HHS Public Access

Author manuscript

Calcif Tissue Int. Author manuscript; available in PMC 2017 November 01.

Published in final edited form as:

Calcif Tissue Int. 2016 November ; 99(5): 481–488. doi:10.1007/s00223-016-0170-2.

Potential Role of DARC-chemokine interaction in the recruitment of osteoclast precursors in response to bacterial lipopolysaccharide challenge

Fatemeh Alemi[1], **Mohamed El Gendy**[1], and **Bouchra Edderkaoui**[1,2]

[1]Musculoskeletal Disease Center, Research Service, Jerry L Pettis Memorial Veterans Administration Medical Center, Loma Linda, CA, USA

[2]School of Medicine, Loma Linda University, Loma Linda, CA, USA

Abstract

Duffy antigen receptor for chemokines (*DARC*) binds to a number of pro-inflammatory chemokines, and since chemokines are known to regulate trafficking of osteoclast (OC) precursors, we predicted that *DARC* would regulate OC recruitment to sites of inflammation by modulating chemokine activity. To test this hypothesis, we evaluated the mRNA expression of *Darc* and the chemokines known to bind to DARC, in endothelial cells treated with bacterial lipopolysaccharide (LPS). The mRNA expression of *Mcp-1*, *Rantes*, *Darc* and *Ccr5* was significantly increased in endothelial cells in response to LPS treatment. Blocking the function of *DARC* with neutralizing antibody partially abrogated the effect of LPS on the mRNA expression of *Mcp-1* and *Rantes. In vivo*, mice with targeted disruption of *Darc* gene (*Darc*-KO) and control wild type (WT) mice were used to assess the role of *DARC* in response to single LPS application on the top of parietal bones. Five hours post-LPS injection, local expression of *Cd14* mRNA (a marker of inflammatory monocytes) was significantly increased in both lines of mice. However, the magnitude of increase was greater in WT mice compared to *Darc*-KO mice suggesting a role for *DARC* in mediating the recruitment of monocytes in response to LPS. Histological staining for tartrate-resistant acid phosphatase (TRAP) in calvaria sections taken from the injection sites revealed a significant reduction in TRAP-labeled surface per bone surface in response to LPS in *Darc*-KO mice compared to WT mice. Based on these findings, we concluded that *DARC* regulates recruitment of OC precursors at the inflammation site, probably through regulation of chemokines transcytosis across endothelial cell barrier.

Keywords

Inflammation; chemokines; Duffy antigen receptor for chemokines; bone

Corresponding author: Bouchra Edderkaoui, Bouchra.Edderkaoui@va.gov. Musculoskeletal Disease Center, Jerry L. Pettis VA Medical Center, 11201 Benton St. (151), Loma Linda, CA 92357. Phone: (909) 825-7084, ext. 2780, Fax: (909)796-1680.

Introduction

Chronic inflammatory diseases such as rheumatoid arthritis, ankylosing spondylitis and periodontitis most frequently lead to increased bone resorption [1]. Therefore, understanding the molecular pathways involved in the recruitment of osteoclast progenitors to the site of inflammation for subsequent bone resorption is crucial for the development of new therapeutic strategies to prevent or mitigate inflammation-induced bone loss. The infiltration of osteoclast precursors from blood circulation to the site of inflammation is a complicated process that involves adhesion molecules, proteolytic enzymes, cytokines, and chemokines [2, 3].

In our previous studies to identify new candidate genes that contribute to variation in peak bone mineral density (BMD), we have identified the **D**uffy **A**ntigen **R**eceptor for **C**hemokines (*DARC*) as a negative regulator of BMD. In these studies, it was found that lack of *Darc* expression led to reduced osteoclastic bone resorption, and increased BMD, in the *Darc*-knock out (KO) mice [4]. Furthermore, we recently reported that lack of *Darc* expression reduced post-fracture inflammation in mice [5].

Darc is mainly expressed in erythrocytes and endothelial cells that are known for their importance in inflammation and wound healing. A study by Pruenster et al., [6] showed that *DARC* plays an important role in chemokine transcytosis through vascular endothelial cells (VEC) to regulate transendothelial migration of monocytes. Therefore, based on the predicted role of *DARC* in the transmigration of monocytes across VEC, and our previously published data on the role of *DARC* in regulating bone resorption and inflammation [4 and 5], we predicted that *DARC would* play a key role in mediating the effects of inflammatory chemokines on the control of transmigration of osteoclast precursors from vascular endothelium to the site of inflammation in bone.

Bacterial lipopolysaccharide (LPS) is known to induce the synthesis of the pro-inflammatory chemokines that bind to *DARC*, such as monocyte chemoattractant protein 1 and RANTES [7]. These two chemokines play important roles in osteoclastogenic leukocyte chemoattraction and bone loss [16, 18]. Thus, in the present study, we have examined the role of *DARC*-chemokine interaction in regulating the recruitment of osteoclast precursors. We induced an inflammatory response via local administration of LPS. Our results showed that *DARC*-chemokine interaction plays an important role in mediating LPS effect on the recruitment of osteoclast progenitors to the inflammatory site.

Materials and Methods

Animal models

All animal experiments were approved by Institutional Animal Care and Use Committee at the VA Loma Linda Healthcare System, and were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. C57BL/6J wild type and *Darc*-knockout (KO) mice were used in the present study to determine the role of *DARC* on osteoclast precursor recruitment in response to local inflammation. *Darc*-KO and control wild type (WT) mice were provided by Dr. A.

Chaudhuri (Laboratory of Cell Biology, New York Blood Center, New York, NY, USA). The animals used in the present study were generated and bred as previously described by Luo et al., [8] and Edderkaoui et al., [4].

Mice of 10–12 weeks old received a single 200 μg LPS (from *Escherichia coli* 055:B5; Sigma-Aldrich Corp. St. Louis, MO 63103, USA) subcutaneous injection on the top of calvaria at the midpoint between the two pinnae. Control animals received phosphate-buffered saline solution (PBS). Animals were then sacrificed at three different time points to evaluate inflammatory cell recruitment and bone resorption markers at the PBS and LPS treated calvaria.

Cell culture assays

To assess the involvement of *DARC* on endothelial cell response to LPS challenge, we used a mouse SVEC4-10 cell line (ATCC, Manassas, VA 20110, USA; catalog No. CRL-2181), an endothelial cell line derived from mouse axillary lymph node vessels. Cells were plated in 6-well plates for 2 days and were grown in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Inc., Flowery Branch, GA30542, USA; Catalog No. S11150), 50 U/ml penicillin, and 50 mg/ml streptomycin (Gibco/Thermo Fisher Scientific, Grand Island, NY 14072, USA; Catalog No. 15140-122). Upon reaching 50–70% confluency, endothelial cells were treated with either 10 μg/ml LPS, or PBS, in DMEM supplemented with 0.5% BSA for 6 and 24 hours (hrs). For the 24-hour assay, either goat polyclonal anti-*DARC* antibody (Santa Cruz Biotechnology Inc. Paso Robles, CA 93446, USA) or IgG control was added to LPS treated cells to evaluate the involvement of *DARC* on the response to LPS challenge.

RNA extraction and Real-Time PCR

SVEC cells were harvested at 6 hrs and 24 hrs after treatment with LPS or PBS and RNA isolated following the protocol provided with the RNeasy mini kit (Qiagen Inc., Valencia, CA 91355, USA; Catalog No. 74104). Mice treated with LPS/PBS were sacrificed at 5 hrs and 24 hrs post LPS injection. Calvarial bone covering the injected area (6–10 mm diameter) was dissected out for RNA extraction using Trizol as per manufacturer's instructions (Life Technology Company; Ref# 15596018). Relative differences in mRNA expression between the groups were measured by real time-PCR using specific primers as previously described [5]. Briefly, Reverse transcription was performed with MMLV Reverse Transcriptase (Promega, San Luis Obispo, CA, USA). Real-time PCR was performed using the SYBR Green master mix (Applied Biosystems, Foster City, CA, USA) with gene-specific primers (Integrated DNA Technologies, Coralville, IA, USA). Changes in gene expression were determined by subtracting the Ct (threshold cycle) of target gene from the Ct value of the housekeeping gene; peptidylprolyl isomerase A (Ppia) (ΔCt = Ct of target gene – Ct of Ppia). Mean ΔCt of replicates was then used to calculate the difference in cycle thresholds between groups (ΔΔCt). Then, the fold-change was calculated as $2^{-\Delta\Delta Ct}$. The genes examined were as follows: interleukin (*Il*)-*6*, tumor necrosis factor α (*Tnfa*), monocyte chemotactic protein-1 (*Mcp-1*), Regulated on Activation, Normal T Expressed and Secreted (*Rantes*) also called *Ccl5*, Cd14, chemokine receptor (Ccr)1, Ccr2, Ccr5 and Darc.

Immunohistochemistry and TRAP staining

Twenty-four and 72 hours post LPS or PBS injections, mice were sacrificed and the entire calvarial bone was dissected and fixed in 10% formalin, then decalcified using EDTA. The anterior half of the frontal bone and the interparietal as well as the occipital bone was trimmed off. The remaining calvarial bone was dehydrated in a graded series of alcohols, embedded in paraffin and each sample sectioned at 5 μm thickness as described by Bancroft [9] and Li et al., [10]. Immunohistochemistry was performed using rat anti-mouse F4/80 (ABD Serotec, A Division of MorphoSys, Raleigh, NC 27609, USA) as previously described [5], and cells were counterstained with hematoxylin [10]. Antigen detection was performed using DAB chromogenic staining (IHC WORLD, LLC, Ellicott City, MD 21042, USA; Catalog No. IW-1600B). Tartrate resistant acid phosphatase (Trap) staining was performed as previously described [10]. Using a 20× microscope objective, 3–4 randomly selected fields within the sutures were analyzed for each section. Reproducibility in multiple sections from the same site was checked through the analysis of duplicates by two blinded independent observers. For F4/80, digital images were captured and analyzed using Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). Data are expressed as the percentage of brown stained area/total field area and are presented as fold change between LPS treated mice and PBS treated WT mice. TRAP stained surfaces were measured using OsteoMeasure software (Osteometrics Inc. GA, USA), the data are expressed as TRAP stained surface/bone surface [10] and are presented as percentage of PBS treated samples from WT mice.

Statistical analysis

For gene expression profiling, data were normalized relative to the housekeeping gene; peptidylprolyl isomerase A (Ppia) and data expressed as a fold change relative to WT samples treated with PBS ± standard error of mean (SEM) as previously described [5].

Histology data were expressed as the mean ± SEM and were reported as percentage of control PBS treated samples. Mean differences were compared and significant differences between groups from all the data were determined by Student's t-test. Differences were considered significant at values of $p \leq 0.05$.

Results

In order to examine the role of DARC-chemokine interaction on local inflammation, we first evaluated the expression level of the chemokines that bind to DARC, as well as the expression of *Darc* gene and other chemokine receptors, in response to LPS treatment *in vitro* and *in vivo*. Treatment of SVEC mouse endothelial cells with LPS increased mRNA expression of two major pro-inflammatory cytokines; *Il-6* and *Tnfa*, as compared to PBS-treated controls (Fig. 1.A). In response to a 6 hr treatment with LPS, mRNA levels for *Mcp-1* and *Rantes*, two inflammatory chemokines known to bind to DARC, were increased by 12 and 3-fold respectively (Fig. 1B.). Evaluation of the expression levels of chemokine receptors in SVEC cells in response to LPS treatment revealed a 23- and 15-fold increase, respectively, in *Ccr5* and *Darc* mRNA levels as compared to cells treated with vehicle alone (Fig. 1C). While the expression level of *Ccr2* remained unchanged, *Ccr1* expression was

increased slightly in response to 6 hrs LPS treatment in SVEC cells (Fig. 1C). After a 24 hr treatment with LPS treatment, the levels of *Rantes* and *Mcp-1* mRNA remained elevated (Fig. 2A) while those of the chemokine receptors, *Ccr5* and *Darc* returned to the levels observed prior to treatment (Fig. 2C).

To determine if *DARC* is involved in mediating changes in the expression levels of chemokines and their receptors in response to LPS treatment, we measured the levels of *Il-6*, *Rantes*, *Mcp-1* and *Ccr5* mRNAs in response to a 24 hr LPS treatment, in the presence of either neutralizing antibody to *DARC* or control IgG. We found significant reductions in mRNA levels for *Il-6* and *Mcp-1*, but not *Rantes*, in cells treated with LPS and *DARC*-Ab, as compared to cells treated with LPS and control IgG (Fig. 2). However, no significant difference in mRNA expression of either *Ccr5* or *Darc* was observed in cells treated with LPS and either *DARC*-Ab or control IgG (Fig. 2).

To determine the role of *DARC* in mediating LPS effects on the expression levels of mediators of inflammatory response *in vivo*, we measured mRNA levels of genes encoding inflammatory cytokines, chemokines and their receptors after local LPS treatment in a mouse calvaria model. As expected, mRNA levels of the two major pro-inflammatory cytokines, *Il-6* and *Tnfa* were significantly enhanced in LPS treated animals as compared to PBS treated animals (Fig. 3A). While the levels of *Mcp-1* and *Rantes* mRNAs, as well as mRNAs for the chemokine receptors *Darc* and *Ccr5* were significantly increased in calvaria treated with LPS compared to PBS treated calvaria (Fig. 3B and 3C), the *Ccr2* mRNA levels were significantly reduced, and there was no significant change in *Ccr1* mRNA levels at 5 hrs post LPS injection (Fig. 3C). Among the four chemokine receptors analyzed in this study, *Darc* gene showed the greatest change in mRNA expression at 5 hrs post LPS injection (Fig. 3C) suggesting that *DARC* plays an important role in local inflammatory response induced by LPS injection.

To test the involvement of *DARC* in response to LPS challenge, we compared mRNA expression of the major inflammatory cytokines, *Tnfa* and *Il-6*, as well as *Cd14*, a marker of macrophages and the most important endotoxin receptor [11 and 12] in *Darc*-KO and WT mice, 24 hrs post-LPS injection. The mRNA levels of all three genes were increased in LPS treated animals compared to PBS treated animals (Fig. 4). However, the magnitude of increase was less in *Darc*-KO as compared to WT mice (Fig. 4). This suggests that the lack of *Darc* expression in *Darc*-KO mice led to a reduction in the recruitment of inflammatory cells to the inflamed area, reducing the inflammatory response in *Darc*-KO mice compared to WT mice. To test this prediction, we quantified the macrophage population within the sutures of calvaria bone, using antibody against F4/80, a specific cell-surface marker for murine macrophages [13 and 14]. At one-day post LPS/PBS injections, the number of F4/80 positive cells, was significantly increased in LPS treated animals compared to controls for both lines of mice. In contrast, in KO mice there were significantly fewer F4/80 positive cells as compared to WT mice (Fig. 5A and 5B). Consistent with the reduced recruitment of macrophage precursors in the LPS treated *Darc*-KO mice; we found significantly fewer TRAP positive osteoclasts in response to LPS treatment in *Darc*-KO mice as compared to WT mice (Fig. 6).

Discussion

Inflammatory reactions observed after bacterial infections have been shown to be powerful activators of monocytes and macrophages, and potent inducers of pro-inflammatory cytokines and chemokines known to play major role in bone loss. However, the molecular pathways involved in the recruitment of osteoclast precursors in response to inflammation challenge are still not well understood. Since *DARC* binds to a number of the pro-inflammatory chemokines known to play a major role in inflammatory cell migration as well as bone resorption, we posited that it might also play a critical role in chemokine-induced osteoclast precursor recruitment that leads to bone resorption.

Since *DARC* is mainly expressed in endothelial cells, we first evaluated the response of *DARC* to bacterial endotoxin (LPS) challenge in endothelial cells. Our results showed that treatment of the murine endothelial cell line SVEC10-4 with LPS resulted in a significant increase in expression of *Darc* and *Ccr5,* as well as the *Mcp-1* and *Rantes* mRNAs by 6 hrs. Both *MCP-1* and *RANTES* bind to *DARC* [15] and to their specific receptors; *CCR2* and *CCR5*, respectively. Interestingly, our results also showed that the expression of *Ccr2* mRNA was not affected by treatment with LPS. This suggests that interactions between *MCP-1*/*DARC* and *RANTES*/*CCR5* are involved in the response to LPS challenge.

When *DARC* function was blocked with anti-*DARC* antibody in LPS treated cells, a reduction in the expression of *Il-6* and *Mcp-1* mRNAs was observed as compared to vehicle treated cells, suggesting that alteration of *MCP-1* binding to *DARC* mitigates inflammation. This finding strengthens the notion *that DARC and MCP-1* play important roles in LPS induced inflammation. A reduction in the expression of *Mcp-1* and *Il-6* mRNAs was also observed after local LPS injection in calvaria derived from *Darc*-KO mice as compared to WT mice.

Other investigators have reported that *DARC* is responsible for the chemokine transcytosis from the apical to the basal side of the vascular endothelial membrane [6]. Overall, such reports and our gene profiling data are consistent with the hypothesis that neutralizing *DARC* function on chemokine transcytosis with anti-*DARC* antibody reduces chemokine synthesis, which in turn reduces the recruitment of inflammatory cells to the area of LPS injection. To test this hypothesis, the expression of the monocyte marker *Cd14* was evaluated post LPS injection. As predicted, the expression of *Cd14* mRNA was found to be reduced in *Darc*-KO mice compared to WT mice. Furthermore, immunohistochemical experiments revealed a significant reduction in the number of F4/80 positive cells and Trap-positive osteoclasts post LPS injection in KO mice as compared to WT mice, suggesting an important role of *DARC* - *MCP-1* interaction on osteoclast precursor recruitment in response to the local inflammation induced by LPS.

Previous studies [16] have shown that a lack of *Ccr5* expression in *Ccr5*-KO mice led to significant reduction, but not total blockade, in F4/80-positive leukocyte migration following bacterial infection. In the present study, we have observed a significant increase in the expression of *Ccr5* mRNA in response to LPS challenge in both SVEC endothelial cells *in vitro,* and mouse calvaria *in vivo*. However, blocking *DARC* function in endothelial cells did

not affect *Rantes* mRNA expression, the specific ligand of *CCR5*, but rather resulted in a decrease in *Mcp-1* mRNA expression, the chemokine known to bind to *DARC*, and a reduction in inflammatory cell infiltration to the area where LPS was injected. Taken together, these data suggest that both *DARC* - *MCP-1* and *CCR5* - *RANTES* interactions play an important role in leukocyte migration in response to bacterially induced inflammation.

One of the key regulators of migration and infiltration of monocytes and macrophages is *MCP-1* [17]. *MCP-1* is also associated with various inflammatory diseases related to bone loss [18, 19]. In *Mcp-1*-KO mice, it has been shown that a lack of *Mcp-1* mRNA expression leads to increased bone mass and a decrease in serum collagen type I fragments (CTX-1), and TRACP 5b [20]. In this study, we have shown that the lack of *Darc* expression leads to a significant reduction in the expression of *Mcp-1* mRNA, and a reduction in the number of Trap-positive osteoclasts per unit bone surface area after LPS challenge as compared to WT mice. Based on these data, we have developed a model as to how LPS treatment induces chemokine expression. Since *DARC* is responsible for the chemokine transcytosis by providing a gradient of chemotactic chemokines to attract osteoclast precursors, our model proposes that blockade of *DARC* function/expression results in reduced *Mcp-1* expression via feedback mechanism, which in turn leads to a reduction in the recruitment of osteoclast precursors to the site of bacterial infection.

Conclusions

The results of this study showed reduced macrophage recruitment to LPS treated bone of *Darc*-KO mice compared to WT mice. This was accompanied by a reduction in the expression of *Il-6* and *Mcp-1* mRNAs in *Darc*-KO mice compared with WT mice injected with LPS. In the present study, *Darc*-KO mice also exhibited reduced TRAP staining on calvaria bone treated with LPS as compared to WT mice, which was likely due to a reduction in the recruitment of inflammatory cells in response to LPS. Taken together, these data suggest that *DARC* - *MCP-1* interaction plays an important role in local bacterial induced inflammation and subsequent bone resorption.

Acknowledgments

The authors thank Dr. Subburaman Mohan for his generosity in providing reagents and equipment to perform this work and for his assistance in writing the manuscript. Many thanks to Dr. Shirwany Najeeb and Dr. Rasmussen Collin for their help in editing the manuscript. Thanks to Ms. Lowen Nancy who did all the histology work from sectioning to staining.

Research reported in this publication was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number R21 AR060452-02. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

All work was performed in facilities provided by the Department of Veterans Affairs in Loma Linda, CA.

References

1. Herman S, Krönke G, Schett G. Molecular mechanisms of inflammatory bone damage: emerging targets for therapy. Trends Mol Med. 2008; 14(6):245–53. Review. [PubMed: 18468489]

2. Lapidot T, Petit I. Current understanding of stem cell mobilization: the roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. Exp Hematol. 2002; 30:973–981. [PubMed: 12225788]
3. Thomas J1, Liu F, Link DC. Mechanisms of mobilization of hematopoietic progenitors with granulocyte colony-stimulating factor. Curr Opin Hematol. 2002; 9:183–189. [PubMed: 11953662]
4. Edderkaoui B, Baylink DJ, Beamer WG, Wergedal JE, Porte R, Chaudhuri A, Mohan S. Identification of mouse Duffy antigen receptor for chemokines (Darc) as a BMD QTL gene. Genome Res. 2007; 17:577–585. [PubMed: 17416748]
5. Rundle CH, Mohan S, Edderkaoui B. Duffy antigen receptor for chemokines regulates post-fracture inflammation. PLoS One. 2013; 8(10):e77362. [PubMed: 24146983]
6. Pruenster M, Mudde L, Bombosi P, Dimitrova S, Zsak M, Middleton J, Richmond A, Graham GJ, Segerer S, Nibbs RJ, Rot A. The Duffy antigen receptor for chemokines transports chemokines and supports their promigratory activity. Nat Immunol. 2009; 10(1):101–8. [PubMed: 19060902]
7. Basso FG, Soares DG, Pansani TN, Turrioni AP, Scheffel DL, de Souza Costa CA, Hebling J. Effect of LPS treatment on the viability and chemokine synthesis by epithelial cells and gingival fibroblasts. Arch Oral Biol. 2015; 60(8):1117–21. [PubMed: 26042620]
8. Luo H, Chaudhuri A, Zbrzezna V, He Y, Pogo AO. Deletion of the murine Duffy gene (Dfy) reveals that the Duffy receptor is functionally redundant. Mol Cell Biol. 2000; 20:3097–3101. [PubMed: 10757794]
9. Bancroft, JD. Enzyme histochemistry and its diagnostic applications. Churchill Livingstone Elsevier; Harcourt: 2002.
10. Li L, Khansari A, Shapira L, Graves DT, Amar S. Contribution of interleukin-11 and prostaglandin(s) in lipopolysaccharide-induced bone resorption *in vivo*. Infect Immun. 2002; 70(7):3915–22. [PubMed: 12065535]
11. Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC. CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science. 1990; 249:1431–1433. [PubMed: 1698311]
12. Ziegler Heitbrock HW, Ulevitch RJ. CD14: cell surface receptor and differentiation marker. Immunol Today. 1993; 14:121–125. [PubMed: 7682078]
13. Austyn JM, Gordon S. F4/80, a monoclonal antibody directed specifically against the mouse macrophage. Eur J Immunol. 1981; 11:805–815. [PubMed: 7308288]
14. Morris L, Graham CF, Gordon S. Macrophages in haemopoietic and other tissues of the developing mouse detected by the monoclonal antibody F4/80. Development. 1991; 112:517–526. [PubMed: 1794320]
15. Szabo MC, Soo KS, Zlotnik A, Schall TJ. Chemokine class differences in binding to the Duffy antigen-erythrocyte chemokine receptor. J Biol Chem. 1995; 270(43):25348–51. [PubMed: 7592697]
16. Ferreira SB Jr1, Repeke CE, Raimundo FM, Nunes IS, Avila-Campos MJ, Ferreira BR, Santana da Silva J, Campanelli AP, Garlet GP. CCR5 mediates pro-osteoclastic and osteoclastogenic leukocyte chemoattraction. J Dent Res. 2011; 90(5):632–7. [PubMed: 21245464]
17. Deshmane SL, Kremlev S, Amini S, Sawaya BE. Monocyte chemoattractant protein-1 (MCP-1): an overview. J Interferon Cytokine Res. 2009; 29:313–326. [PubMed: 19441883]
18. Jiang X, Sato T, Yao Z, Keeney M, Pajarinen J, Lin TH, Loi F, Egashira K, Goodman S, Yang F. Local delivery of mutant CCL2 protein-reduced orthopaedic implant wear particle-induced osteolysis and inflammation in vivo. J Orthop Res. 2016; 34(1):58–64. Epub 2015 Jul 29. DOI: 10.1002/jor.22977 [PubMed: 26174978]
19. Hofmann SR, Kubasch AS, Range U, Laass MW, Morbach H, Girschick HJ, Hedrich CM. Serum biomarkers for the diagnosis and monitoring of chronic recurrent multifocal osteomyelitis (CRMO). Rheumatol Int. 2016 Epub ahead of print.
20. Sul OJ, Ke K, Kim WK, Kim SH, Lee SC, Kim HJ, Kim SY, Suh JH, Choi HS. Absence of MCP-1 leads to elevated bone mass via impaired actin ring formation. J Cell Physiol. 2012; 227(4):1619–27. [PubMed: 21678414]

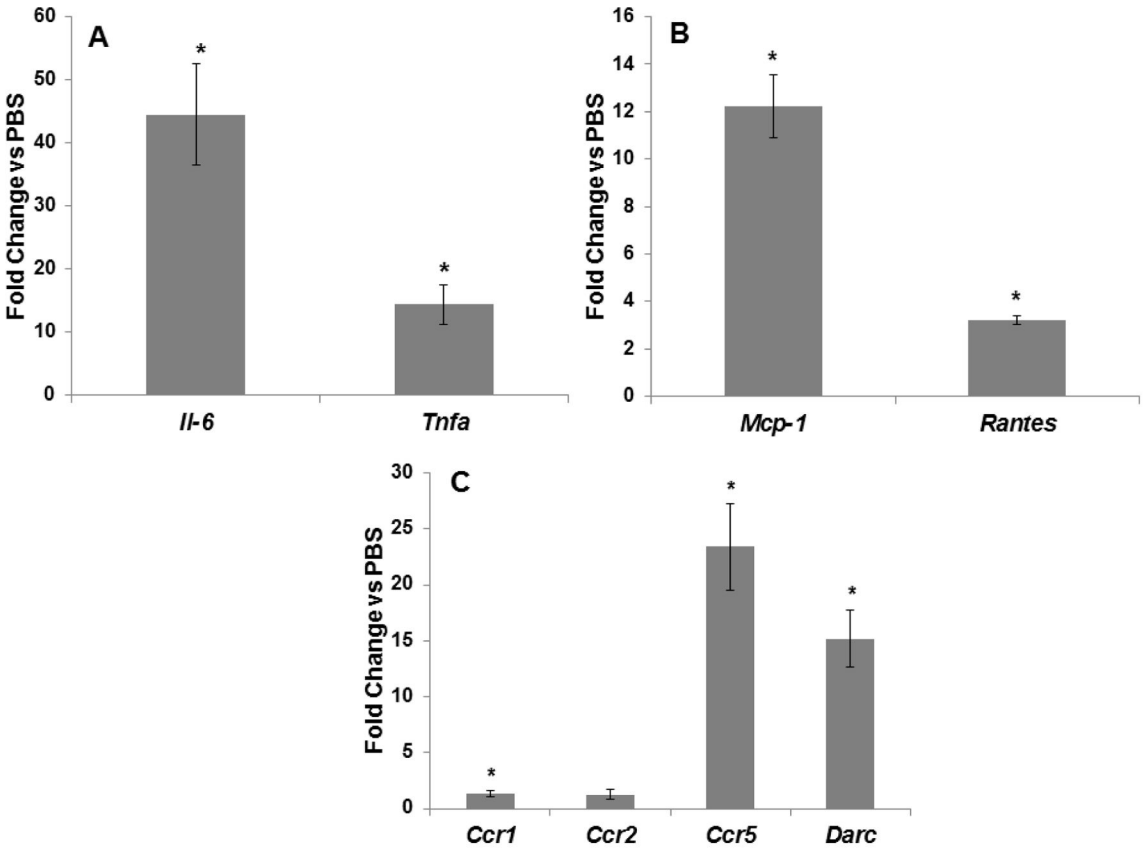

Figure 1. The Changes in the mRNA expression of two major pro-inflammatory cytokines (A), chemokines (B) and four chemokine receptors (C) post-LPS challenge *in vitro*

Mouse endothelial SVE cells were treated with 10 μg/ml LPS or PBS. At 6 hrs time point, the cells were harvested and the change in gene expression was evaluated by real-time PCR. n=3–4. Data are presented as Fold change vs PBS treated samples ± SEM. *p<0.05 vs PBS treated samples

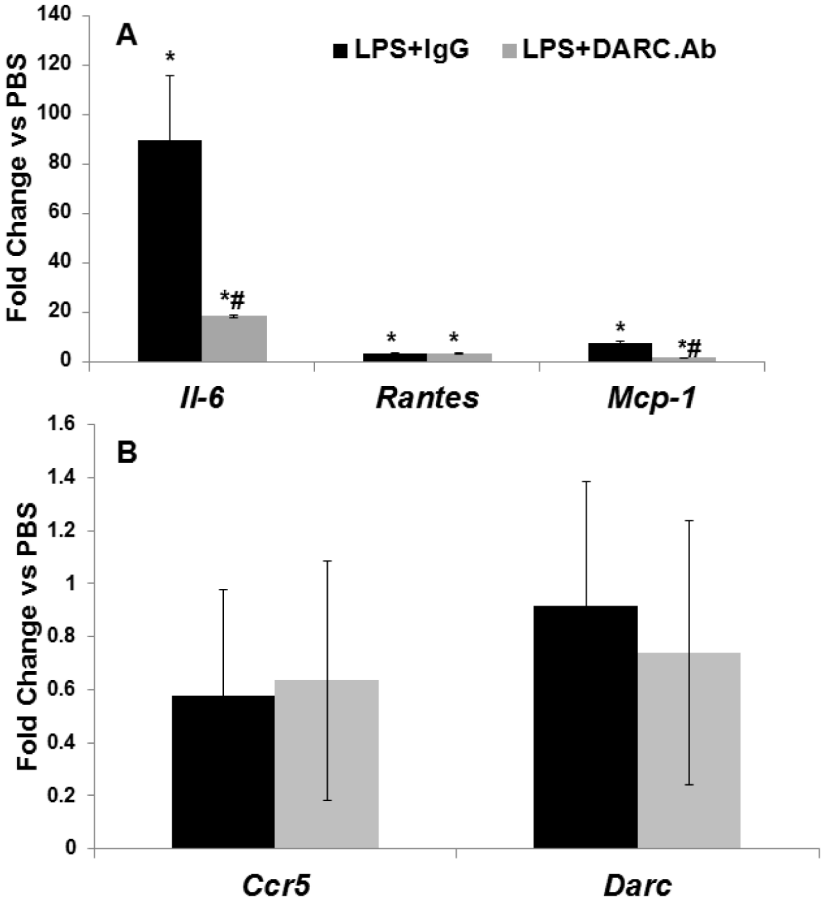

Figure 2. The changes in mRNA expression of the major pro-inflammatory effectors after blockade of *DARC* function, in response to LPS treatment

(A) and (B) Mouse endothelial cells were treated with 10 μg/ml LPS or PBS in the presence of 2 μg/ml anti-*DARC* antibody or control IgG, the cells were harvested at 24 hrs time point, and the change in gene expression was evaluated by real-time PCR. n=4–5. Data are presented as Fold change vs PBS treated samples ± SEM. **p*<0.05 vs PBS treated samples, # LPS. IgG vs LPS. *DARC*-Ab.

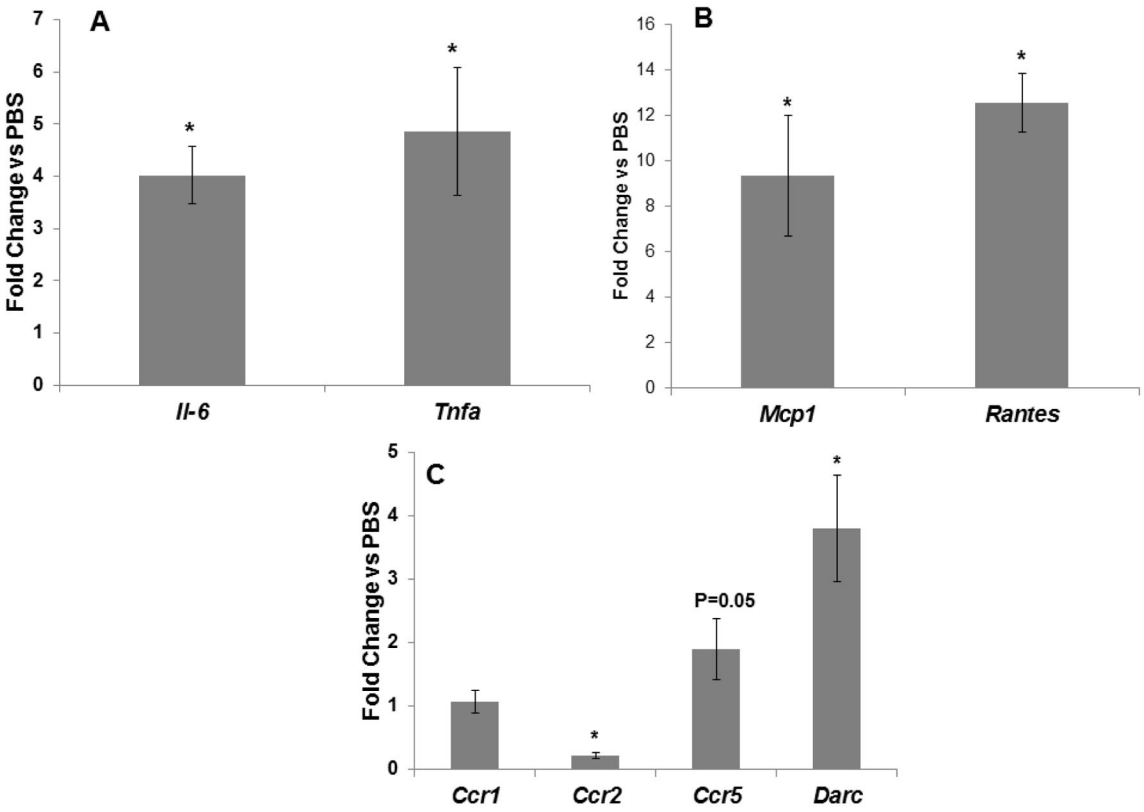

Figure 3. The Changes in the mRNA expression of two major pro-inflammatory cytokines (A), chemokines (B) and four chemokine receptors (C) post-LPS challenge *in vivo*

200 μg LPS or PBS was injected subcutaneously on the top of calvaria bone. Animals were sacrificed at 5 hrs post LPS injection, and gene expression was evaluated by real-time PCR from calvaria. n=5–6.Data are presented as Fold change vs PBS treated WT mice ± SEM. *p<0.05 vs PBS treated samples.

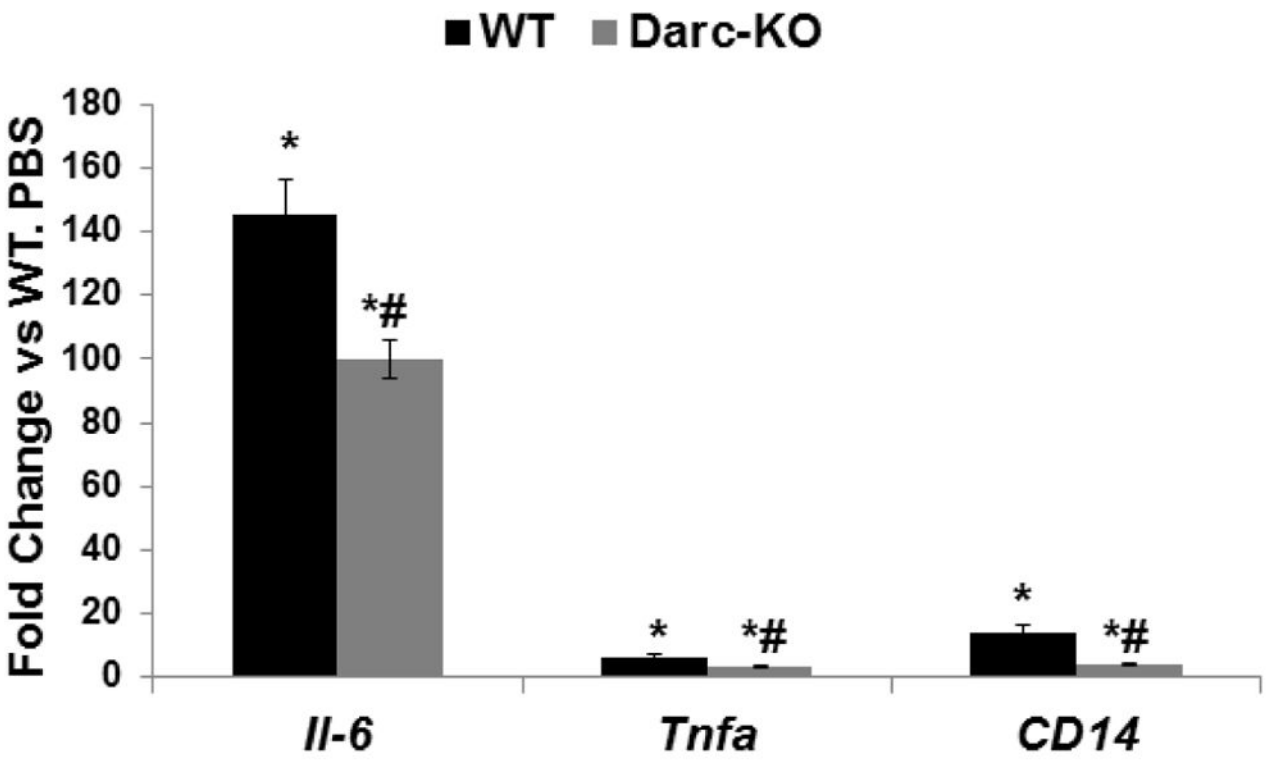

Figure 4. Changes in the mRNA expression levels of monocyte marker *Cd14* and the two pro-inflammatory cytokines in response to LPS challenge, in the presence and in the absence of DARC

200 μg LPS or PBS was injected subcutaneously on the top of calvaria bone. Animals were sacrificed at 24 hrs post LPS/PBS injection, and gene expression was evaluated by real-time PCR. n=6–7. Data are presented as Fold change vs PBS treated WT mice ± SEM. *P<0.05 vs PBS treated animals, # LPS treated WT mice compared to LPS treated KO mice.

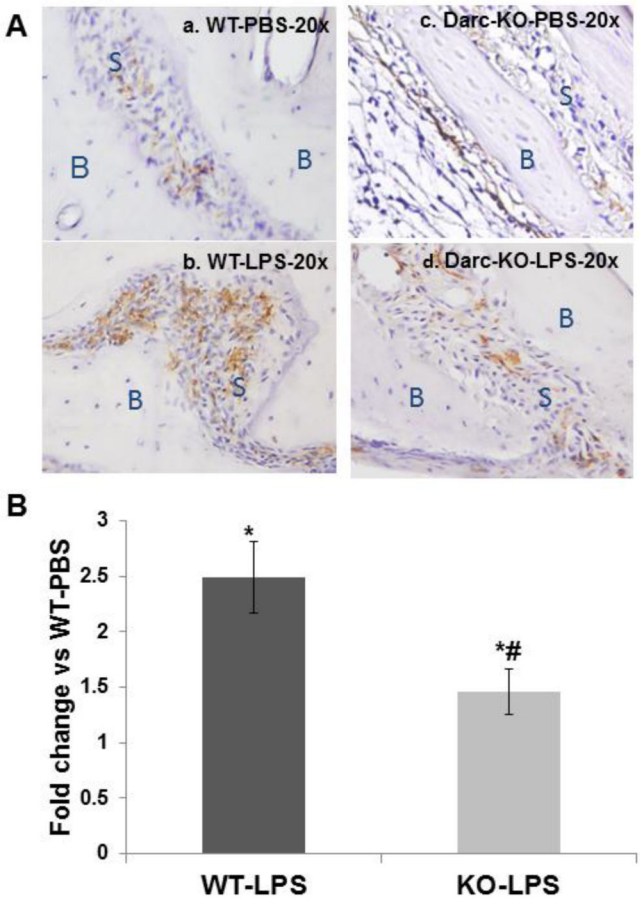

Figure 5. Evaluation of the infiltration of macrophages to the inflamed bone in *Darc*-KO and WT mice, 24 hrs post LPS injection

A. Representative images of the macrophage population at the sutures in calvaria bone from PBS (**a, c**) and LPS (**b, d**) treated WT (**a, b**) and *Darc*-KO (**c, d**) mice. Immunohistochemical detection of the macrophage-specific antigen F4/80 (brown color) using DAB chromogen staining. The sections were counterstained with hematoxylin. S. suture, B, bone. **B.** Quantification of the macrophage population at the sutures of calvaria bones. Data are expressed as stained surface/total field area in *Darc*-KO and WT mice and are presented as fold change vs PBS treated WT mice ± SEM. We have examined 7–8 animals/mouse strain. The macrophages were identified by rat anti mouse antibody F4/80. *$p<0.05$ *vs* WT.PBS, #P<0.05 WT-LPS *vs Darc*-KO-LPS.

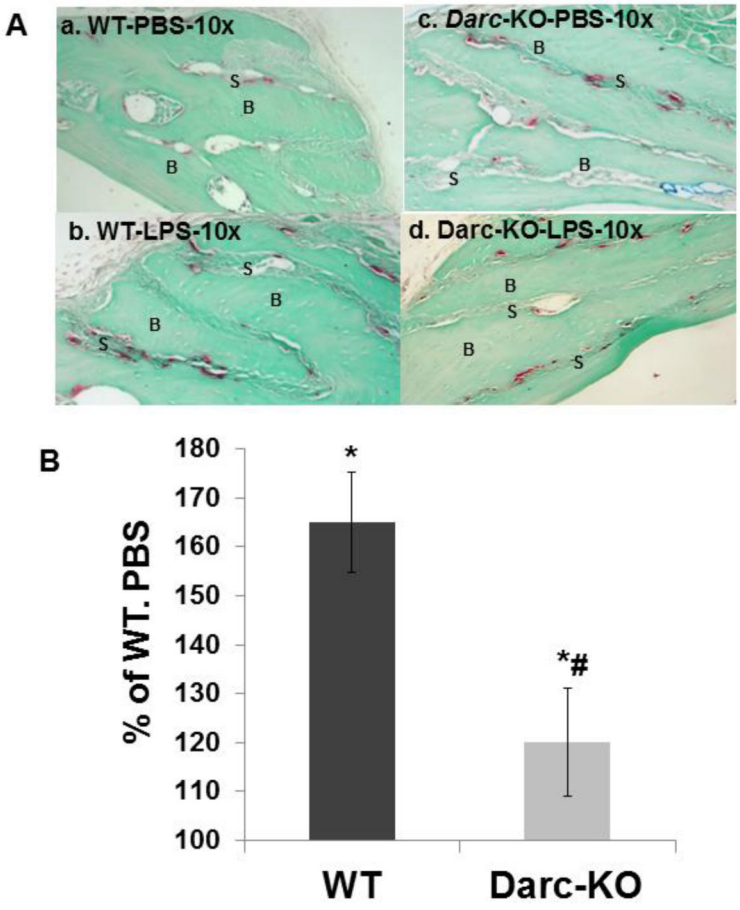

Figure 6. Quantitation of TRAP positive cells on treated calvaria three days post LPS and PBS injections

A. Representative images of Trap stained clavaria bones at the sutures, from PBS (**a**, **c**) and LPS (**b**, **d**) treated WT (**a**, **b**) and *Darc*-KO (**c**, **d**) mice. S. suture, B, bone. **B.** Data are expressed as Trap positive surface by bone surface at the sutures of calvaria bone treated with 200 μg LPS compared to WT mice calvaria treated with PBS and are presented as a percentage of WT mice treated with PBS. n=7–8, **p*<0.05 *vs* WT.PBS, #*p*<0.05 WT-LPS *vs* *Darc*-KO-LPS.