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Oxidized lipids enhance RANKL production by T lymphocytes: Implications for lipid-induced bone loss

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Abstract Osteoporosis is a systemic disease that is associated with increased morbidity, mortality and health care costs. Whereas osteoclasts and osteoblasts are the main regulators of bone homeostasis, recent studies underscore a key role for the immune system, particularly via activation-induced T lymphocyte production of receptor activator of NFκB ligand (RANKL). Well-documented as a mediator of T lymphocyte/dendritic cell interactions, RANKL also stimulates the maturation and activation of bone-resorbing osteoclasts. Given that lipid oxidation products mediate inflammatory and metabolic disorders such as osteoporosis and atherosclerosis, and since oxidized lipids affect several T lymphocyte functions, we hypothesized that RANKL production might also be subject to modulation by oxidized lipids. Here, we show that short term exposure of both unstimulated and activated human T lymphocytes to minimally oxidized low density lipoprotein (LDL), but not native LDL, significantly enhances RANKL production and promotes expression of the lectin-like oxidized LDL receptor-1 (LOX-1). The effect, which is also observed with 8-*iso*-Prostaglandin E2, an inflammatory isoprostane produced by lipid peroxidation, is mediated via the NFκB pathway, and involves increased RANKL mRNA expression. The link between oxidized lipids and T lymphocytes is further reinforced by analysis of hyperlipidemic mice, in which bone loss is associated with increased RANKL mRNA in T lymphocytes and elevated RANKL serum levels. Our results suggest a novel pathway by which T lymphocytes contribute to bone changes, namely, via oxidized lipid enhancement of RANKL production. These findings may help elucidate clinical associations between cardiovascular disease and decreased bone mass, and may also lead to new immune-based approaches to osteoporosis.

Introduction

Osteoporosis and osteopenia are asymptomatic systemic pathologies that are characterized by decreased bone mass

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and diminished skeletal integrity. Reduced bone mass is associated with greater risk of fracture, as well as increased morbidity and mortality and elevated health care expenditures [1,2]. The dynamic process of bone remodeling had heretofore been assumed to mainly involve osteoblasts, bone forming cells that secrete organic matrix molecules, and bone-resorbing osteoclasts, which are derived from hematopoietic precursors. However, it is becoming increasingly evident that the immune system also plays a significant role in bone homeostasis [3]. Indeed, the term "osteoimmunology" was coined to reflect the intricate bidirectional communication between the immune and skeletal systems [4].

A central component of the interaction between the immune and skeletal systems is the tumor necrosis factor (TNF) receptor family molecule, receptor activator of NFκB ligand (RANKL). This protein (also known as TNF-related activation cytokine, TRANCE) was actually first identified as a T lymphocyte surface marker that had regulatory effects on immune cell function [5]; only later was it shown to also be essential for the differentiation, activation, and survival of osteoclasts [6–8]. RANKL, which is now known to be secreted by activated T lymphocytes, has been studied extensively in the context of bone loss associated with rheumatoid arthritis [9,10], periodontal disease [11,12], and osteoporosis [3], where it has been shown to bind to RANK on osteoclasts, and induce these bone-resorbing cells to mature and become active. The regulation of T lymphocyte RANKL production in response to physiologic stimuli that are distinct from those that mediate activation via the antigen receptor has not been studied.

The notion that products of lipid and lipoprotein oxidation may contribute to the pathophysiology of osteoporosis is suggested by a variety of studies [13–15]. Epidemiological analyses have repeatedly shown associations between osteoporosis and hyperlipidemia [16–22], and patients with lower bone density and osteoporosis have higher lipid levels, more severe coronary atherosclerosis and increased risk of stroke [18,20,23–25]. Conversely, lipid-lowering treatments are associated with both reduced coronary vascular calcification [26] and fewer osteoporotic fractures [27–30]. *In vitro* studies demonstrate that minimally oxidized LDL (MM-LDL) inhibits the differentiation of osteoblasts [31] and can induce differentiation of osteoclasts [32]. Within the immune system, oxidized LDL is an inducer of apoptosis in T lymphocytes [33], and mildly oxidized LDL decreases early production of IL-2 by T lymphocytes [34]. In addition, dendritic cell function is inhibited in a mouse model of high-fat diet-induced dyslipidemia [35].

Since bone development and repair require adequate vascularization [36], it is likely that both circulating lipoproteins and immune cells are in close contact with cortical and trabecular bone. Therefore, in the present study, we sought to examine whether oxidized lipids, and particularly, MM-LDL, which is highly inflammatory, might have a direct effect on T lymphocyte RANKL production. Our results show that exposure of both *ex vivo*, unmanipulated human T lymphocytes, as well as *in vitro*-activated T lymphocytes to MM-LDL, results in enhanced RANKL secretion, involving the NFκB pathway and increased RANKL transcripts. Oxidized lipids also induced expression of the lectin-like oxidized LDL receptor-1 (LOX-1) on T lymphocytes. Complementary *in vivo* data from a mouse model of high-fat diet-induced osteopenia show that these mice have elevated serum levels of RANKL as well as increased T lymphocyte RANKL mRNA. These observations support a role for oxidized lipids in immune-mediated bone loss, and provide a possible mechanistic link for the well-documented association between dyslipidemia and osteoporosis.

Materials and methods

Cell isolation and culture

Human peripheral blood mononuclear cells (PBMCs) from healthy donors with normal lipid levels, as documented by the UCLA Clinical Labs, were isolated by density-gradient centrifugation on Ficoll-Hypaque (Cellgro). T lymphocytes were purified by immunomagnetic depletion using a mixture of biotinylated mAbs against CD14, CD16, CD19, CD56, CD36, CD123, and CD235a, which were subsequently labeled with anti-biotin microbeads (Miltenyi Biotec). Purity of T lymphocytes (>98%) was confirmed by flow cytometry (Becton Dickson) using anti-CD3 antibody (BD Pharmingen). Cell viability following purification was consistently >90%, as assessed by trypan blue exclusion. Cells were plated at a concentration of 1×10^6/ml in AIM-V serum-free medium (Gibco) for cell culture experiments. For experiments testing activated cells, the T lymphocytes were stimulated with either CD2/CD3/CD28-antibody-coated beads (Miltenyi Biotec) at a bead:cell ratio of 1:1 or 6 μg/ml PHA (Sigma).

Lipoprotein preparation and oxidation

Human LDL was isolated by density-gradient centrifugation of serum, and stored in phosphate-buffered 0.15 M NaCl containing 0.01% EDTA [37]. Minimally oxidized LDL was prepared by iron oxidation of human LDL, as described in Parhami et al. [31]. Minimal oxidation of LDL resulted in a 3- to 4-fold increase in conjugated dienes. The lipoproteins were tested pre- and post-oxidation for lipopolysaccharide levels and found to have <30 pg of lipopolysaccharide/ml of medium. In some experiments, components of MM-LDL were tested, including 8-*iso*-Prostaglandin E2 (*iso*-PGE2; Cayman Chemical) and oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine (Ox-PAPC), which was a generous gift of Dr. Judy Berliner, UCLA. The concentrations of lipoproteins used in this study are reported in micrograms of protein.

Cytokine measurement

Supernatants from cultured cells were harvested after 72 h of incubation with 25 μg/ml nLDL, MM-LDL, 25 μM *iso*-PGE_2 or control buffer, centrifuged, and immediately frozen at −80 °C until further analysis. All samples were thawed immediately prior to ELISA evaluation. The following ELISA kits were used: RANKL, (Peprotech), IFN γ (Biotrack ELISA System, Amersham), IL-6, TGF-β and TNFα (Quantikine, R and D Systems). All measurements were performed in triplicate wells, and in accordance to the manufacturer's instructions.

Flow cytometry

Cell activation status and viability were assessed by flow cytometry. Cells were harvested after 72 h of incubation with 25 μg/ml nLDL, MM-LDL or buffer control, centrifuged

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and washed twice with 1× PBS, then resuspended in 1× binding buffer (BD Biosciences) prior to the staining. Briefly, cells were incubated with Annexin V-FITC, propidium iodide (PI), CD38 PerCp-Cy5.5, CD25 APC-Cy7, HLA-DR APC, CD8 PE-Cy7 (BD Biosciences) and CD3 Pacific Blue (Invitrogen) for 15 min at room temperature in the dark, washed and resuspended in 1× binding buffer. The 7-color staining flow cytometry data was acquired immediately by BD LSRII and FACS Diva software (BD Biosciences). Data analysis was performed using FlowJo (Tree Star). Cells that were negative for Annexin V and PI were considered as viable; apoptotic cells (Annexin V+) and necrotic cells (both Annexin V+/PI+ or PI+ alone) were detected and quantified as a percentage of the entire population [38]. Data from 4 independent experiments, each performed on triplicate samples, were pooled to determine the mean cell viability. For LOX-1 measurement, cells were harvested after 72 h of incubation with 25 μg/ml nLDL, MM-LDL, 25 μM *iso*-PGE_2, or control buffer, centrifuged and washed twice with 1× PBS, then resuspended with staining buffer (BD Biosciences). Cells were incubated with biotinylated LOX-1 (Hycult Biotechnology), CD8 PerCp, and CD3 APC (BD Biosciences) for 30 min at 4 °C in the dark, washed and resuspended in staining buffer for secondary staining for 30 min at 4 °C in the dark. Cell acquisition and data analysis were performed as described above.

Mice and diets

Male C57Bl/6 (atherosclerosis-susceptible strain) mice (Jackson Laboratory) were placed on either a control chow diet (National Institute of Health [NIH]-31 Mouse/Rat Diet 7013 containing 6% fat) or high-fat (atherogenic) diet (Teklad TD90221; Harlan Tekland; containing 1.25% cholesterol, 15.8% fat, and 0.5% cholate) starting at 1 month of age. This diet causes significant hypercholesterolemia [39,40] and osteopenia [41] in C57Bl/6 mice. Spleen and serum were collected from control and high-fat diet fed animals at 11 months of age. T lymphocytes were isolated from splenocytes by immunomagnetic negative selection, using a mixture of mAbs of biotinylated CD14, CD16, CD19, CD56, CD36, CD123, CD235a and subsequently labeled with anti-biotin microbeads (Miltenyi Biotec). Purity of T lymphocytes (>98%) was confirmed by flow cytometry (Becton Dickson) using anti-CD3 antibody (BD Pharmingen).

Quantitative computed tomographic scanning

Femurs were carefully cleaned of soft tissue and fixed in 95% ethanol. Peripheral quantitative computed tomographic (pQCT) scans were performed on the left femur from each mouse. Scanning was done with a Stratec XCT-Research M (Norland Medical Systems) and associated software (version 5.4; Stratec Medizintechnik), and Bone Mineral Content (BMC) analysis was performed as previously described [42]. The mean of 3 evenly-spaced slices located in the mid-diaphysis was selected for data analysis.

Pathway inhibitors

The following pathway inhibitors were used: Akt inhibitors (124005 or 124009 — Calbiochem; both at 25 μM), MAPK inhibitor (PD98059 — Calbiochem at 10 μM), NFκB inhibitors (sc3060 — Santa Cruz Biotechnology at 75 μg/ml (inhibitor 1) or Bay 11-7082 — BioMol at 2 μM (inhibitor 2)), PKA inhibitor (H89 — Calbiochem at 5 μM), and PKC inhibitor (RO-31-7549 — Calbiochem at 1 μM). All inhibitors were dissolved in DMSO, according to the manufacturer's instruction, except for inhibitor 1 (sc-3060), which was dissolved in 1× PBS supplemented with 0.2% BSA and 0.1% NaN3. Prior to treatment with oxidized lipids or control buffer, T lymphocytes, at a concentration of 1×10^6/ml, were pre-incubated with inhibitors or appropriate vehicle for 2 h at 37 °C.

NFκB activity assay

Purified T lymphocytes were incubated with 25 μg/ml MM-LDL for 1 h or up to 2 h, then harvested, and the pellet washed with ice-cold PBS/Phosphatase inhibitors. Nuclear extraction and analysis were performed according to the manufacturer's instructions (Active Motif). The nuclear fraction was analyzed using a TransAM NFκB family transcription factor assay kit. Briefly, in a 96-well plate, the active form of NFκB in the nuclear extract binds specifically to the oligonucleotide containing the NFκB consensus binding site (GGGACTTTCC) that has been immobilized on the plate. To monitor the specificity of the assay, the wild-type consensus oligonucleotide competitor for NFκB binding is added to the well prior to addition of cell extract. Activated NFκB was detected by the addition of primary antibody for the p65 subunit, and measurement was achieved with a HRP-conjugated secondary antibody, providing a colorimetric readout at 450 nm with a reference wavelength of 655 nm.

Quantitative real-time PCR (QT-PCR) for RANKL expression

Purified T lymphocytes were incubated with 25 μg/ml of nLDL or MM-LDL, or 25 μM *iso*-PGE_2 for 24 h. Total RNA was extracted using RNeasy Mini Kit (Qiagen), and RNA concentrations were determined using the Quant-iT Ribogreen RNA Assay Kit (Molecular Probes). Template cDNAs were synthesized with the iScript cDNA synthesis kit (Bio-Rad) using 500 ng of RNA. The QT-PCR assays were performed using the iQ SYBR Green SuperMix and IQCycler (Bio-Rad). As an internal control, QT-PCR for the housekeeping gene, GAPDH, was performed. The sequences of the primers were designed with the aid from the Beacon software. hRANKL — 3′-TATCGTTGGATCACAGCACATCAGAG; 5′-GAGGACAGACTCACTTTATGGGAACC. hOPG — 3′-TGTGAGGAGGCATTCTTCAGGTTTG; 5′-TCTCTACACTCTCTGCGTTTACTTTGG. hLOX-1 — 3′-TGGGAAAAGAGCCAAGAGAA; 5′-TAAGTGGGGCATCAAAGGAG. hGAPDH — 3′-CCTCAAGATCATCAGCAATGCCTCCT; 5′-GGTCATGAGTCCTTCCACGATACCAA. mRANKL — 3′-GCCTCCCGCTCCATGTTCC; 5′-TGAGTGCTGTCTTCTGATATTCTGTTAG. mGAPDH — 3′-ATTGTCAGCAATGCATCCTG; 5′-ATGGACTGTGGTCATGAGCC. Samples were run in triplicate in a 96-well plate using the settings of 95 °C for 15 s, 61 °C for 30 s and 72 °C for 30 s (single fluorescence measurement).

Statistical analysis

Data in the graphs indicate the mean ± S calculated from independent experiments. The Wilcoxon rank sum test, a

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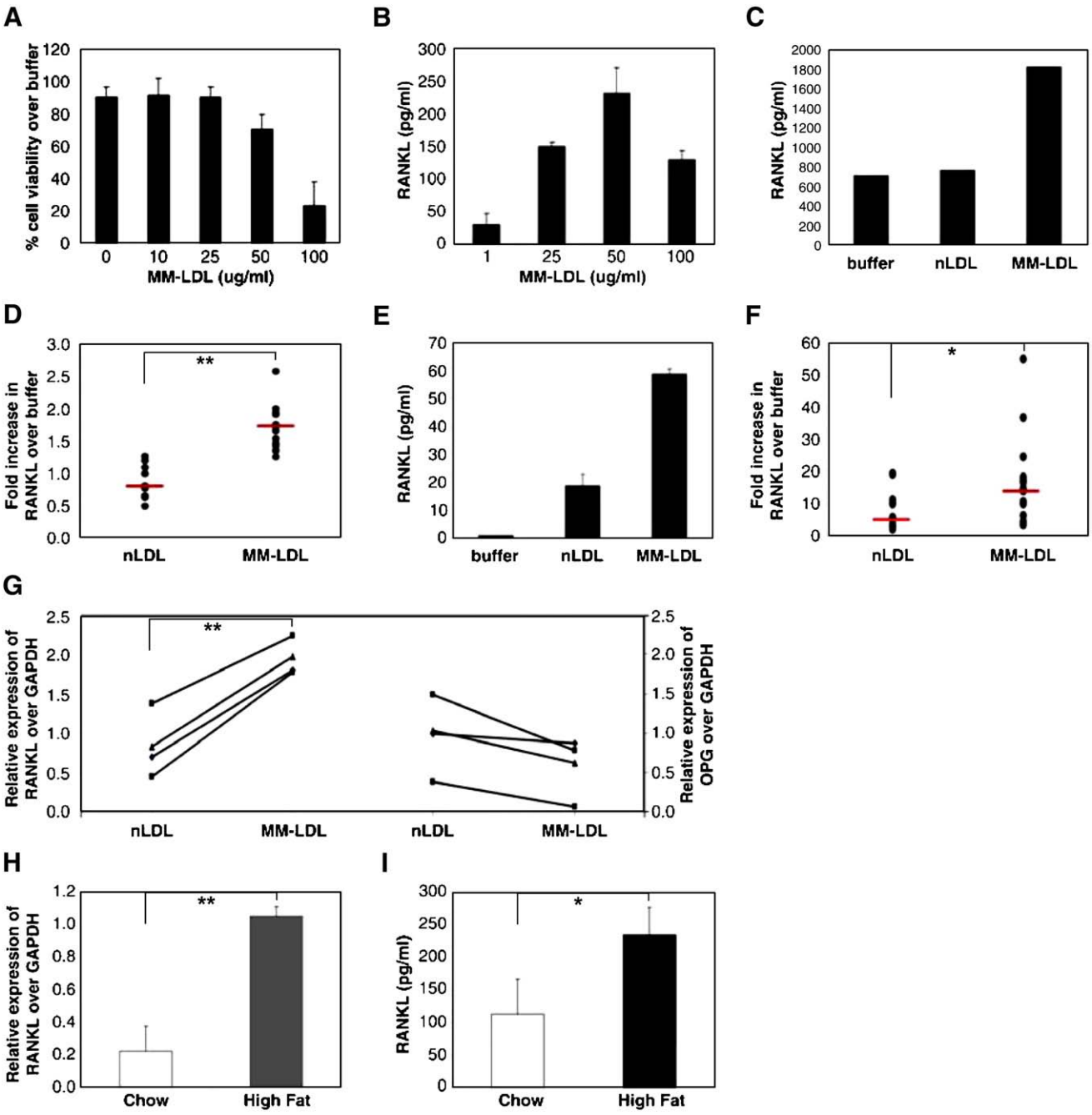

Figure 1 RANKL production by T lymphocytes exposed to oxidized lipids. A. Unstimulated T lymphocytes were plated in culture medium with various concentrations of MM-LDL (0, 1, 10, 25, 50, 100 μg/ml), and incubated for 72 h. Cell viability was determined by counting the number of live cells under light microscope analysis and trypan blue exclusion ($n=8$). B. Measurement of RANKL production by ELISA of unstimulated T lymphocytes exposed to various concentrations of MM-LDL (0, 1, 25, 50, 100 μg/ml) ($n=4$). Error bars indicate SD. C. T lymphocytes were stimulated with CD2/CD3/CD28 antibody-coated beads in the presence of 25 μg/ml of either nLDL or MM-LDL, and after 72 h, RANKL (pg/ml) in the culture supernatant was evaluated by ELISA. Representative ELISA results from one donor. D. Fold-increase (compared to buffer control) of RANKL production by activated T lymphocytes treated with oxidized lipids ($n=18$). Error bars indicate SD. E. Representative ELISA results for RANKL (pg/ml) production by unstimulated T lymphocytes cultured for 72 h in the presence of 25 μg/ml of either nLDL or MM-LDL. F. Fold-increase (compared to buffer control) in RANKL production by unstimulated T lymphocytes treated with oxidized lipids ($n=15$). Error bars indicate SD. G. Relative expression (compared to GAPDH) of RANKL (left) and OPG (right) transcript in unstimulated T lymphocytes treated with 25 μg/ml nLDL or MM-LDL for 24 h, as detected by QT-PCR ($n=4$). Error bars indicate SD. H. Relative expression (compared to GAPDH) of RANKL transcript in T lymphocytes from mice fed either chow ($n=4$) or high fat diet ($n=4$) for 11 months as detected by QT-PCR. Error bars indicate SD. I. Serum levels of RANKL (pg/ml), detected by ELISA from mice fed either chow ($n=4$) or high fat ($n=4$) for 11 months. Error bars indicate SD. * $-$ $p \leq 0.05$. ** $-$ $p \leq 0.01$.

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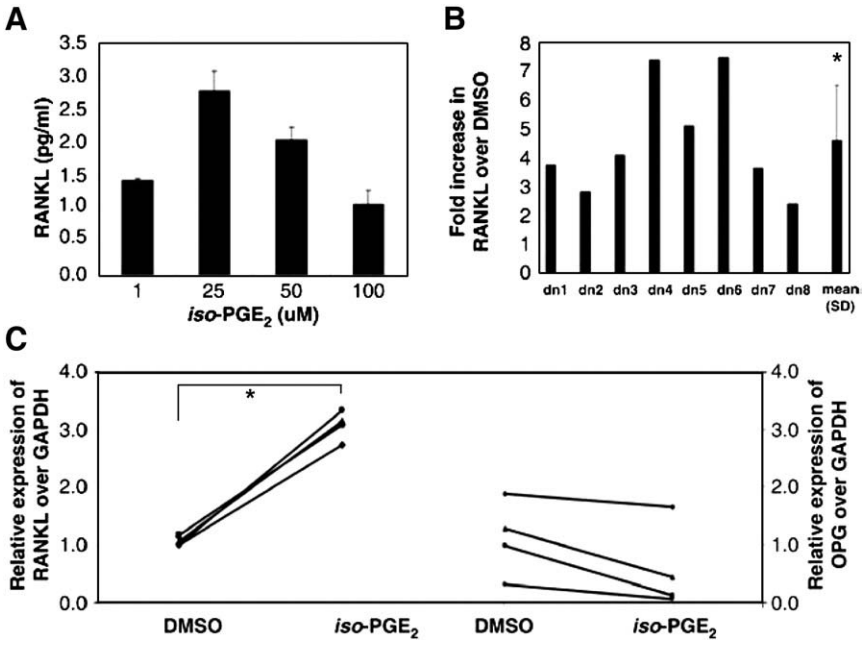

Figure 2 *iso*-PGE_2 induces RANKL production by unstimulated T lymphocytes. A. T lymphocytes were exposed to various concentrations of *iso*-PGE_2 (0, 10, 25, 50 μM) and incubated for 72 h. RANKL production in culture supernatant was measured by ELISA. Graph represents fold-increase over DMSO control ($n=3$). Error bars indicate SD. B. T lymphocytes were cultured with 25 μM *iso*-PGE_2 for 72 h and RANKL measurement in culture supernatant was evaluated by ELISA. Graph represents fold-increase over DMSO control ($n=8$). Error bar indicate SD. C. Relative expression (compared to GAPDH) of RANKL (left) and OPG (right) transcript in unstimulated T lymphocytes treated with 25 μM *iso*-PGE_2 for 24 h, as detected by QT-PCR ($n=4$). Error bars indicate SD. * — $p \leq 0.01$.

non-parametric test that is powerful in analyzing small sample sizes [43], was used to compare groups. Statistical significance was defined as $p \leq 0.05$.

Results

RANKL production is induced in human T lymphocytes by oxidized lipids

Consistent with previous reports [44], we observed that T lymphocytes stimulated with CD2/CD3/CD28 antibodies produce high levels of RANKL, which can be detected by ELISA (data not shown). To address the potential effect of oxidized lipids on T lymphocyte RANKL production, we selected MM-LDL for these experiments since it is known to be highly inflammatory, yet does not induce the cytotoxicity associated with highly oxidized LDL. Preliminary titration experiments were performed to identify the optimum concentration of MM-LDL required to induce maximum RANKL production without compromising cell viability. Highly purified human T lymphocytes were exposed to various concentrations of MM-LDL (0, 1, 10, 25, 50, 100 μg/ml) for 72 h. As shown in Figure 1A, MM-LDL concentrations up to 25 μg/ml had no effect on cell viability, whereas the higher concentrations were toxic, as determined by trypan blue dye exclusion. Flow cytometric Annexin V and PI staining confirmed that exposure of T lymphocytes to 25 μg/ml of

Table 1 Bone mineral content (BMC) of femur from C57Bl/6 mice after 11 months on a control chow or high-fat diet.

Slice	Chow	BMC (mg)	Mean±SD	High Fat	BMC (mg)	Mean±SD	Percent change	*p*-value
6	1-Chow	1.33		5-High Fat	1.09			
6	2-Chow	1.05		6-High Fat	0.86			
6	3-Chow	1.42		7-High Fat	0.98			
6	4-Chow	1.24	1.3±0.16	8-High Fat	0.99	1.0±0.09	22.2	0.03
7	1-Chow	1.48		5-High Fat	1.21			
7	2-Chow	1.24		6-High Fat	0.97			
7	3-Chow	1.61		7-High Fat	1.00			
7	4-Chow	1.32	1.4±0.17	8-High Fat	1.10	1.1±0.11	23.7	0.02
8	1-Chow	1.75		5-High Fat	1.47			
8	2-Chow	1.42		6-High Fat	1.13			
8	3-Chow	1.82		7-High Fat	1.25			
8	4-Chow	1.54	1.6±0.19	8-High Fat	1.36	1.3±0.15	20.2	0.03

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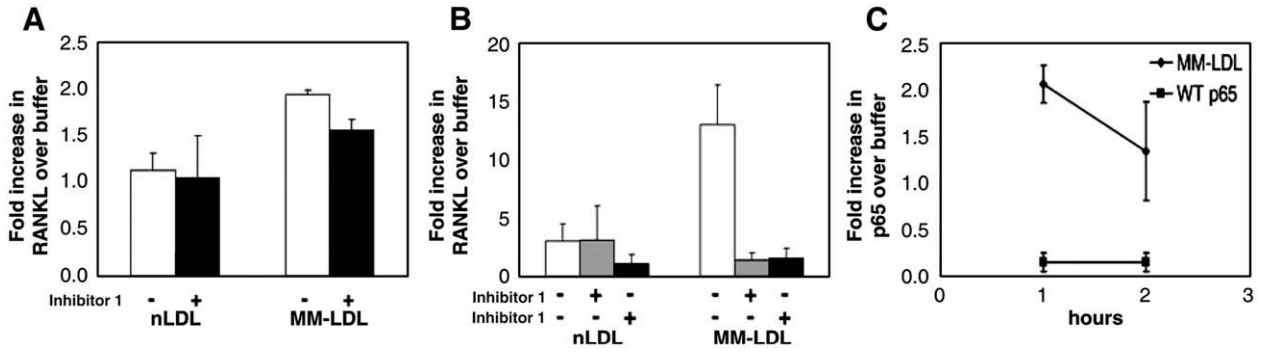

Figure 3 Mechanism of oxidized lipid-induced RANKL production in T lymphocytes. A. Activated T lymphocytes: T lymphocytes were pre-incubated with NFκB inhibitor 1 for 1 h prior to stimulation with CD2/CD3/CD28 antibody-coated beads in the presence of 25 μg/ml nLDL or MM-LDL. Cells were harvested after 72 h of treatment, and RANKL in culture supernatant was evaluated by ELISA. Graph represents fold-increase over buffer ($n=4$). Error bars indicate SD. $p=0.052$ for MM-LDL no inhibitor vs. MM-LDL with inhibitor 1. B. Unstimulated T lymphocytes: T lymphocytes were pre-incubated with either NFκB inhibitor 1 or inhibitor 2 prior to treatment with oxidized lipids. RANKL (pg/ml) in culture supernatant was evaluated by ELISA after 72 h of treatment. Data is presented as fold-increase over buffer ($n=4$). Error bars indicate SD. $p \leq 0.05$ for MM-LDL no inhibitor vs. MM-LDL with inhibitor 1 and MM-LDL no inhibitor vs. MM-LDL with inhibitor 2. C. Nuclear localization of activated NFκB: Unstimulated T lymphocytes were incubated with 25 μg/ml of MM-LDL for 1 or up to 2 h, and nuclear extracts were tested in wells containing the NFκB consensus binding site. Assay specificity was determined by competition with wild-type NFκB oligonucleotide (WT p65). Data is presented from 4 independent experiments. Error bars indicate SD. $p \leq 0.05$ for buffer vs. MM-LDL.

MM-LDL did not cause significant cell death (data not shown). Thus, although 50 μg/ml MM-LDL induced the highest level of RANKL production, due to the dramatic loss in cell viability at this concentration, all subsequent experiments were performed using 25 μg/ml (Fig. 1B). Nevertheless, to further control for any minor differences in cell viability over the 72-hour incubation period, RANKL production by T lymphocytes was also normalized based on the production per million viable cells; these values were similar to those displayed in Figures 1C, D, E, F and 2A, B.

The first set of experiments evaluated whether MM-LDL had an additive effect on the already robust levels of activation-induced T lymphocyte RANKL production. We observed that the concentration of RANKL in the culture supernatants of purified T lymphocytes stimulated with CD2/CD3/CD28 antibodies was significantly increased in the presence of MM-LDL, as compared to buffer or native LDL (nLDL). Figure 1C shows representative ELISA data for one donor, and in Figure 1D, the results of experiments on purified T lymphocytes from 18 individual donors are presented. Unexpectedly, even in the absence of an activation stimulus, exposure of T lymphocytes to MM-LDL resulted in a significant increase in RANKL production (Fig. 1E). Indeed, this pattern was seen in T lymphocytes from all 15 healthy individuals tested, with fold-increases (over buffer alone) ranging from 4 to 55, and a mean fold-increase of 16 (Fig. 1F). The effect of MM-LDL on unstimulated T lymphocyte RANKL production was significantly greater than that of nLDL, which had only a modest effect (Fig. 1F). Flow cytometry evaluation of the CD38, CD25 and HLA-DR activation markers confirmed that the RANKL production by unstimulated T lymphocytes was not due to possible MM-LDL induced activation (data not shown). QT-PCR experiments showed that the increased RANKL protein induced in unstimulated T lymphocytes exposed to MM-LDL was associated with a significant increase in RANKL mRNA (Fig. 1G). Osteoprotegerin (OPG) is a natural decoy receptor that blocks RANKL from binding to its receptor (RANK) and the ratio of RANKL to OPG is considered a key parameter in regulating bone metabolism. Unstimulated T lymphocytes exposed to MM-LDL had lower OPG expression compared to nLDL (Fig. 1G), and the RANKL: OPG ratio for nLDL and MM-LDL is 0.9 and 3.4, respectively. To determine whether MM-LDL exposure induced unstimulated T lymphocytes to produce osteoclastogenic factors in addition to RANKL, ELISA assays were performed. These assays, which detect concentrations as low as 0.7 pg/ml, 1.6 pg/ml, and 4.6 pg/ml of IL-6, TNFα, and TGFβ, respectively, did not show any increases in these cytokines (data not shown).

In parallel with our *in vitro* experiments on human T lymphocytes, we evaluated an *in vivo* model of bone loss induced in C57BL/6 mice that become hyperlipidemic when treated with a high-fat diet [41]. Consistent with our earlier studies [41], pQCT analysis of femoral bones from high-fat fed mice showed a significant reduction in bone mineral content compared to their chow-fed counterparts (Table 1). Importantly, T lymphocytes from these mice, tested immediately *ex vivo*, in the absence of any stimulation, expressed RANKL mRNA that was significantly greater than that of T lymphocytes from the chow-fed mice (Fig. 1H). The hyperlipidemic mice also had significant increases in serum levels of RANKL protein (Fig. 1I). Overall, the *in vitro* data on human T lymphocytes, and the murine *in vivo* model of hyperlipidemia-induced bone loss indicate that exposure to oxidized lipids enhances T lymphocyte RANKL production.

iso-PGE_2 also induces RANKL production by unstimulated human T lymphocytes

The prostaglandin-like isoprostane, 8-*iso*-Prostaglandin E2 (*iso*-PGE_2), is produced *in vivo* as one of the by-products of free radical-catalyzed lipid peroxidation, and may contribute to oxidative injury [45]. Previous studies have shown

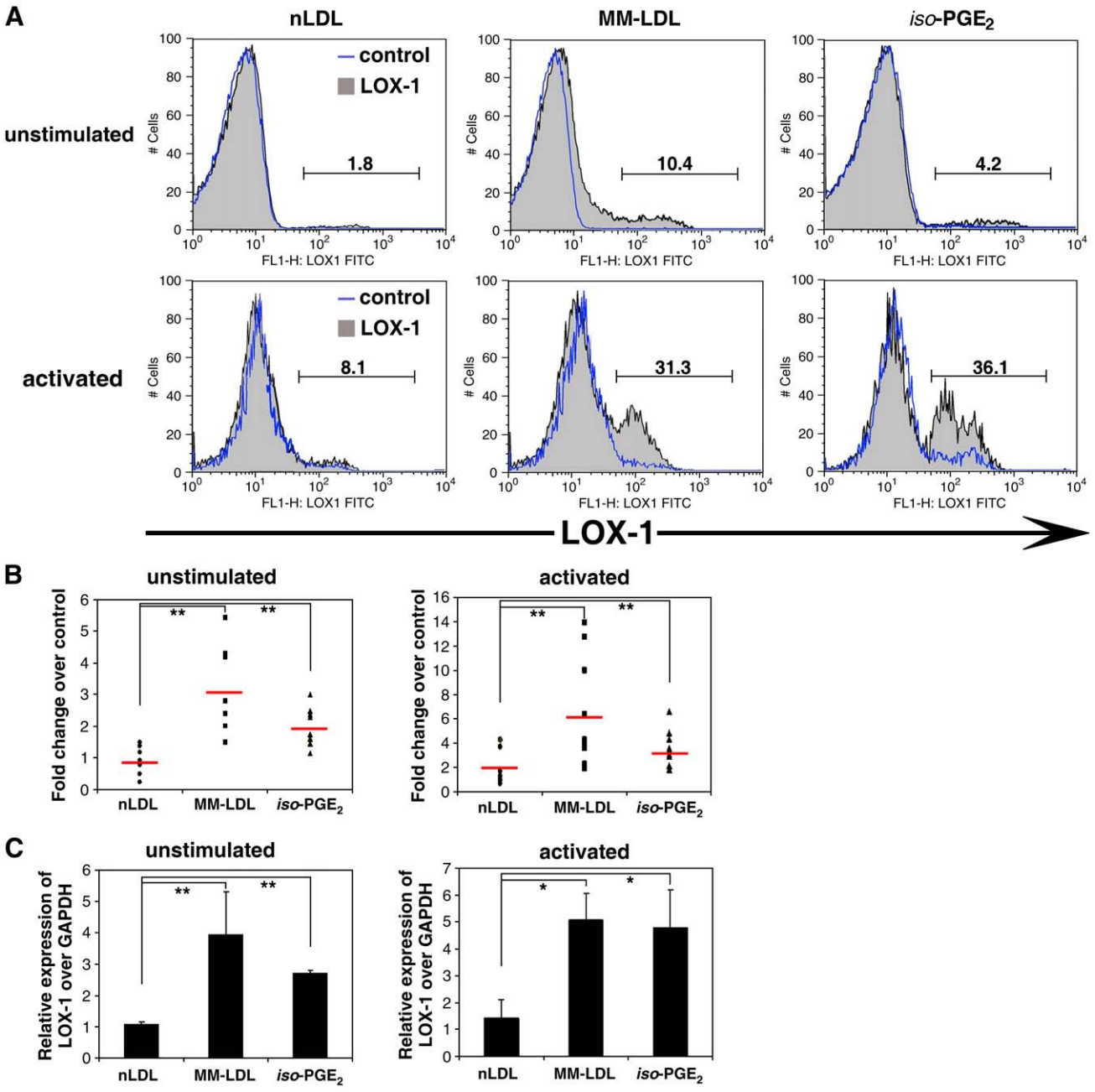

Figure 4 LOX-1 expression on T lymphocytes treated with oxidized lipids. A. T lymphocytes, unstimulated or activated with 6 μg/ml PHA, were exposed to 25 μg/ml nLDL, 25 μg/ml MM-LDL or 25 μM *iso*-PGE_2 for 72 h. Representative histogram analysis of LOX-1 expression from one donor. B. Fold-increase (compared to buffer control) of LOX-1 expression by T lymphocytes, unstimulated ($n=9$) and activated ($n=10$), treated with oxidized lipids. Error bars indicate SD. C. Relative expression (compared to GAPDH) of LOX-1 transcripts in unstimulated ($n=5$) and activated ($n=4$) T lymphocytes treated with 25 μg/ml nLDL, 25 μg/ml MM-LDL or 25 μM *iso*-PGE_2 for 24 h as detected by QT-PCR. Error bars indicate SD. * — $p \leq 0.05$. ** — $p \leq 0.01$.

that oxidized lipids, including *iso*-PGE_2, inhibit differentiation of preosteoblasts [31,32,46,47]. Titration experiments on unstimulated T lymphocytes showed that the maximum RANKL production was induced in the presence of 25 μM *iso*-PGE_2 (Fig. 2A). We found that unstimulated T lymphocytes exposed to *iso*-PGE_2 produced a mean increase in RANKL protein that was 4-fold greater than that of control cultures exposed to the DMSO diluent (Fig. 2B). QT-PCR experiments showed that the increased RANKL protein induced in unstimulated T lymphocytes exposed to *iso*-PGE_2 was preceded by increased RANKL mRNA (Fig. 2C). We also found that T lymphocytes exposed to *iso*-PGE_2 had lower OPG expression as compared to DMSO, with RANKL:OPG ratios of 0.9 and 5.3 for DMSO and *iso*-PGE_2, respectively. Ox-PAPC (oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine), a biologically active component of MM-LDL, which is known to have pleiotropic effects on human endothelial cells [46] and also affects T lymphocyte

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activation [48], was found to be toxic to unstimulated human T lymphocytes, even at concentrations as low as 0.5 μg/ml (data not shown).

MM-LDL induction of T lymphocyte RANKL requires NFκB signaling

To elucidate the mechanism involved in the oxidized lipid-induced RANKL production in T lymphocytes, we tested a panel of pathway inhibitors, using concentrations that did not affect cell viability. We first investigated the NFκB pathway, based on previous reports showing that this pathway is central to the maturation and activation of osteoclast precursor cells induced by RANK/RANKL binding [44]. Furthermore, the biological activity of MM-LDL and oxidized lipids in several other cell types is mediated, at least in part, through the NFκB pathway [49]. Two different NFκB inhibitors were used, one that blocks the translocation of the active NFκB complex from the cytoplasm into the nucleus (inhibitor 1), and one that prevents the phosphorylation of IκBα (inhibitor 2). We found that the MM-LDL-induced RANKL production by the stimulated T lymphocytes was only modestly reduced when the NFκB pathway was inhibited. (Fig. 3A). By contrast, in the unstimulated T lymphocytes, RANKL production induced by MM-LDL was nearly completely abolished in the presence of the NFκB inhibitors (Fig. 3B). The NFκB pathway role in this effect was further confirmed using an assay that measures the presence of the NFκB p65 subunit in the nucleus, a downstream effect of NFκB activation. A one-hour incubation of unstimulated T lymphocytes with MM-LDL resulted in a 2-fold increase in nuclear p65; this activity decreased after 2 h (Fig. 3C). No inhibition of the MM-LDL induction of RANKL production by unstimulated T lymphocytes was observed in the presence of specific MAPK (10 μM), PKA (5 μM) and PKC (1 μM) pathway inhibitors (data not shown). Thus, our data indicate that unstimulated T lymphocytes exposed to MM-LDL upregulate RANKL message and protein via an NFκB-mediated pathway. The very modest reduction in RANKL seen in the stimulated T lymphocytes exposed to MM-LDL (Fig. 3A) suggests that RANKL induction in activated T lymphocytes may be mediated by both NFκB-dependent and -independent pathways. Other reports on oxidized lipid effects in several cell types suggest varying degrees of NFκB pathway involvement in response to oxidized lipids, ranging from total absence of NFκB signaling [46,47] to inhibitory effects on the NFκB pathway [35,50], depending on cell type, function being assessed and the type of oxidized lipids tested.

Oxidized lipids induce T lymphocyte LOX-1 expression

The lectin-like oxidized LDL receptor-1 (LOX-1) has been identified as the primary Ox-LDL receptor on endothelial cells [51] and is associated with cardiovascular disease [52,53]. Macrophages also express LOX-1 when exposed to TNF-α [54]. A recent report documented LOX-1 mRNA in the Jurkat T cell tumor line following exposure to a component of oxidized lipids [55], suggesting that normal T lymphocytes, which infiltrate and accumulate in the vascular wall during the development of atherosclerosis [56], and are therefore exposed to Ox-LDL *in vivo* might also express LOX-1. Purified T lymphocytes were exposed to 25 μg/ml MM-LDL, 25 μg/ml nLDL, 25 μM *iso*-PGE_2 or control buffers for 72 h and then analyzed by flow cytometry. Figure 4A shows a flow cytometry histogram for a representative donor, and in Figure 4B, the results of experiments on purified T lymphocytes from 9 unstimulated and 10 stimulated samples. Our data show that the percentage of LOX-1 positive T lymphocytes treated with oxidized lipids ranged from 1 to 10% for unstimulated and 10–39% for activated cells (Fig. 4B), and both CD4 and CD8 T lymphocytes were found to express LOX-1(data not shown). The effect of oxidized lipids on LOX-1 expression was significantly higher than that of nLDL on both unstimulated and PHA-activated T lymphocytes. These data show that unstimulated T lymphocytes exposed to either MM-LDL or *iso*-PGE_2 had fold-changes in surface LOX-1 expression (over control buffers) of 6.1 and 3.5, respectively; the fold-changes for the activated T lymphocytes were 3.0 and 2.0, respectively. QT-PCR experiments showed that increased LOX-1 expression on cell surface of T lymphocytes exposed to oxidized lipids was associated with increased LOX-1 mRNA (Fig. 4C).

Discussion

The present study provides the first demonstration that oxidized lipids can induce human T lymphocytes to secrete RANKL, a cytokine that is a key mediator of osteoclast differentiation and bone loss. We show that exposure of human T lymphocytes to either the lipoprotein oxidation product, MM-LDL, or to *iso*-PGE_2, a by-product of the free radical-catalyzed peroxidation of essential fatty acids, significantly increases RANKL production. The effect is observed in unstimulated T lymphocytes tested immediately *ex vivo*, as well as in T lymphocytes stimulated via activatory antibodies. The MM-LDL induction of RANKL protein is preceded by upregulation of RANKL mRNA, and the effect on the unstimulated T lymphocytes is mediated through the NFκB pathway. Additionally, T lymphocytes exposed to oxidized lipids had higher RANKL:OPG ratios, which might further disrupt bone homeostasis *in vivo*. Our study also provides the first evidence that LOX-1, previously documented on endothelial cells and macrophages, is also expressed on primary T lymphocytes. Consistent with our *in vitro* observations on human T lymphocytes, splenic T lymphocytes from hyperlipidemic mice, which show both bone loss and increased levels of oxidized lipids [13,57], also express significantly greater RANKL mRNA. Thus T lymphocytes may be one of the sources of the significantly higher serum levels of RANKL protein in these mice. The results of our study provide a novel pathway by which T lymphocytes may contribute to bone loss, and suggest that the well-established association between cardiovascular disease and bone loss [13,16,58,59] may involve T lymphocytes, along with other known sources of RANKL.

Our results expand upon the growing repertoire of diverse biological effects mediated by oxidized lipids on cells of the immune system. For example, human peripheral blood T lymphocytes are strongly inhibited in anti-CD3/CD28-induced proliferation and *de novo* synthesis of activation markers by certain concentrations of Ox-PAPC, an oxidized

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phospholipid [48], consistent with the proposed link between sterol metabolism and T cell proliferative responses to antigens [60]. Oxidized lipids have also been shown to negatively regulate dendritic cell maturation. Human dendritic cells that are pretreated with Ox-PAPC are inhibited in LPS-induced upregulation of a variety of surface markers, including the CD40 and CD80 costimulatory molecules, the ICAM-1 cell adhesion marker, and MHC Class I and II transplantation antigens [50]. Finally, in macrophages, MM-LDL is able to offset the apoptosis induction by more extensively oxidized LDL [61]. Thus, oxidized lipids have diverse and important regulatory functions within both the innate and adaptive immune systems.

LOX-1, the specific receptor for oxidized forms of lipids [62] has been studied in a variety of non-immune cell types, such as cells within the placenta [63], chondrocytes, [64] breast cancer cells [65] and endothelial cells, in which it not only mediates uptake of Ox-LDL, but also functions as a scavenger receptor that interacts with a variety of distinct ligands such as platelets, apoptotic cells, heat shock proteins, and phosphatidylserine [51,54,66]. To our knowledge, our study is the first to demonstrate that LOX-1 is induced on primary T lymphocytes. Interestingly, C-reactive protein (CRP), an acute phase serum marker that is a strong predictor of cardiovascular disease, hypertension and diabetes [67,68] also binds to LOX-1. Ongoing studies in our laboratory are, therefore, examining the potential involvement of CRP in the MM-LDL induction of T lymphocyte-mediated bone loss.

Our study provides another example of the interplay of immune cells and oxidized lipids in a disease that has major implications for human health. This interaction has already been documented for atherosclerosis, in which one of the initiating events is the uptake of oxidized lipids by macrophages, leading to the formation of the foam cells that are involved in the actual plaque formation [69–72]. Oxidized lipids may also stimulate autoreactive T lymphocytes, which could further contribute to atherosclerosis. Indeed, adoptive transfer of Ox-LDL-reactive CD4 T lymphocytes into mice that are genetically engineered to develop atherosclerosis leads to accelerated lesion progression [73]. Moreover, human CD8 T cells can mediate Ox-LDL-specific cytotoxicity, thus contributing to the inflammatory process associated with atherosclerosis [74]. A role for oxidized phospholipids has recently been documented in human leprosy, where host-derived oxidized phospholipids accumulate in macrophages within lepromatous lesions, a process that is strikingly similar to observations in atherosclerotic lesions [75]. Finally, recent research has also suggested an association between lipid levels and both telomerase activity and telomere length in PBMC [76,77], findings which have important implications for immunosenescence. Thus, immune-mediated pathogenic changes in a variety of diseases have been documented to be modulated by oxidized lipids. Our ongoing *in vitro* studies on the effects of long-term exposure of T lymphocytes to oxidized lipids may identify additional effects that relate to age-related pathologies in addition to bone loss.

It has been well-established that chronic immune activation plays a key role in bone homeostasis. Indeed, skeletal changes are observed in a variety of clinical situations involving chronic, long-term immune activation. For example, reduced bone mass is being increasingly reported in HIV/AIDS, even in the absence of anti-retroviral drugs [78]. RANKL production by activated T lymphocytes has also been shown to play a central role in inflammatory bone loss, particularly in rheumatoid arthritis. The current study provides the first demonstration that not only activated, but also resting, unstimulated T lymphocytes are potential sources of RANKL, if they are exposed to oxidized lipids. In summary, our results underscore the interaction between T lymphocytes and oxidized lipids in osteoporosis and osteopenia, and provide a possible pathway for the documented improved bone mass seen in patients who are treated with lipid-lowering medications [26,28].

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