2.5% to 70±16.5%, (*t*-test *P*=0.001, Figure 3B). The RANKL MFI was also increased to comparable levels. Our data demonstrates that agal induces iNKT cells to overexpress RANKL.

Discussion

Osteoporosis is considered today to be a major health problem worldwide and it is estimated that the national direct health care costs for osteoporotic fractures are 12.2 to 17.9 billion US dollars per year, with costs rising [22].

Bisphosphonates are still considered the mainstay of osteoporosis treatment but new-targeted therapies against RANKL or the Wnt-signaling pathway are evolving. Denosumab, a fully humanized anti-RANKL monoclonal antibody has been approved as the best second-line treatment for overt osteoporosis, denoting the importance of RANKL targeting in the therapy of osteoporosis [23].

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The role of iNKT cells in inflammatory osteoporosis is well established [24]. In the synovial fluid from patients with immune arthritis, iNKT cells constitute more than 20% of the total lymphocytes [25]. In the synovium, iNKT cells activate T cells and macrophages to secrete TNF-a, a strong pro-osteoclastogenic cytokine that acts indirectly through RANKL [26]. Having the ability to secrete RANKL and MCSF, iNKT cells induce the commitment of hemopoietic progenitors to the osteoclast lineage and thus they promote bone resorption [27]. In this study, we showed that iNKT cells overexpress RANKL in patients with overt osteoporosis, a phenomenon that is increasing with the severity of bone loss. Additionally, RANKL overexpression is not linked to clonal expansion of the iNKT cells, while the rest of the T cells are not turned to RANKL overexpression. It seems that after estrogen loss, cellular or humoral mediated events turn specifically the iNKT cells, among other T cells, to RANKL production without expanding their number, causing finally, an immune deregulation that promotes immunoporosis. We recently showed that in myeloma patients, iNKT cells are also turned to RANKL expression and they migrate towards the bone marrow from the peripheral blood. In the same study, we showed that iNKT cells are more capable, compared to conventional T cells, to secrete RANKL after T cell antigen receptor (TCR) ligation and they promote more efficiently than T cells the osteoclast development [28].We propose that, in both osteoporosis and myeloma, the iNKT cells are reacting against a novel antigen that induces an abnormal immune phenotype characterized by RANKL overexpression.

It is well known that iNKT cells recognize and react against glycolipid antigens in the context of CD1d/glycolipid/invariant TCR ligation. Depending on the length of lipid tail that fills CD1d binding loop and also on the immune environment, iNKT cells react variably to novel exogenous or endogenous glycolipid antigens [29]. In animal models, therapeutic infusion of glycolipids can alleviate immune destruction of organs in an iNKT dependent manner, like in cases of immune colitis [30]. In mouse models of immune arthritis, the effects of the prototypical glycolipid (agal) are variable according to the protocol of administration [16,17]. We speculate the existence of a glycolipid antigen that could ameliorate RANKL expression on iNKT cells in patients with osteoporosis. In that case, we could offer protection against the development of osteoporosis. For this purpose, we activated human iNKT cells *in vitro* with agal, but interestingly we found that agal enhances RANKL expression on iNKT cells. In osteoporosis and in myeloma probably an agal-like antigen is overexpressed that turns iNKT cells towards RANKL production. This abnormal immune phenotype contributes to the bone destruction seen in these diseases. We speculate the existence of other glycolipids antigens that could turn this fire off.

β-CTx is a direct marker of osteoclast-driven bone resorptive activity. In myeloma patients, we found a strong correlation between β-CTx levels in serum and the levels of RANKL expression by iNKT cells. In contrast, we could not demonstrate such a correlation in our normal donors' group. In osteoporosis patients, the correlation between instant bone resorption, as measured by β-CTx levels, and RANKL expression on iNKT cells is weak. We suppose that this is an effect of low strength or an even load of antigen triggering of iNKT cells in patients with a degenerative disease like osteoporosis. In contrast, in patients with myeloma bone disease, tumor cells secrete a more robust triggering factor of iNKT cells and probably this explains why RANKL-expressing iNKT cells correlate with osteoclast activation.

Conclusions

Herein, we enforce the role of iNKT cells in the pathogenesis of immunoporosis. This immunoporotic phenotype of iNKT cells is characterized by RANKL overexpression on their surface without clonal expansion of them. The RANKL upregulation is independent from the instant level of osteoclastic activity, as determined by β-CTx levels, probably representing a low burden of antigenic stimulation by a still unknown glycolipid antigen. Interestingly, in our study, the glycolipid antigens agal emphatically upregulate RANKL expression on iNKT cells. This arises the possibility that proper targeting of iNKT cells with another glycolipid could downregulate RANKL expression and thus can become a valuable supplement to the established therapeutic interventions for osteoporosis in the future.

Conflict of interests

None.

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