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Chemotactic and immunoregulatory properties of bone cells are modulated by endotoxin-stimulated lymphocytes

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Running title: Immunoregulatory properties of bone cells

Abstract

In our study we explored the bidirectional communication, via soluble factors, between bone cells and endotoxin-stimulated splenic lymphocytes in a co-culture model that mimics the inflammatory environment *in vitro*. Both the ability of lymphocytes to affect differentiation and immune properties of bone cells, osteoblasts (OBL) and osteoclasts (OCL), and of bone cells to modulate cytokine and activation profile of endotoxin-stimulated lymphocytes were tested. LPS-pulsed lymphocytes enhanced OCL, but inhibited OBL differentiation and increased the RANKL/OPG ratio, and, at the same time, up-regulated chemotactic properties of bone cells, specifically CCL2, CCL5 and CXCL10 in OCL, and CCL5 and CXCL13 in OBL. In parallel, bone cells had immunosuppressive effects by down-regulating the lymphocyte expression of IL-1, IL-6, TNF-α and co-stimulatory molecules. OCL stimulated the production of osteoclastogenic cytokine RANKL in T lymphocytes. Anti-inflammatory effect, especially of OBL, suggests a possible compensatory mechanism to limit the inflammatory reaction during infection.

Keywords: cytokines, lipopolysaccharide, lymphocytes, osteoblasts, osteoclasts

Introduction

Immune and bone cells originate from bone marrow and are closely functionally and anatomically related, defining the bone marrow microenvironment [1-3]. Osteoblasts (OBL) are bone forming cells responsible for the synthesis of extracellular proteins and regulation of the mineralization process. Together with adipocytes, chondrocytes and myocytes, OBL originate from mesenchymal stem cells [2]. Osteoclasts (OCL) are cells of hematopoietic origin which differentiate from myeloid progenitors, upon stimulation by receptor-activator of NF-κB ligand (RANKL) and monocyte-macrophage colony-stimulation factor (M-CSF), into cells with the ability to decalcify and degrade bone matrix [3]. Bone homeostasis is maintained by a delicate balance between bone resorption by OCL and bone formation by OBL. However, inflammatory processes, including acute and chronic bacterial infections, can alter the bone microenvironment to promote misbalances in the remodeling processes resulting in irreversible bone destruction [4-6].

Lipopolysaccharide (LPS), a major component of the Gram-negative bacteria cell wall, activates innate immunity and causes infiltration of polymorphonuclear leukocytes, T lymphocytes and monocytes into the infected site [7, 8]. LPS promotes bone resorption in mice, by stimulating pro-inflammatory cytokines, antigen presentation and OBL expression of osteoclastogenic cytokines RANKL and tumor necrosis factor (TNF)-α [9-12]. B lymphocytes, highly susceptible to LPS, additionally contribute to osteoclastogenic effect of LPS *in vitro* and *in vivo* [13-14]. T lymphocytes within an inflamed site support bone destruction by stimulating RANKL expression and OCL precursors [15-16].

Although the influence of immune system on bone cells has been intensively investigated, the mechanisms of the reverse effects of bone cells on the activity and cytokine profile of

immune cells are still unclear. Some studies suggested that OBL may have immunocompetent properties similar to that of macrophages or dendritic cells, including the expression of immunoreactive molecules, presentation of superantigens and IL-6 secretion [18, 19]. A subpopulation of human OBLs expresses the major histocompatibility complex class II (MHC-II) and costimulatory molecules, thus participating in antigen presentation and T lymphocyte activation [17]. On the other hand, OBL lineage cells as well as their ancestry, bone-marrow derived mesenchymal stem cells (MSC) may exhibit immunosuppressive properties by secreting immunosuppressive cytokines and stimulating regulatory T lymphocytes [20-22]. It has been recently proposed that MSC may be polarized toward either the proinflammatory or the immunosuppressive subpopulation depending on the toll like receptor (TLR) triggered by an exogenous danger signals [23]. OCL, expressing MHC-I/II and costimulatory molecules, can uptake soluble antigens serving as antigen-presenting cells for alloreactive T lymphocytes [24]. In response to immune cells these bone cells produce cytokines, chemokines, extracellular matrix proteins and other inflammatory mediators [25-27].

Since multiple aspects of bone homeostasis depend on complex interactions between bone cells and immune cells, including T and B lymphocytes, we aimed to define their interaction in the inflammatory environment using a co-culture system of bone cells overlaid with endotoxin-stimulated lymphoid cells. Besides the effects of LPS-pulsed splenocytes on bone cell differentiation, we specifically focused on the chemotactic effects of bone cells and their ability to directly modulate the lymphocyte cytokine and activation profile. We hypothesized that bone cells have chemotactic and immunomodulatory effects, properties that could consequently contribute to the local pathological milieu as a key feature of persistent bacterial infections and prolonged inflammation associated with caries, periodontitis or osteomyelitis.

Materials and methods

Mice

Female 10-week-old C57BL/6J mice were used as a source of splenic and bone marrow cells. All animal protocols were approved by the Ethics Committee of the University of Zagreb School of Medicine (Zagreb, Croatia). Maintenance of animals and experimental procedures followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

MTT assay

Cells were isolated from mouse spleen, pulsed with *E.Coli* LPS (0111:B4; Sigma-Aldrich, St Louis, MO, USA) in a dose of 50 μg/mL for 15 minutes at 37°C, washed twice in PBS, plated in quadruplicates into a 96-well flat bottom plate at a density of 0.5×10^6 cells/well in 0.2 mL/well of RPMI containing 10% fetal calf serum (FCS; Invitrogen, Carlsbad, CA, USA) and cultured at 37°C with 5% CO_2 in air. Proliferation was assessed 2 and 4 days following LPS pulse by the colorimetric MTT (3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) assay as recommended by the manufacturer. Optical density (OD) of the dissolved formazan crystals formed from the tetrazolium salts was spectrophotometrically read at 595 nm wavelength (Bio-Rad Laboratories, Hercules, CA, USA). Differences in the OD of LPS-pulsed and unstimulated cultures were presented as stimulation indices.

Flow cytometry and cell sorting

Separation of B and T lymphocytes from spleen cell suspensions was performed in a BD FACSAria I (BD Biosciences, Franklin Lakes, NJ, USA) instrument. Erythrocytes were

lysed with red blood cell lysing buffer (Sigma-Aldrich) and suspensions were labeled using the lymphoid lineage markers (anti-CD3 for T lymphocytes, anti-CD19 for B lymphocytes). Sorted cells were collected in minimum essential medium α (α-MEM) supplemented with 20% FCS and used for co-culture experiments. Sorting purity was determined by a reanalysis of fractioned populations and was greater than 99% for all experiments.

For the phenotype characterization of LPS-pulsed splenic lymphocytes we used commercially available directly conjugated monoclonal antibodies for LPS receptor TLR4, lymphoid lineage markers (CD3 for T lymphocytes and CD19 for B lymphocytes) and activation markers (CD69, CD86 and MHC-II) (e-Biosciences; San Diego, CA, USA). Stained cells (10^5 cells/sample) were analyzed in a FACSCalibur (BD Biosciences) instrument, and the data analyzed using CellQuest software (BD Biosciences).

Bone cell cultures

For OBL generation bone marrow cells were flushed out from the femoral medullar cavity and plated into 6-well plates at a density of 3×10^6 cells/well in 3 mL/well of α-MEM/10% FCS. OBL differentiation was induced from culture day 7 by the addition of 50 μg/ml ascorbic acid and 8 mM β-glycerophosphate (Sigma-Aldrich) [28]. At day 14, OBL colonies were detached from plate surface by trypsin/EDTA (Sigma-Aldrich) digestion and replated for co-cultures into 24-well plates at a density of 0.3×10^6/well in 1 mL/well using the same differentiation media. At day 5 following replating, OBL cultures were stained cytochemically for the activity of alkaline phosphatase (ALP) using a commercially available kit (Sigma-Aldrich) and red color intensity was measured by custom-made software. Cell lysates were additionally collected for ALP-activity assay.

For OCL generation bone marrow was cultured overnight with 5 ng/mL recombinant mouse (rm)M-CSF (R&D Systems, Abingdon, UK) in α-MEM/10% FCS, to stimulate the

monocyte/macrophage lineage followed by harvesting of non-adherent cells as enriched hematopoietic monocyte/macrophage progenitors [29]. Non-adherent bone marrow cells were replated for co-cultures into 24-well plates at a density of 0.5×10^6/well in 1 mL/well of α-MEM/10% FCS supplemented with 15 ng/mL rmM-CSF and 30 ng/mL rmRANKL (R&D Systems, Abingdon, UK). At day 5 following replating, tartrate-resistant acid phosphatase (TRAP) positive multi-nucleated cells (with ≥three nuclei/cell) were identified using a commercially available kit (Sigma-Aldrich) and counted by light microscopy. Cell lysates were additionally collected for TRAP-activity assay.

Enzyme activity assays

The activity of ALP and TRAP enzymes in cell lysates of OBL and OCL cell cultures was determined by a colorimetric method with chromogenic *p*-nitrophenylphosphate (PNPP) used as enzyme substrate. ALP and TRAP hydrolyzes PNPP at 37°C to form *p*-nitrophenol (PNP) at alkaline or acidic conditions respectively. The OD of the formed yellow-colored PNP was spectrophotometrically read at 405 nm wavelength (Bio-Rad Laboratories). The intensity of the yellow color correlates with the amount of PNP (mmol/min) and represents the enzyme activity in solution (U/L).

Co-culture system

Total splenocytes or sorted B and T lymphocytes were pulsed with LPS (50 μg/mL) for 15 minutes at 37°C, washed twice in PBS and plated over the bone cells (in the appropriate differentiation media) using the transwell culture inserts (4 μm pore size, BD Biosciences) in 24-well plate at a density of 0.5×10^6 cells/transwell. Culture media and traswell inserts containing splenocytes were changed at day 2.5. At several co-culture time-points (as indicated by the experiments), lymphocytes were subjected to RNA extraction and flow

cytometric analysis, while bone cells were used for cytochemistry, enzyme activity assay and RNA extraction.

Gene expression analysis

Total RNA from cultured OBL, OCL and transwell inserts (containing splenocytes, T or B lymphocytes) was extracted using TRI Reagent (Applied Biosystems, Foster City, CA, USA), reversely transcribed (1 µg) to cDNA and amplified (20 ng/well in triplicates) by quantitative (q)PCR using an AB7500 (Applied Biosystems) instrument. Expressions of OBL (ALP, osteocalcin (OC), bone-sialoprotein (BSP), runt-related transcription factor 2 (RUNX2), osterix (OSTX), distal-less homeobox 3 (DLX3), osteoprotegerin (OPG) and RANKL), and OCL differentiation genes (cFMS (CD115), cFOS, RANK), CCL (-2, -5, -10) and CXCL chemokines (-10, -12, -13), cytokines (IL-1, -6, -18, TNF-α) and activation-associated molecules (CD80, CD83, CD86, MHC-II (I-Aα)) were determined using commercially available TaqMan Gene Expression Assays (Applied Biosystems). The expression of specific gene was calculated according to the relative standard curve of gene expression in the calibrator sample (cDNA from lymphocytes, OBL or OCL cultures), and then normalized to the expression level of the ß-actin gene as an endogenous control.

Statistics

Experiments were performed at least three times and the representative data for bone cell quantification, OD readings and relative gene expression are presented as mean value ± standard deviation. Statistical analysis of the group difference was performed using the analysis of variance (ANOVA) with Bonferroni's correction for multiple testing. The methodological studies of quantitative PCR analysis suggest that the least difference in gene expression that could be reproducibly detected is around 100% [30]. Therefore we

assumed the change in the gene expression that is statistically significant (ANOVA, $p<0.05$) and more than 2-fold different compared with control, repeating through all experiments, as biologically significant. Statistical analysis was performed using the MedCalc software package (Mariakerke, Belgium).

Results

Effects of LPS pulse on splenic T and B lymphocytes

To confirm the splenocyte responsiveness to LPS pulse we first evaluated the splenic expression of TLR4, a component of the LPS receptor complex (Fig 1A). We also measured relative quantity of TLR4 RNA in different populations (23.72±0.94 for total splenocytes, 104.01±4.38 for B lymphocytes, 1.36±0.10 for T lymphocytes). In addition we found that splenocytes exhibited a proliferative response to LPS pulse *in vitro* (Fig 1A). Further on, we aimed to determine the LPS effect on the expression of surface molecules associated with lymphocyte activation. As expected [14, 31], T lymphocyte response to LPS pulse was weak, although an early increase in the CD69 expression was found (Fig 1B). In contrast, a strong increase in the membrane expression of CD69, CD86 and MHC-II within 48 hours following the LPS pulse was confirmed on B lymphocytes (Fig 1C). Gene expression of pro-inflammatory cytokines and activation markers was analyzed in sorted T and B lymphocytes (Fig 1D). Following LPS pulse, we observed increase for several pro-inflammatory cytokines (IL-1, IL-6, TNF-α, IL-18) and the MHC-II molecule in T lymphocytes as well as IL-6, IL-18, co-stimulatory molecules (CD80 and CD86) and the activation marker CD83 in B lymphocytes (Fig 1E and 1F).

Effects of LPS-pulsed splenocytes on bone cell differentiation

The effect of LPS-pulsed splenocytes on the differentiation of bone marrow derived bone cell progenitors *in vitro* was assessed in bone cell cultures overlaid with the splenic cells using the transwell inserts.

OBL co-cultured with LPS-pulsed splenocytes exhibited a lower ALP-activity and lower ALP-staining intensity compared with the control untreated OBL cultures and OBL co-

cultured with splenocytes that were not pulsed with LPS (Fig 2A). Similar effect was seen with LPS-pulsed sorted lymphocytes, specifically T lymphocytes. The expression of early (BSP, DLX3, RUNX2) and mature (OSTX, OC, OPG) OBL differentiation genes was significantly lower in co-cultures with LPS-pulsed splenocytes (Fig 2B). Only the expression of RANKL was enhanced in OBL co-cultured with LPS-pulsed splenocytes, creating an approximately 10-fold increase in the RANKL/OPG ratio compared to the control co-cultures (Fig 2C).

In contrast to the suppressed OBL differentiation, co-cultures of OCL with LPS-pulsed splenocytes exhibited enhanced TRAP-activity compared to the control cultures or co-cultures with non LPS-pulsed splenocytes (Fig 3A). The total number of TRAP-positive cells was not different between groups, but OCL differentiated in co-cultures with LPS-pulsed splenocytes were larger and with more nuclei/cell than the control co-cultures (231.5±16.3 per well of OCL with ≥10 nuclei/cell in co-cultures with the LPS-pulsed splenocytes compared to 114.3±7.8 in the control cultures, $p<0.001$). Increase in TRAP-activity was observed in co-cultures with LPS-pulsed sorted lymphocytes, although to the lesser extend compared to LPS-pulsed total splenocytes. The expression of OCL-specific differentiation genes RANK and cFOS, normalized to total RNA extracted from wells, did not show the increase compared to the control groups (Fig 3B), due to the enhanced proliferation of monocyte/macrophage lineage-derived cells other than OCL in LPS-pulsed splenocytes (numerous TRAP-negative cells in wells co-cultured with LPS-pulsed splenocytes compared with control cultures, Fig 3A).

Chemoattractant properties of *in vitro* differentiated bone cells

To evaluate the chemotactic properties proposed for bone cells, gene expression of selected CXCL and CCL chemokines was assessed in a time-curse of co-cultures of bone cells with LPS-pulsed splenocytes (Fig 4A).

Compared to OBL, OCL exhibited several fold higher expression for CCL5 and CXCL10, but lower for CXCL12 and CXCL13, while the expression of CCL20 was barely detected in both OCL and OBL (Fig 4B). Time-kinetic profile in non-treated cultures showed that CXCL10 and CXCL13 increased in the expression with OCL differentiation. LPS-pulsed splenocytes strongly induced OCL expression of all selected chemokines except CXCL12, specific for mesenchymal lineages including OBL. In addition, several pro-inflammatory cytokines (IL-1, IL-6, IL-18) were induced in OCL co-cultured with LPS-pulsed lymphoid cells (not shown).

OBL exhibited increase in gene expression of CXCL12, whereas decreases in gene expression of CCL5, CXCL10 and CXCL13 with co-culture duration. In response to LPS-pulsed splenocytes OBL up-regulated the expression of CCL5 and CXCL13 in all tested time-points (Fig 4B).

Immunomodulatory effects of *in vitro* differentiated bone cells

Beside chemotactic properties, we also determined the immunomodulatory effect that bone cells have on LPS-pulsed lymphocytes in a co-culture setting (Fig 5A).

Several pro-inflammatory cytokines were down-regulated in total splenic cells as well as sorted lymphocytes, specifically IL-6 (Fig 5B). In general, OBL had a stronger immunosuppressive effect than OCL, reducing the expression of IL-1 and TNF-α mostly in T lymphocytes. In contrast, OBL induced the expression of IL-18 in LPS-pulsed B lymphocytes (Fig 5B). The expression of osteoclastogenic cytokine RANKL in LPS-pulsed T lymphocytes was increased by co-culturing with OCL. Finally, the expression of co-

stimulatory molecules was down-regulated in LPS-pulsed B lymphocytes co-cultured with bone cells, specifically with OBL that suppressed both CD80 and CD86.

Flow cytometric analysis of CD69 and CD86 expression confirmed the immunosuppressive effect of bone cells on co-cultured lymphoid cells, more prominent in B lymphocytes co-cultured with OBL cells (Fig 5C).

Discussion

Although several recent studies confirmed immunological properties of bone cells [19-22, 24], direct chemotactic and immunomodulatory effects of OBL and OCL have not been precisely determined. In the co-culture model we demonstrated both the ability of lymphocytes to affect bone cell differentiation and their chemotactic properties, and of bone cells to modulate the cytokine and activation profile of lymphoid cells.

The course of infection mediated by Gram-negative bacteria is largely determined by immune responses to LPS that signal dominantly by an interaction with the TLR4/MD-2/CD14 complex expressed mostly by innate immune cells [7, 32]. As a complementation to these studies, we aimed to determine whether there was a specific response of lymphocytes to LPS and to what extent this response may affect immune and bone system interactions during inflammation. We used spleen as a lymphocytes' source, since it consists of approximately 50% B lymphocytes and 40% T lymphocytes of total nucleated cells. Spleen also includes, in low percent, LPS-susceptible antigen presenting cells (APCs) such as macrophages, which in response to LPS release proinflammatory cytokines and induce Th1-type response in T lymphocytes [31, 35]. To exclude this indirect effect of LPS in total splenocyte population, we separately analyzed the activation profile of sorted B and T lymphocytes. LPS pulse had a more prominent effect on splenic B than T lymphocytes, assessed by the expression of activation markers, such as CD69. Nevertheless, several activation-induced molecules, including cytokines, were strongly up-regulated in both B and T lymphocytes following LPS treatment. LPS has a mitogenic effect on murine B lymphocytes, mostly by binding to TLR4 [33], although TLR engagement may produce an alternate pathway for B cell receptor-triggered signal propagation [34]. T lymphocytes respond to LPS in both indirect, involving LPS stimulation of APC [35] and direct manner, through TLR-mediated signaling pathways [36].

Activation of the immune system during a microbial invasion is generally protective, but an excessive reaction to LPS may harm the host by endogenously generated inflammatory mediators, which consequently have important effects on bone metabolism [9-13, 25, 29]. We found that OBL differentiation was suppressed by endotoxin-stimulated splenocytes and, in addition, OBL exhibited an increased RANKL/OPG ratio, considered to determine OCL activity and bone resorption [3, 25]. Moreover, LPS-pulsed splenocytes enhanced OCL differentiation, confirming that inflammatory environment lead to bone destruction as a serious complication of bacterial infections of bones or adjacent tissues [6, 9, 12-16, 37].

Bone cells, as bone tissue constituents, physiologically maintain bone homeostasis, but many studies suggest a functional role of principal bone cells beyond bone. Immunomodulatory properties attracted great attention, but most studies primarily investigated effects of immune cells on bone cells but not vice versa. Among others, chemokines have been recognized as essential signals for the trafficking of cells regulating the bone metabolism [27, 39-46]. Increased expression of CCL2 and CCL5 by OCLs co-cultured with LPS-stimulated splenocytes may attract monocyte/macrophage lineage cells, including OCL progenitors, as suggested by several studies [27, 39, 43]. Enhanced OCL expression of CXCL10 may further stimulate infiltration of osteoclast precursors and T lymphocytes [44]. In addition, OBLs co-cultured with LPS-stimulated splenocytes had enhanced expression of CXCL13, mediating B lymphocyte chemotaxis [27]. IL-1β has been shown to induce CXCL13 in differentiated OBLs, attracting preosteoclast cells and stimulating osteoclast differentiation [45]. Those results indicate that bone calls participate in creating the inflammatory proresorptive microenvironment.

Although we showed that bone cells are strongly chemotactic for lymphocytes, their direct immunomodulatory effects on lymphoid cells are rather immunosuppressive and anti-inflammatory. In co-cultured total splenic cells we could not exclude the effect of bone cells

on cytokine expression in LPS-activated monocytes/macrophages [31, 32, 35], which could account for the discrepancy in the results on total splenocytes compared to sorted T and B lymphocytes. The expression of several pro-inflammatory cytokines, including IL-1, IL-6 and TNF-α, are suppressed in lymphocytes co-cultured with bone cells, especially OBL. Immunosuppressive properties of OBL lineage cells are confirmed by several studies through their ability to suppress dendritic cell maturation, lymphocyte proliferation and cytotoxic T lymphocyte generation [21, 23]. Moreover, B lymphocyte expression of activation and co-stimulatory molecules was down-regulated by co-culturing with OBL lineage cells, which contributed to their immunosuppressive function. We can postulate that those immunosuppressive effects represent a compensatory mechanism to limit the pro-inflammatory signals during infection. Only IL-18, known to be induced by LPS [15, 25], was up-regulated in B lymphocytes co-cultured with OBL, possibly having a role in linking the Th1-response to inflammation. OCL immunomodulatory effect on endotoxin stimulated lymphocytes was equivocal, exhibiting down-regulation of IL-1 and IL-6 and up-regulation of RANKL in T lymphocytes. Enhanced RANKL expression indicates that OCL-to-T lymphocytes interactions during endotoxemia modulate T lymphocyte activity to cause an exacerbated OCL function [16].

In conclusion, our results demonstrate that there is a bidirectional communication during endotoxin-induced inflammation between bone and immune cells. We are aware of the limitation of our co-culture system being *in vitro* model to mimic the inflammatory environment, but it provides a specific setting in which we excluded the direct effects that LPS has on bone cells [9, 12, 26, 47]. LPS-pulsed lymphocytes stimulate bone resorption by enhancing OCL and suppressing OBL differentiation with increased RANKL/OPG ratio, while bone cells create a chemotactic gradient and enhance the production of RANKL by T lymphocyte. These chemotactic and proresorptive effects may help in co-localization of

inflammatory cells at sites of infection and contribute to the inflammation-induced bone loss. Furthermore, OBL exhibit immunosuppressive effect on the expression of pro-inflammatory cytokines and costimulatory molecules, as a possible compensatory mechanism to limit the inflammatory reaction in pathological conditions.

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Figure legends

Figure 1. Lipopolysaccharide pulse activates lymphoid cells and stimulates expression of proinflammatory cytokines. (A) Expression of lipopolysaccharide (LPS) membrane receptor – toll-like receptor 4 (TLR4) on splenocytes assessed by flow cytometry and splenocyte proliferative response *in vitro* following LPS-pulse assessed by MTT assay. Proliferation index represents the difference between OD readings in LPS-pulsed and non-stimulated wells at days 2 and 4 of culture. (B and C) Flow cytometric analysis of the expression of activation marker CD69 on T lymphocytes and CD69, CD86 and MHC-II on B lymphocytes following LPS-pulse. Cells were first gated by the lineage marker (CD3 for T lymphocytes and CD19 for B lymphocytes), then the expression of activation markers was assessed in the gated population. Expression was followed up to 48 hours and expressed as mean fluorescent intensity (MFI). T lymphocytes did not show changes in the expression of CD86 and MHC-II (not shown). (D) FACS separation of B and T lymphocyte from total splenic population. Splenocytes were labeled with lineage markers as above. Sorted populations were reanalyzed for sorting purity and used for subsequent co-culture experiments. (E and F) Gene expression analysis of activation-associated molecules in LPS-pulsed (+LPS) T and B lymphocyte populations by RT-qPCR. Lymphocytes were cultured for 2.5 days in the same conditions as for subsequent co-culture experiments, but without the bone cell layer. RNA relative quantity was calculated using the relative standard curve of the calibrator sample and normalized to the endogenous control (ß-actin). For LPS pulse, lymphocytes were incubated with LPS (50 μg/mL) for 15 minutes at 37°C, and washed twice in PBS prior to culture. IL, interleukin; TNF, tumor necrosis factor; MHC, major histocompatibility complex.

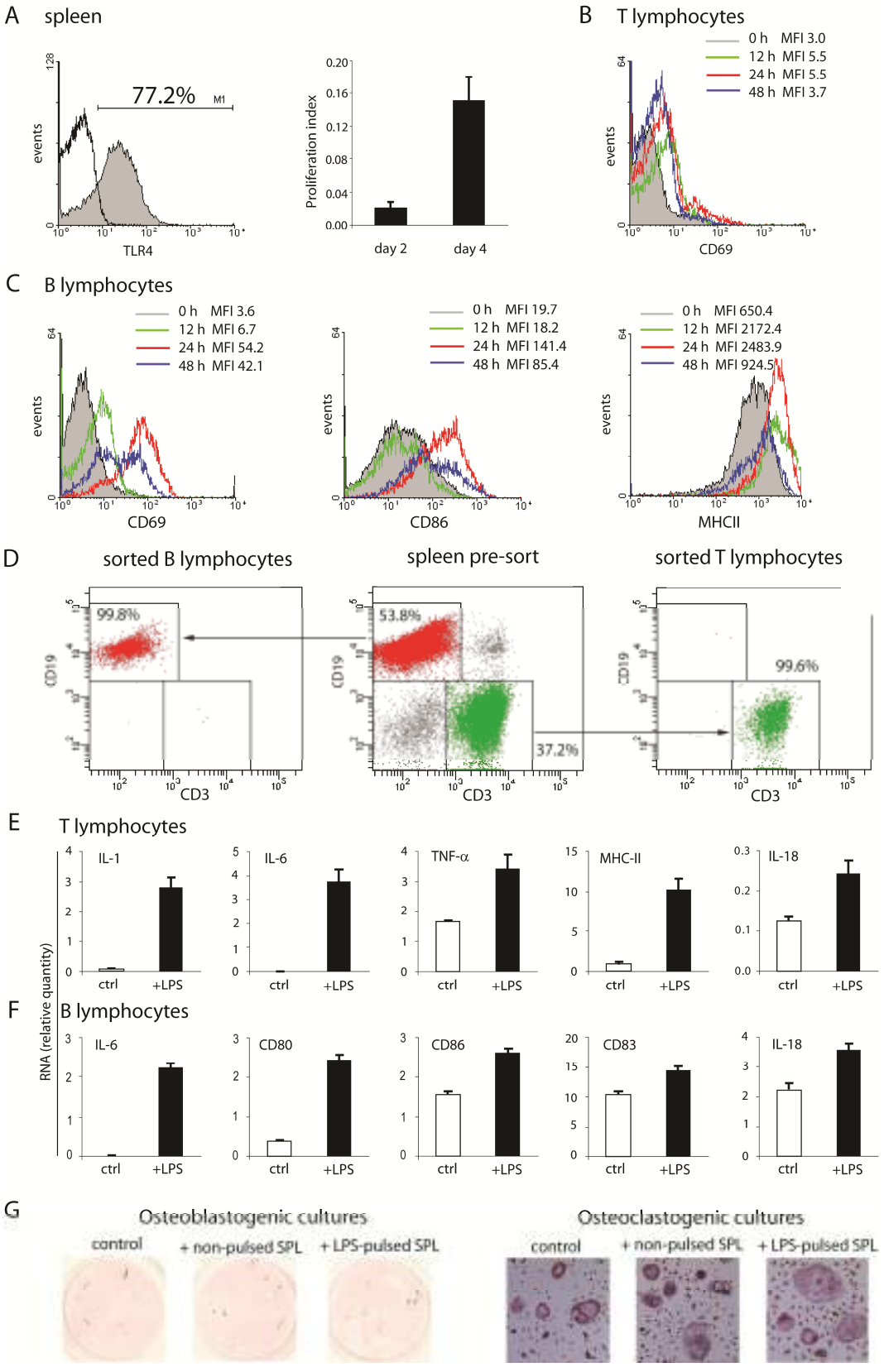
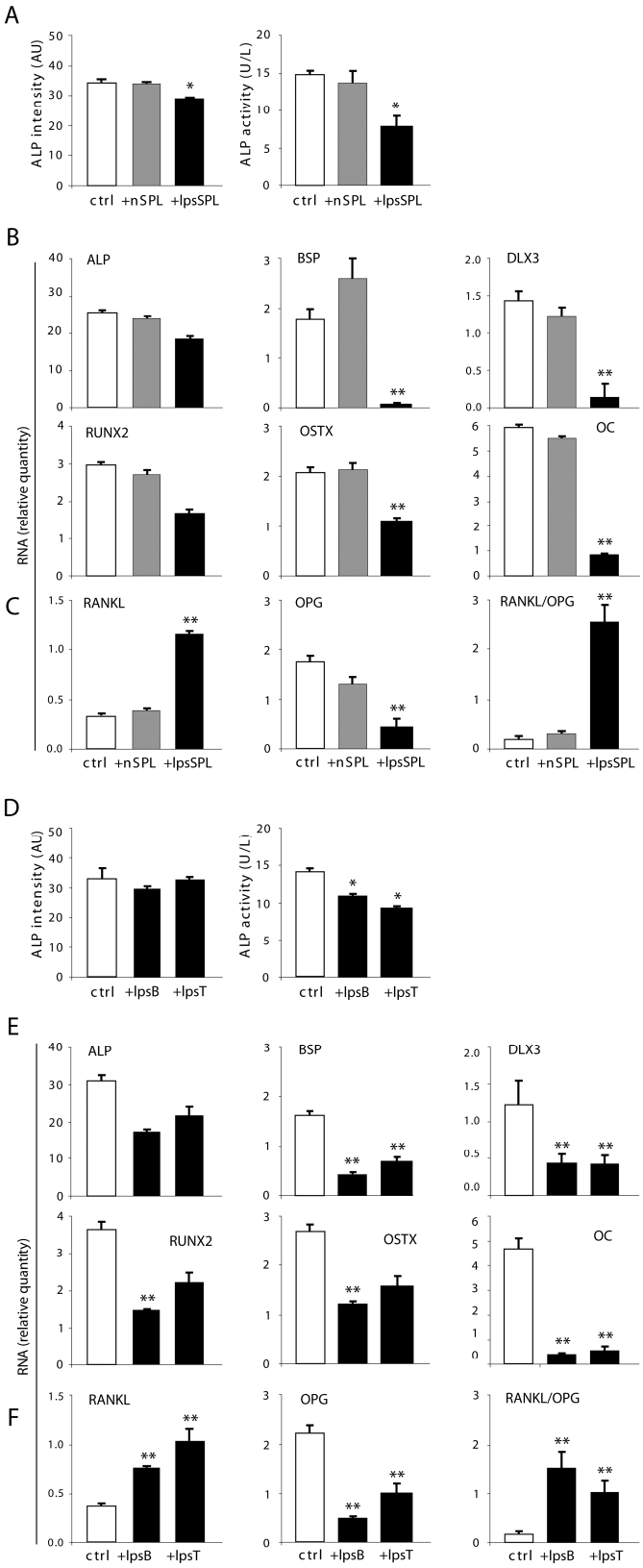

A spleen
77.2%
M1
events
TLR4
Proliferation index
day 2
day 4
B T lymphocytes
0 h MFI 3.0
12 h MFI 5.5
24 h MFI 5.5
48 h MFI 3.7
CD69
C B lymphocytes
0 h MFI 3.6
12 h MFI 6.7
24 h MFI 54.2
48 h MFI 42.1
CD69
0 h MFI 19.7
12 h MFI 18.2
24 h MFI 141.4
48 h MFI 85.4
CD86
0 h MFI 650.4
12 h MFI 2172.4
24 h MFI 2483.9
48 h MFI 924.5
MHCII
D
sorted B lymphocytes
spleen pre-sort
sorted T lymphocytes
99.8%
53.8%
37.2%
99.6%
CD19
CD3
E T lymphocytes
IL-1
IL-6
TNF-α
MHC-II
IL-18
RNA (relative quantity)
ctrl
+LPS
F B lymphocytes
IL-6
CD80
CD86
CD83
IL-18
G
Osteoblastogenic cultures
control
+ non-pulsed SPL
+ LPS-pulsed SPL
Osteoclastogenic cultures
control
+ non-pulsed SPL
+ LPS-pulsed SPL

Figure 2. Lipopolysaccharide-pulsed lymphoid cells inhibit osteoblast differentiation. (A) Differentiated osteoblastogenic cells (OBL) were co-cultured with non-treated (+NTspl) and lipopolysaccharide (LPS)-pulsed splenocytes (+LPSspl), or LPS-pulsed sorted B (+LPSlyB) or T lymhocytes (+LPSlyT) using transwell culture inserts. For LPS pulse, splenocytes/lymphocytes were incubated with LPS (50 μg/mL) for 15 minutes at 37°C, and washed twice in PBS prior to co-culture. Differentiation of the osteoblast lineage was quantified using the color intensity of cytochemical alkaline phosphatase (ALP) staining and ALP-activity assay of cell lysates at the co-culture end-point (day 5) after the removal of culture inserts. *, indicates statistically significant difference in ALP color-intensity or ALP-activity compared with control groups (ANOVA, $p<0.05$). (B) RT-qPCR analysis of the expression of osteoblastogenic differentiation genes ALP, bone sialoprotein (BSP), distal-less homeobox 3 (DLX3), runt-related transcription factor 2 (RUNX2), osterix (OSTX) and osteocalcin (OC) in osteoblastogenic cells co-cultured with non-treated and LPS-pulsed splenocytes. (C) RT-qPCR analysis of the expression of tumor necrosis factor (TNF)-family genes receptor activator of NF-κB ligand (RANKL) and osteoprotegerin (OPG) in OBL co-cultured with non-treated and LPS-pulsed splenocytes. Ratio of the RNA relative quantity for receptor activator of NK-κB ligand and osteoprotegerin (RANKL/OPG) was calculated to determine the osteoclastogenic effect of OBL. OBL co-cultured with empty inserts (ctrl) and non-treated splenic cells were used as controls. RNA was extracted from OBL at the co-culture end-point (day 5) after removal of culture inserts. RNA relative quantity was calculated using the relative standard curve of the calibrator sample and normalized to the endogenous control (ß-actin). Data for a representative of 3 repeated experiments with similar results are shown. **, indicates change in the gene expression

that is statistically significant (ANOVA, $p<0.05$) and more than 2-fold different (considered as biologically significant) compared with control groups.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ALP activity (U/L)
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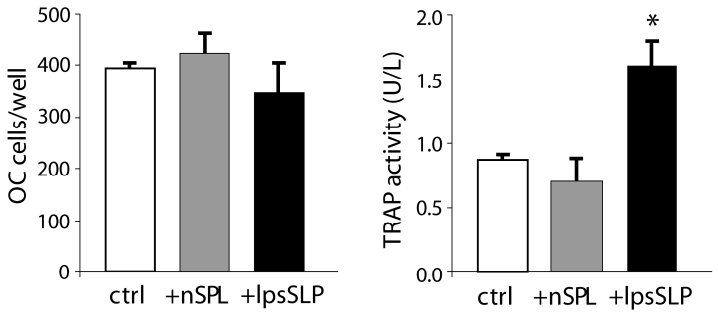
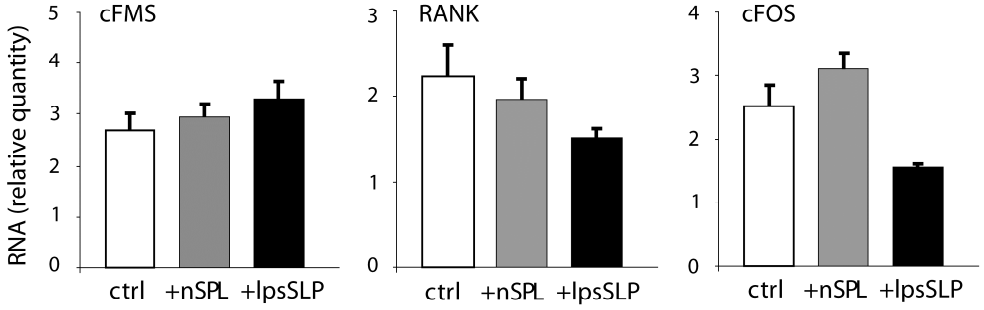
B
ALP
BSP
DLX3
RUNX2
OSTX
OC
RNA (relative quantity)
C
RANKL
OPG
RANKL/OPG
ctrl +nSPL +lpsSPL
D
ALP intensity (AU)
ALP activity (U/L)
ctrl +lpsB +lpsT
E
ALP
BSP
DLX3
RUNX2
OSTX
OC
RNA (relative quantity)
F
RANKL
OPG
RANKL/OPG
ctrl +lpsB +lpsT

Figure 3. Lipopolysaccharide-pulsed lymphoid cells enhance osteoclast differentiation. (A) Differentiated osteoclastogenic cells (OCL) were co-cultured with non-treated (+NTspl) and lipopolysaccharide (LPS)-pulsed splenocytes (+LPSspl), or LPS-pulsed sorted B (+LPSlyB) or T lymhocytes (+LPSlyT) using transwell culture inserts. For LPS pulse, lymphocytes were incubated with LPS (50 μg/mL) for 15 minutes at 37°C, and washed twice in PBS prior to co-culture. Differentiation of OCL lineage was quantified by counting tartrate-resistent acid phosphatase (TRAP)-positive multinucleated cells and by measuring TRAP-activity in cell lysates at the co-culture end-point (day 5) after removal of culture inserts. *, indicates statistically significant difference in TRAP activity compared with control groups (ANOVA, $p<0.05$). (B) RT-qPCR analysis of the expression of OCL differentiation genes cFMS (CD115, M-CSF receptor), receptor activator of NF-κB (RANK) and cFOS in OCL co-cultured with non-treated and LPS-pulsed splenocytes. OCL co-cultured with empty inserts (ctrl) and non-treated splenic cells were used as controls. RNA was extracted from OCL at the co-culture end-point (day 5) after removal of culture inserts. RNA relative quantity was calculated using the relative standard curve of the calibrator sample and normalized to the endogenous control (ß-actin). Data for a representative of 3 repeated experiments with similar results are shown.

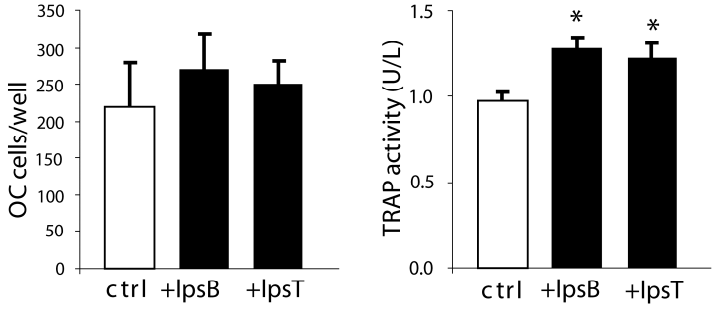
A


OC cells/well
500
400
300
200
100
0
ctrl
+nSPL
+lpsSLP
TRAP activity (U/L)
2.0
1.5
1.0
0.5
0.0
*
ctrl
+nSPL
+lpsSLP

B


RNA (relative quantity)
cFMS
RANK
cFOS
ctrl
+nSPL
+lpsSLP

C


OC cells/well
350
300
250
200
150
100
50
0
ctrl
+lpsB
+lpsT
TRAP activity (U/L)
1.5
1.0
0.5
0.0
*
*

D

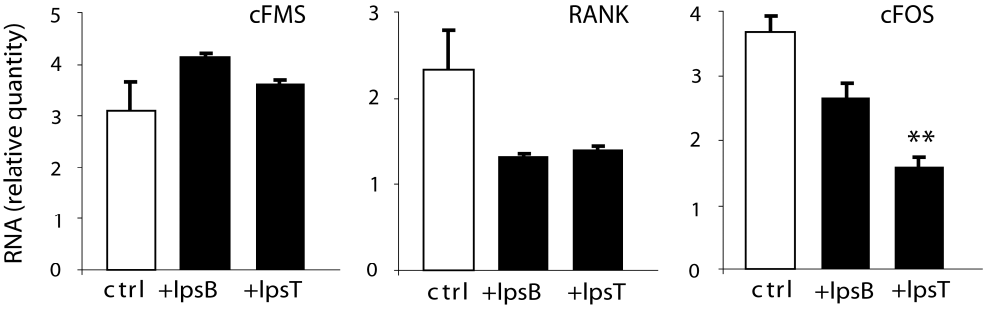

RNA (relative quantity)
cFMS
RANK
cFOS
**
ctrl
+lpsB
+lpsT

Figure 4. Chemoattractant properties of bone cells are enhanced by co-culturing with lipopolysaccharide-pulsed splenic cells *in vitro*. (A) Schematic diagram of the co-culture model. Bone marrow cells were pretreated for a week with 50 µg/ml ascorbic acid (AA) and β-glycerophosphate (β-GP) for osteoblast (OBL), or over-night with M-CSF for osteoclast (OCL) prior to co-culture. At the co-culture start-point (day 0), OCL and OBL were seeded in plates in the appropriate differentiation media (AA/β-GP or RANKL/M-CSF respectively) and growth for 5 days. (B) RT-qPCR analysis of the gene expression of selected CCL (CCL2, CCL5, CCL20) and CXCL (CXCL10, CXCL12, CXCL13) chemokines in OCL and OBL co-cultured with lipopolysaccharide (LPS)-pulsed splenocytes. For LPS pulse, splenocytes were incubated with LPS (50 µg/mL) for 15 minutes at 37°C, and washed twice in PBS prior to co-culture. OCL and OBL co-cultured with empty inserts (ctrl) and non-treated splenic cells (not shown) were used as controls. RNA was extracted from OCL and OBL at several co-culture time-points (day 0.5, 1, 2.5 and 5) to assess the time-kinetic of chemokine expression during bone cell differentiation. RNA relative quantity was calculated using the relative standard curve of the calibrator sample and normalized to the endogenous control (ß-actin). Data for a representative of 3 repeated experiments with similar results are shown. *, indicates change in the gene expression that is statistically significant (ANOVA, $p<0.05$) and more than 2-fold different (considered as biologically significant) compared with control groups.

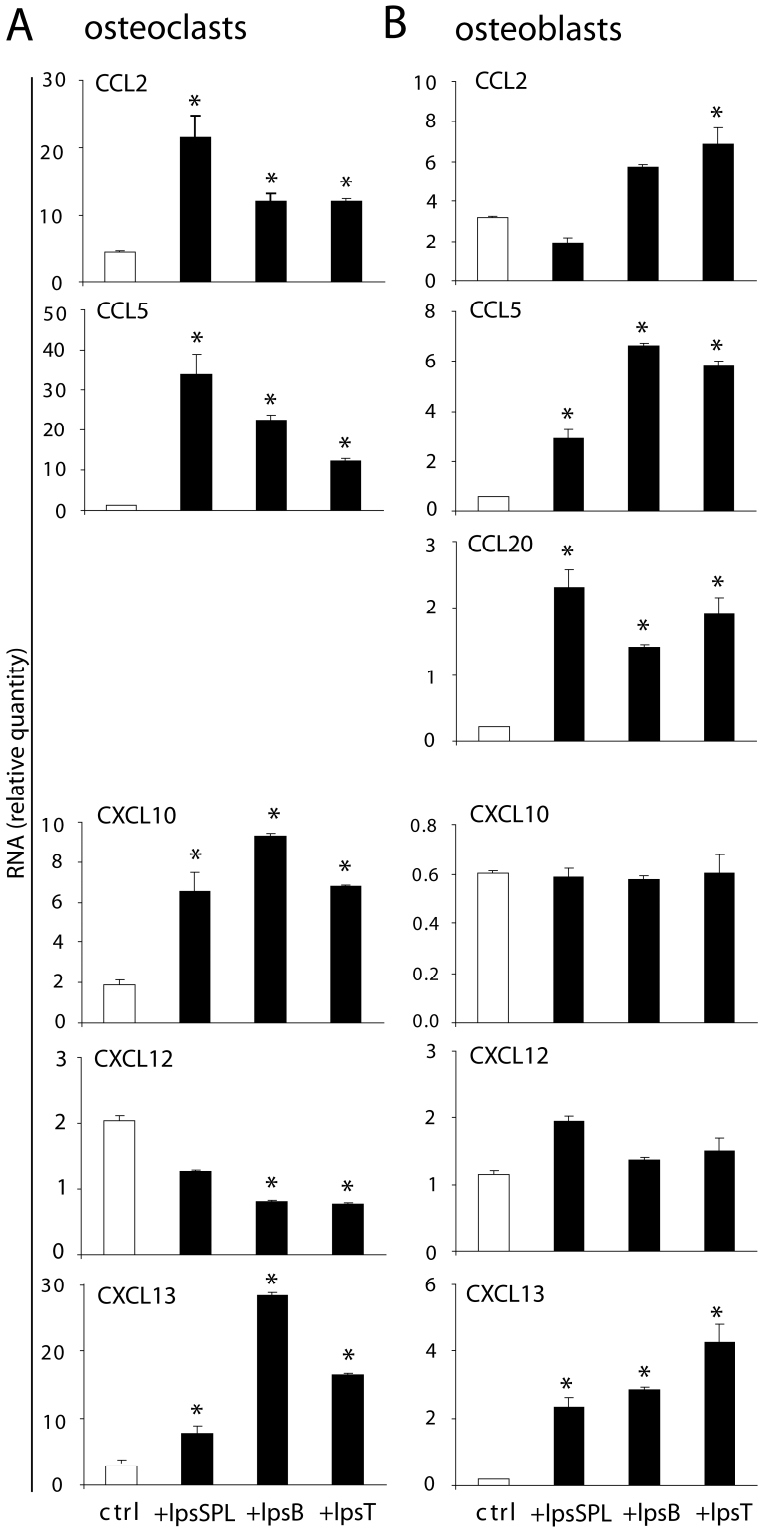
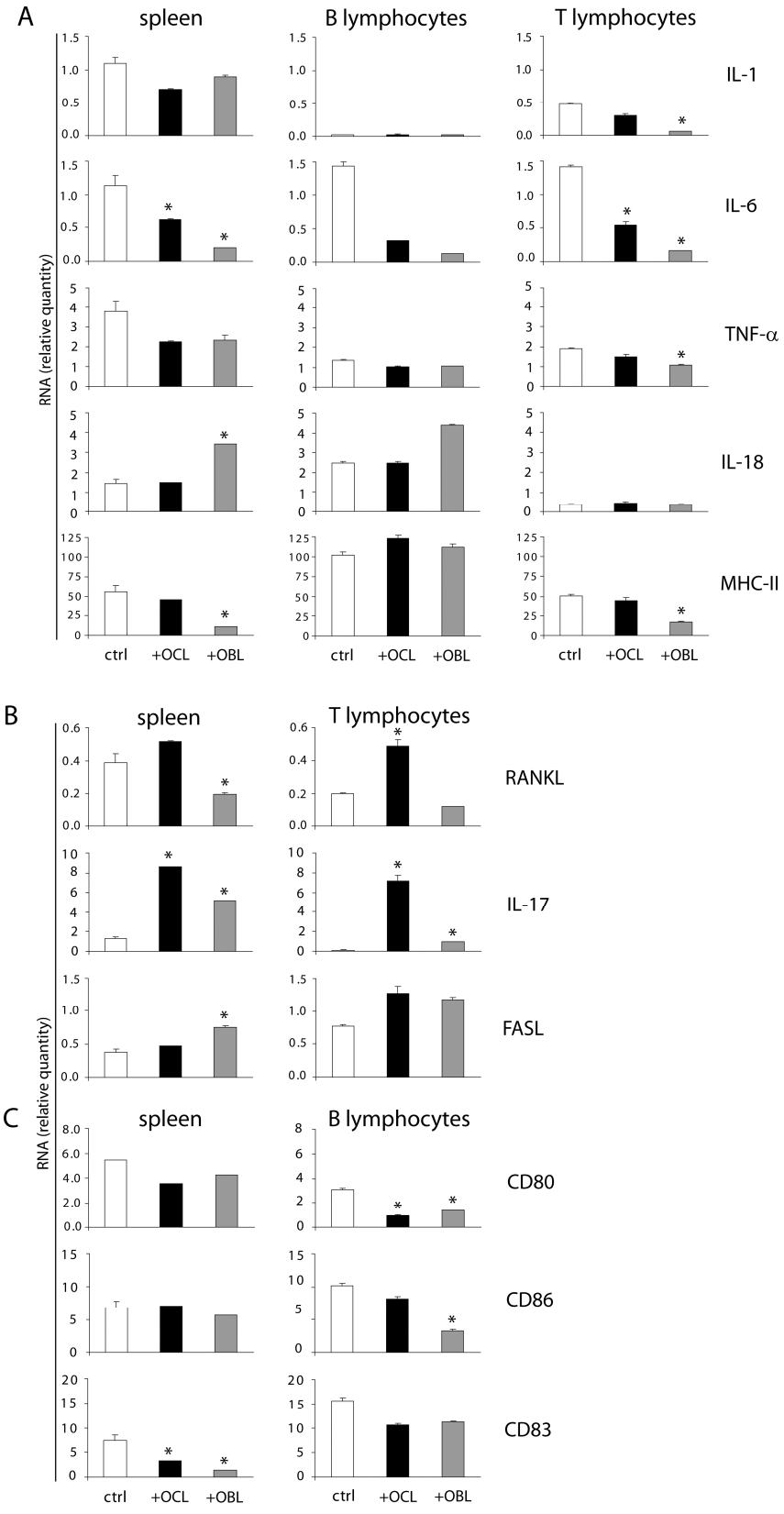

A osteoclasts
B osteoblasts
RNA (relative quantity)
CCL2
CCL5
CCL20
CXCL10
CXCL12
CXCL13
ctrl
+lpsSPL
+lpsB
+lpsT

Figure 5. Bone cells have immunomodulatory effects on lymphoid cells primed with the lipopolysaccharide pulse. (A) Schematic diagram of the co-culture model. LPS-pulsed splenocytes or sorted B and T lymphocytes were added to transwell inserts at the co-culture start-point (day 0). Traswell inserts together with LPS-pulsed splenocytes were changed at day 2.5 of co-culture. For LPS pulse, splenocytes/lymphocytes were incubated with LPS (50 μg/mL) for 15 minutes at 37°C, and washed twice in PBS prior to co-culture. (B) RT-qPCR analysis of the gene expression of costimulatory molecules CD80 and CD86, interleukin (IL)-1 and receptor activator of NF-κB ligand (RANKL), tumor necrosis factor-α (TNF-α), IL-6, and IL-18 in lipopolysaccharide (LPS)-pulsed total splenocytes, and spleen derived B and T lymphocytes cultured without bone cell layer (control) or co-cultured with osteoclastogenic (+ OCL) and osteoblastogenic (+ OBL) cells. RNA was extracted from splenocytes/lymphocytes harvested out of culture inserts at day 0.5 and 2.5 following LPS pulse. RNA relative quantity was calculated using the relative standard curve of the calibrator sample and normalized to the endogenous control (ß-actin). Data for a representative of 3 repeated experiments with similar results are shown. *, indicates change in the gene expression that is statistically significant (ANOVA, $p<0.05$) and more than 2-fold different (considered as biologically significant) compared with control group. (C) Flow-cytometric analysis of lymphocyte expression of activation markers CD69 and CD86 at day 1 following LPS pulse. Splenocytes were cultured without bone cell layer (control) or co-cultured with osteoclastogenic (+ OCL) and osteoblastogenic (+ OBL) cells. Splenocytes were first gated by the lineage marker (CD3 for T lymphocytes and CD19 for B lymphocytes), then the expression of activation markers was assessed in the gated population).


A
spleen
B lymphocytes
T lymphocytes
IL-1
IL-6
TNF-α
IL-18
MHC-II
RNA (relative quantity)
ctrl
+OCL
+OBL
B
spleen
T lymphocytes
RANKL
IL-17
FASL
RNA (relative quantity)
C
spleen
B lymphocytes
CD80
CD86
CD83
RNA (relative quantity)
ctrl
+OCL
+OBL