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Brucella abortus–Infected Macrophages Modulate T Lymphocytes to Promote Osteoclastogenesis via IL-17

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The pathogenic mechanisms of bone loss caused by *Brucella* species have not been completely deciphered. Although T lymphocytes (LTs) are considered important to control infection, the mechanism of *Brucella*-induced T-cell responses to immunopathological features is not known. We present *in vitro* and *in vivo* evidence showing that *Brucella abortus*–induced inflammatory response leads to the activation of LTs, which further promote osteoclastogenesis. Pre-activated murine LTs treated with culture supernatant from macrophages infected with *B. abortus* induced bone marrow–derived monocytes (BMMs) to undergo osteoclastogenesis. Furthermore, osteoclastogenesis was mediated by $CD4^+$ T cells. Although *B. abortus*–activated T cells actively secreted the pro-osteoclastogenic cytokines RANKL and IL-17, osteoclastogenesis depended on IL-17, because osteoclast generation induced by *Brucella*-activated T cells was completely abrogated when these cells were cultured with BMMs from IL-17 receptor knockout mice. Neutralization experiments indicated that IL-6, generated by *Brucella* infection, induced the production of pro-osteoclastogenic IL-17 from LTs. By using BMMs from tumor necrosis factor receptor p55 knockout mice, we also demonstrated that IL-17 indirectly induced osteoclastogenesis through the induction of tumor necrosis factor-α from osteoclast precursors. Finally, extensive and widespread osteoclastogenesis was observed in the knee joints of mice injected with *Brucella*-activated T cells. Our results indicate that activated T cells, elicited by *B. abortus*–infected macrophages and influenced by the inflammatory milieu, promote the generation of osteoclasts, leading to bone loss. ***(Am J Pathol 2012, 181: 887–896; http://dx.doi.org/10.1016/j.ajpath.2012.05.029)***

Bone loss has been consistently reported in the three most frequent forms of osteoarticular brucellosis (sacroiliitis, spondylitis, and peripheral arthritis).[1–5] The pathogenic mechanisms of bone loss caused by *Brucella* species have not been completely deciphered. Recently, however, we have described a putative immune mechanism for inflammatory bone loss that may occur in response to infection by *Brucella abortus*. Our results revealed an important contribution of the macrophage in response to infection by *B. abortus* and the resulting induction of osteoclastogenesis.[6]

During chronic inflammatory bone diseases, cellular recruitment also contributes to bone loss. RANKL and pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-1β, and IL-6, have been important for disease progression and bone loss.[7–11] T and B lymphocytes have also contributed to the acceleration of bone resorption.[12–14] Specifically, activated T cells undermine bone homeostasis and induce bone destruction under pathological conditions, such as estrogen deficiency,[15–19] and in inflammatory conditions,[18,19] as they become a significant source of RANKL[18] and TNF-α.[16] Although RANKL has been indicated as the major cytokine that regulates osteoclast differentiation,[20] most of the T-cell cytokines, including interferon (IFN)-γ, IL-4, and IL-10, inhibit osteoclastogenesis.[20] However, the seminal work of Sato et al[21] identi-

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fied a new subset of IL-17–producing T-helper (Th17) cells as the exclusive osteoclastogenic T-cell subset. IL-17 also enhances local inflammation and increases the production of inflammatory cytokines, which further promote osteoclastogenic activity.[20] Thus, the interactions of T cells and osteoclasts have a crucial role in the pathogenesis of inflammatory bone loss.

Interestingly, chronic brucellar lesions of bones and joints characteristically reveal, at the histological level, an inflammatory response with varying degrees of bone destruction and the presence of infiltrating lymphocytes.[3] Also, synovial tissue typically reveals a lymphocytic infiltrate.[22] We have recently demonstrated that *Brucella* species can infect and survive within human osteoblasts and that this infection elicits the secretion of pro-inflammatory cytokines and chemokines that might be involved in the osteoarticular manifestations of brucellosis. Such a response was further amplified by subsequent interactions between osteoblasts and monocytes in the face of *B. abortus* infection.[6,23] Thus, it seems conceivable to speculate that these infiltrating T cells, surrounded by the inflammatory microenvironment generated by the bacterium, might activate and promote the generation of osteoclasts, the only cells known to be able to degrade bone. To investigate such a hypothesis, we developed an *in vitro* model in which pre-activated murine T lymphocytes (LTs), influenced by culture supernatants (CSs) of *Brucella*-infected macrophages, were able to drive bone marrow–derived monocytes (BMMs) to undergo osteoclastogenesis. By using this model, we detailed a mechanism by which Th17 T cells increased the production of TNF-α from osteoclast precursors that further promoted osteoclastogenesis.

Materials and Methods

Ethics Statement

All animal procedures were performed according to the rules and standards for the use of laboratory animals of the NIH (Bethesda, MD). Animal experiments were approved by the Ethical Committee of the Institute for the Study of Humoral Immunity.

Animals

Female IL-17 receptor A (IL-17RA) knockout (KO; kindly provided by Amgen Inc., Munich, Germany), TNF receptor (TNFR) p55 KO mice,[24] and wild-type littermate C57BL/6 mice (provided by the University of La Plata, La Plata, Argentina), aged 6 to 8 weeks, were housed in groups of five animals, under a controlled temperature (22°C ± 2°C) and artificial light under a 12-hour cycle period. Mice were kept under specific pathogen-free conditions in positive-pressure cabinets and provided with sterile food and water ad libitum.

Bacteria

Brucella abortus S2308 was grown overnight in 10 mL of tryptic soy broth (Merck, Buenos Aires, Argentina) with constant agitation at 37°C. Bacteria were harvested by centrifugation for 15 minutes at 6000 × *g* at 4°C and washed twice in 10 mL of PBS. The numbers of bacteria in stationary-phase cultures were determined by comparing the optical densities at 600 nm with a standard curve obtained in our laboratory.[6]

Cells and Media

All experiments were performed at 37°C in a 5% CO_2 atmosphere in α-minimum essential medium supplemented with 2 mmol/L L-glutamine, 10% heat-inactivated fetal bovine serum (Gibco-BRL, Life Technologies, Grand Island, NY), 100 U/mL of penicillin, and 100 μg/mL of streptomycin (complete medium). Thioglycolate-elicited peritoneal macrophages were isolated, as previously described,[25] from wild-type mice. LTs were obtained from murine spleens using a $CD3^+$, $CD4^+$, or $CD8^+$ LT-negative isolation kit (BD Biosciences, San Diego, CA), following the manufacturer's instructions. The purity of the isolated $CD3^+$, $CD4^+$, and $CD8^+$ populations was later confirmed by flow cytometry and was >98%, >90%, and >78%, respectively. The viability of cells was >95%, as measured by a trypan blue exclusion test.

Infection

Murine peritoneal macrophages were cultured in 24-well plates at a density of 5×10^5 cells per well in complete medium without the addition of antibiotics. Cells were infected with *B. abortus* at different multiplicities of infection (MOIs) for 2 hours in medium containing no antibiotics. Cells were extensively washed to remove uninternalized bacteria, and infection was maintained for an additional 24 hours in the presence of antibiotics (100 μg/mL gentamicin and 50 μg/mL streptomycin) to kill remaining extracellular bacteria. After a 24-hour culture, CSs were harvested, sterilized by filtration through a 0.22-μm nitrocellulose filter, and stored at −70°C until used for stimulation of LT or cytokine determination. To monitor *Brucella* intracellular replication, cells infected in parallel were washed and lysed at several intervals after infection with 0.1% (v/v) Triton X-100. The number of intracellular viable bacteria (in colony-forming units per well) was determined by plating serial dilutions onto tryptic soy broth agar plates. The number of bacteria internalized after 24 hours of infection into peritoneal macrophages was as follows: MOI 25, 9500 ± 707 bacteria; MOI 50, 38,500 ± 4949; and MOI 100, 72,500 ± 3535.

LT Activation

Purified LTs (1×10^6 cells/0.5 mL per well) were initially stimulated with 5 μg/mL of plate-bound anti-CD3ε monoclonal antibody (BD Biosciences) for 24 hours. Then, cells were washed and further stimulated with 0.2 mL of CS from *B. abortus*–infected or noninfected peritoneal macrophages for an additional 24 hours. Afterwards, T cells were washed and added into BMM cultures or were cultured in fresh media for an additional 24 hours to

produce LT-derived CS (LTCS) to be used to stimulate BMM cultures or to determine cytokine secretion.

Osteoclast Formation Assay

BMMs were induced to undergo osteoclastogenesis, as previously described,[6] with slight modifications. Briefly, bone marrow cells from C57BL/6 wild-type, TNFRp55, or IL-17R KO mice were cultured in complete medium containing 5 ng/mL of recombinant murine macrophage-specific colony-stimulating factor (M-CSF; R&D Systems, Minneapolis, MN) for 12 hours in 24-well plates. Nonadherent cells were harvested and cultured with 30 ng/mL of M-CSF in 24-well plates for an additional 24 hours. Nonadherent cells were washed out, and adherent cells were collected and used as BMMs. The BMMs (5×10^4 cells/0.5 mL per well) were seeded onto glass coverslips in 24-well plates for 6 days and cultured in complete medium containing 30 ng/mL of M-CSF, together with 1×10^6 stimulated T cells or LTCS (0.2 mL). As positive controls of osteoclast formation, BMM cultures received 50 ng/mL of RANKL (R&D Systems). On day 3, the culture media and all reagents were replaced. To identify osteoclasts, cells were fixed in 4% paraformaldehyde and stained for tartrate-resistant acid phosphatase (TRAP; Sigma Aldrich, Buenos Aires, Argentina). In addition, vitronectin receptor (CD51) and calcitonin receptor (CR) expression was determined by fluorescent microscopy using a phosphatidylethanolamine-labeled anti-mouse CD51 or a fluorescein isothiocyanate–labeled anti-mouse CR (BioLegend, San Diego). TRAP-CD51– or CR-positive multinucleated (more than three nuclei) cells were defined as osteoclasts, and their number was determined by microscopic counts.

Determination of MMP-9 Activity

Matrix metalloproteinase (MMP)-9 activity in BMM cultures was determined by zymography, as previously described.[26,27] Briefly, CSs from differentiated osteoclasts were mixed with 5 μL of five times loading buffer [0.25 mol/L Tris (pH 6.8), 50% glycerol, 5% SDS, and bromophenol blue crystals] and loaded onto 10% SDS-PAGE gels containing 1 mg/mL gelatin (Sigma Aldrich). After electrophoresis, gels were washed with a solution containing 50 mmol/L Tris-HCl (pH 7.5) and 2.5% Triton X-100 (buffer A) for 30 minutes and with buffer A added with 5 mmol/L $CaCl_2$ and 1 mol/L $ZnCl_2$ for 30 minutes and were later incubated with buffer A with an additional 10 mmol/L $CaCl_2$ and 200 mmol/L NaCl for 48 hours at 37°C. This denaturation/renaturation step promotes MMP activity without the proteolytic cleavage of pro-MMP-9. Gelatin activity was visualized by the staining of the gels with 0.5% Coomassie Blue. Unstained bands indicated the presence of gelatinase activity, and their positions indicated the molecular weights of the enzymes involved.

Cytokines and RANKL Assays

Concentrations of TNF-α, IFN-γ, IL-17, and IL-10 (BD Pharmingen, San Diego) and RANKL (R&D Systems) in CS and LTCS were determined by using commercially available enzyme-linked immunosorbent assay (ELISA) kits.

Pit Formation Assay

BMMs (2×10^4 cells/0.25 mL per well) were plated on dentine disks (BD BioCoat Osteologic, San Diego) in 96-well culture dishes and cultured in complete medium containing stimulated T cells with 30 ng/mL of M-CSF for 6 days. Medium and all reagents were replaced every day to avoid acidification. After culture with cells, dentine disks were washed with 1 mol/L NH_4OH to remove adherent cells. After rinsing with water, dentine disks were visualized by light microscopy to determine and enumerate resorption lacunae.

Neutralization Experiments

Neutralization experiments were performed using neutralizing antibodies to IL-6 or IL-17A (BD Biosciences) or osteoprotegerin (OPG), RANKL's decoy receptor. The appropriate isotype control was used in each case. CS from *Brucella*-infected cells or LTCS was pre-incubated with the corresponding antibody (or isotype control) or decoy receptor for 1 hour at 37°C before being used to stimulate other cell types.

Evaluation of Osteoclast Formation in an in Vivo *Model*

C57BL/6 mice, aged 6 to 8 weeks, were anesthetized with ketamine chlorhydrate (150 mg/kg) and xylazine (15 mg/kg) and then injected intra-articularly in the knee joint with 1×10^6 stimulated T cells, *Escherichia coli* lipopolysaccharide (LPS; 500 ng) as a positive control, or vehicle (PBS). Mice were sacrificed at 5 days after administration, and whole knee joints were removed. Knee joints were fixed for 4 days using 4% paraformaldehyde, followed by decalcification in 10% EDTA in 1 mmol/L Tris-HCl (pH 7.4) for up to 2 weeks at 4°C. Decalcified specimens were processed for paraffin embedding.[28] Histological sections of the proximal tibiae (7 μm thick) were stained for TRAP, as described. TRAP-positive multinucleated (more than three nuclei) cells were defined as osteoclasts.

Statistical Analysis

Statistical analysis was performed with one-way analysis of variance, followed by a post hoc Tukey test, using GraphPad Prism 4.0 software (San Diego, CA). Data are represented as mean ± SD.

Results

CSs from B. abortus*–Infected Macrophages Activate T Cells to Induce BMM-Derived Osteoclastogenesis*

We investigated whether activation of T cells within the inflammatory milieu generated by *B. abortus* promoted

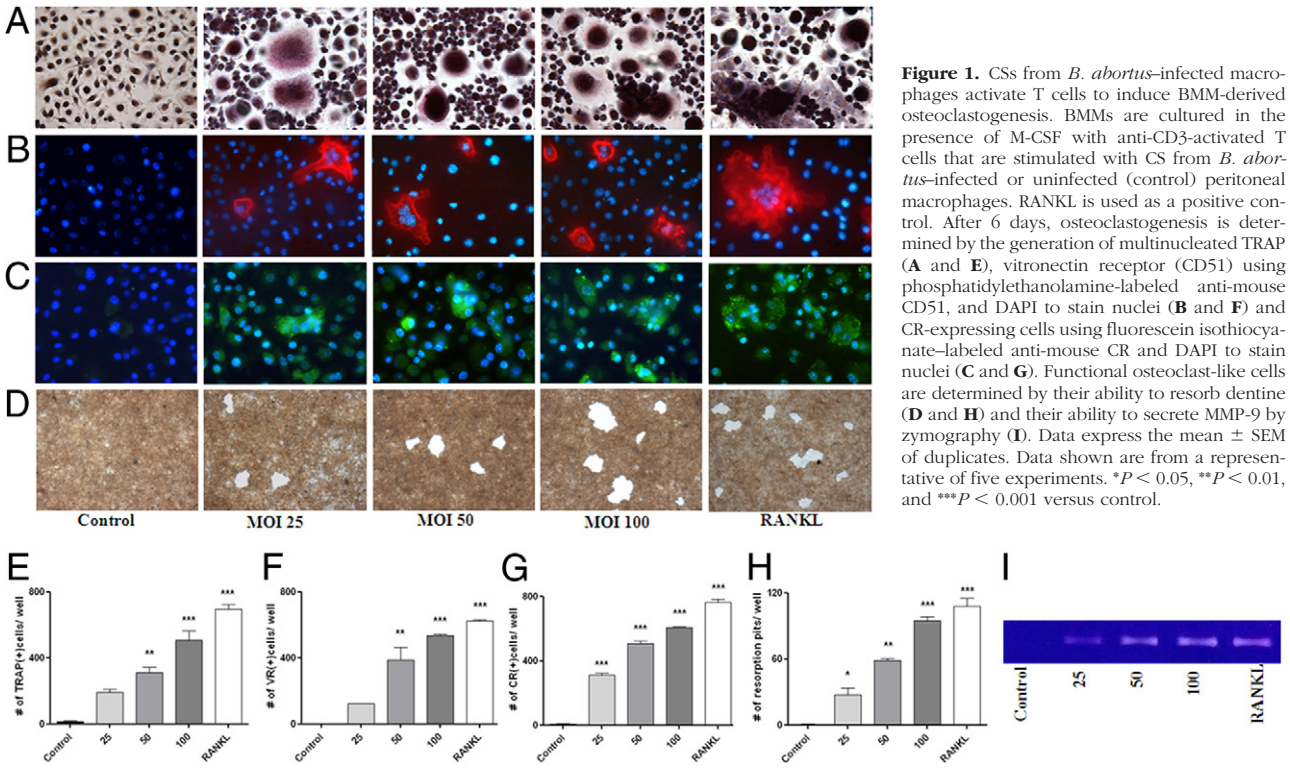

Figure 1. CSs from *B. abortus*–infected macrophages activate T cells to induce BMM-derived osteoclastogenesis. BMMs are cultured in the presence of M-CSF with anti-CD3-activated T cells that are stimulated with CS from *B. abortus*–infected or uninfected (control) peritoneal macrophages. RANKL is used as a positive control. After 6 days, osteoclastogenesis is determined by the generation of multinucleated TRAP (**A** and **E**), vitronectin receptor (CD51) using phosphatidylethanolamine-labeled anti-mouse CD51, and DAPI to stain nuclei (**B** and **F**) and CR-expressing cells using fluorescein isothiocyanate–labeled anti-mouse CR and DAPI to stain nuclei (**C** and **G**). Functional osteoclast-like cells are determined by their ability to resorb dentine (**D** and **H**) and their ability to secrete MMP-9 by zymography (**I**). Data express the mean ± SEM of duplicates. Data shown are from a representative of five experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control.

the generation of osteoclasts from BMMs. To test our hypothesis, BMMs were cultured in the presence of M-CSF with activated T cells that were stimulated with CS from *B. abortus*–infected peritoneal macrophages and, *ex vivo*, osteoclastogenesis was determined by the generation of multinucleated TRAP-, CR-, or vitronectin receptor–expressing cells. RANKL was used as a positive control. The formation of osteoclast-like cells was significantly ($P < 0.01$) induced by T cells that were activated with CS from *B. abortus*–infected macrophages but not by those of uninfected macrophages (Figure 1, A–C and E–G). Because our hypothesis was that *B. abortus* infection might generate a microenvironment that would promote the generation of osteoclasts, leading to bone loss, we also assessed the functional activity of *Brucella*-induced osteoclast-like cells by their ability to resorb dentine. BMMs treated with *B. abortus*–activated LT induced significant ($P < 0.05$) dentine resorption. On the contrary, LT cultured with CS from uninfected macrophages did not induce significant dentine resorption (Figure 1, D and H). The functional activity of osteoclasts was also demonstrated by their ability to secrete MMP-9, an enzyme specifically secreted by functional osteoclasts that is able to degrade organic matrix (Figure 1I). Activated T cells were unable to secrete MMP-9 (data not shown). For every marker investigated, the magnitude of the osteoclastogenesis induced by activated LT was directly related to the MOI used to infect macrophages. CSs derived from activated T cells (LTCS) also induced osteoclastogenesis (data not shown). Taken together, these results indicated that *Brucella*-activated LT can promote functional osteoclast formation from BMMs.

Osteoclastogenesis Induced by Brucella*-Activated T Cells Is Mediated by CD4$^+$ T Cells*

Experiments were then conducted to evaluate the contribution of CD4$^+$ and CD8$^+$ T cells in LT-induced osteoclastogenesis. Purified CD4$^+$ or CD8$^+$ LTs were activated in the presence of CS from *Brucella*-infected macrophages and co-cultured with BMMs in the presence of M-CSF. Osteoclastogenesis was determined by the generation of TRAP-expressing cells with the ability to resorb dentine and secrete MMP-9. RANKL was used as a positive control. Such as the whole LT population, activated CD4$^+$ T cells also induced significant ($P < 0.05$) osteoclast formation from BMMs, as determined by the generation of TRAP-expressing cells that secreted MMP-9 and resorbed dentine. Again, the magnitude of the osteoclastogenesis induced by activated CD4$^+$ T cells was directly related to the MOI used to infect macrophages, whereas the activation of LT in the presence of CS from uninfected macrophages rendered no osteoclastogenesis. On the contrary, under the same experimental conditions, activated CD8$^+$ LTs were unable to induce osteoclast-like cells (Figure 2, A–C). These results indicated that osteoclastogenesis was induced by T cells of the helper lineage.

Brucella*-Activated T Cells Secrete Pro-Osteoclastogenic Cytokines*

Because LTCS induced osteoclastogenesis and T cells produced various pro-osteoclastogenic cytokines,[29] we investigated the cytokines secreted by LT that were treated

with CS from *Brucella*-infected macrophages. The activation of LT with anti-CD3, in the presence of CS from *Brucella*-infected macrophages, resulted in significant ($P < 0.01$) secretion of RANKL and IL-17. Production of these cytokines depended on the MOI used to infect macrophages (Figure 3, A and B). On the contrary, treatment of T cells with anti-CD3ε in the presence of CS from uninfected macrophages resulted in copious production of IFN-γ. This production was significantly ($P < 0.001$) diminished when LTs were treated with CSs that were infected with increasing amounts of *B. abortus* (Figure 3C). TNF-α production was not detected when LTs were treated with CSs from infected or uninfected macrophages (data not shown). Altogether, these results suggested that the effect of T cells on osteoclastogenesis depended on the balance between RANKL/IL-17 and IFN-γ that was driven by CSs from *Brucella*-infected macrophages.

IL-17 Drives Osteoclastogenesis Induced by Brucella*-Activated T Cells*

Both RANKL and IL-17 were the major osteoclastogenic cytokines.[29] Thus, we decided to decipher their role in the osteoclastogenesis induced by *Brucella*-activated T cells. BMMs were cultured with M-CSF and activated T cells that were stimulated with CS from *B. abortus*–infected peritoneal macrophages in the presence of anti-IL-17A antibody or OPG, a RANKL decoy receptor, and osteoclastogenesis was evaluated by the generation of TRAP-expressing cells. When compared with untreated cells (Figure 4A), IL-17A–blocking antibody completely abrogated osteoclastogenesis induced by *Brucella*-activated T cells (Figure 4B); however, the isotype control had no effect (Figure 4C). The neutralizing antibody also

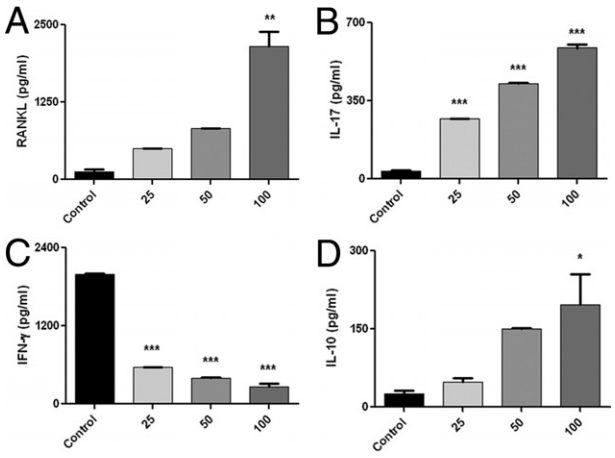

Figure 3. *Brucella*-activated T cells secrete pro-osteoclastogenic cytokines. Anti-CD3-activated T cells that are stimulated with CS from *B. abortus*–infected or uninfected (control) peritoneal macrophages. After 24 hours, RANKL (**A**), IL-17 (**B**), IFN-γ (**C**), and IL-10 (**D**) are determined in CSs by ELISA. Data express the mean ± SEM of duplicates. Data shown are from a representative of five experiments performed. Numbers underneath figures represent the MOI used to infect macrophages. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control.

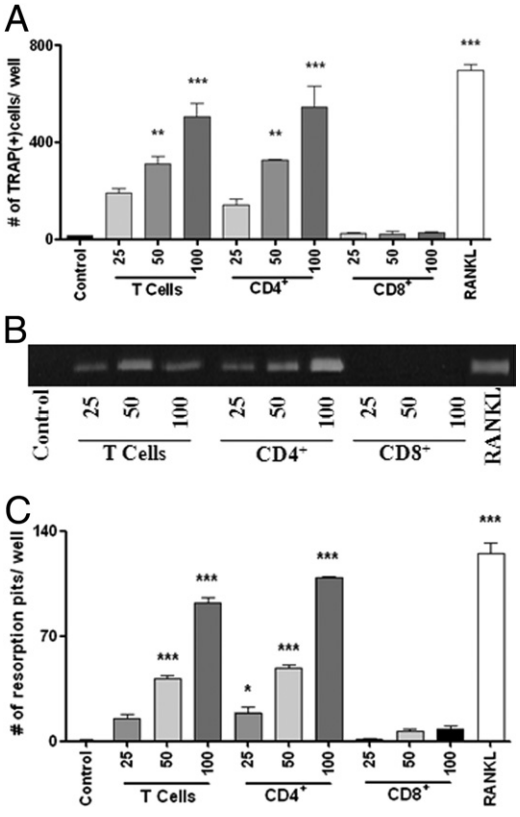

Figure 2. $CD4^+$ T cells mediate osteoclastogenesis induced by *B. abortus*. Unpurified or purified $CD4^+$ or $CD8^+$ LTs are activated in the presence of CS from *Brucella*-infected or uninfected (control) macrophages and co-cultured with BMM in the presence of M-CSF. Osteoclastogenesis is determined by the generation of TRAP-expressing cells (**A**) with the ability to secrete MMP-9 (**B**) and resorb dentin (**C**). RANKL is used as a positive control. Data express the mean ± SEM of duplicates. Data shown are from a representative of five experiments. Numbers underneath figures represent the MOI used to infect macrophages. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control.

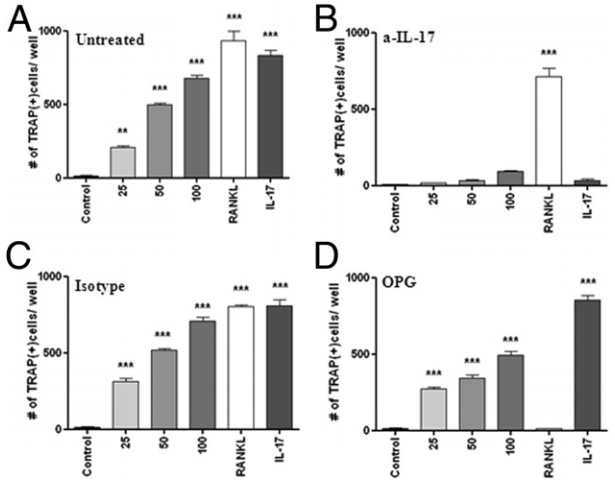

Figure 4. IL-17, not RANKL, drives osteoclastogenesis induced by *Brucella*-activated T cells. BMMs are cultured with M-CSF and activated T cells that are stimulated by CS from *B. abortus*–infected or uninfected (control) peritoneal macrophages. Cultures are left untreated (**A**) or are co-cultured in the presence of anti-IL-17A (a-IL-17; **B**), its respective isotype control (**C**), or OPG (**D**), and osteoclastogenesis is evaluated by the generation of TRAP-expressing cells. RANKL and IL-17A are used as positive controls. Data express the mean ± SEM of duplicates. Data shown are from a representative of three experiments performed. Numbers underneath figures represent the MOI used to infect macrophages. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control.

blocked the generation of TRAP-positive cells induced by recombinant IL-17, whereas it had no effect in RANKL-induced osteoclastogenesis (Figure 4B). On the contrary, OPG only slightly reduced the formation of osteoclast-like cells induced by *Brucella*-activated T cells, whereas abrogated RANKL-induced osteoclastogenesis had no effect on IL-17–driven osteoclastogenesis (Figure 4D). The fact that RANKL (present in LTCS) was unable to induce TRAP-positive cells when IL-17 was blocked was puzzling. Yet, the presence of IL-10, a powerful inhibitor of RANKL/RANK-induced osteoclastogenesis (Figure 3D), would help to explain this phenomenon. Confirming the role of IL-17, osteoclastogenesis induced by *Brucella*-activated T cells was dramatically reduced when these cells were cultured with BMMs from IL-17R KO mice. As expected, BMMs from IL-17R KO mice were unable to become osteoclasts in response to IL-17, whereas RANKL induced TRAP-positive cells in IL-17R KO BMMs (Figure 5A). Altogether, these results indicated that osteoclastogenesis induced by *Brucella*-activated T cells depended on IL-17.

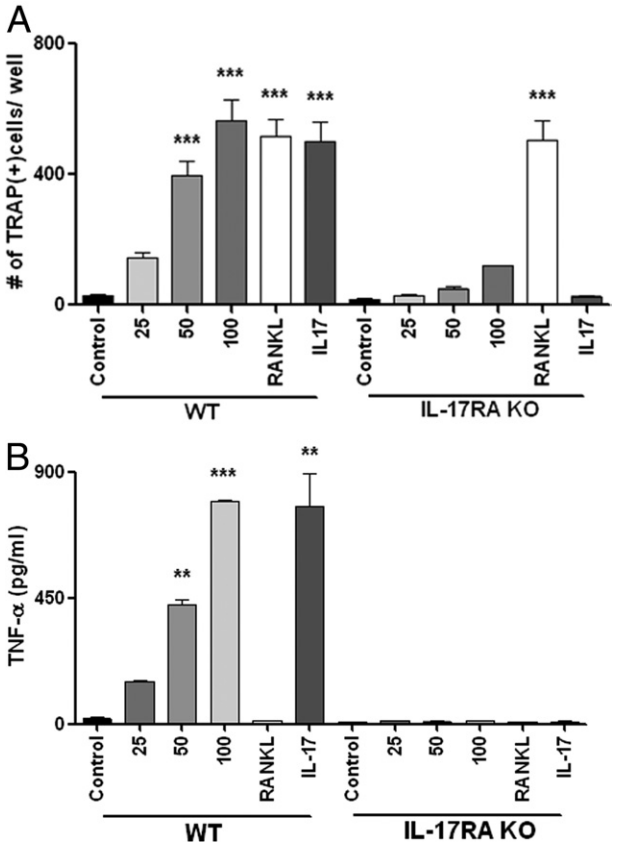

Figure 5. *Brucella*-activated T cells are unable to induce osteoclastogenesis or TNF-α secretion from BMMs from IL-17R KO mice. Wild-type (WT) or IL-17R KO BMMs are cultured with M-CSF and activated T cells that are stimulated by CS from *B. abortus*–infected or uninfected (control) peritoneal macrophages. Osteoclastogenesis is evaluated by the generation of TRAP-expressing cells (**A**), and TNF-α secretion (**B**) is determined in CSs by ELISA. RANKL and IL-17A are used as positive controls. Data express the mean ± SEM of duplicates. Data shown are from a representative of five experiments. Numbers underneath figures represent the MOI used to infect macrophages. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control.

IL-6 Secreted by B. abortus*–Infected Macrophages Determine IL-17 Production by LT and Concomitant Osteoclastogenesis*

We previously demonstrated the ability of *B. abortus* infection to induce the secretion of pro-inflammatory cytokines from macrophages and a variety of other cell types.[6,25,30–36] Among them, IL-6 was abundantly secreted on infection. Because IL-6 was a pivotal cytokine in the induction of Th17 cells,[37] we investigated its role in the production of IL-17 by *Brucella*-activated LT and also in the osteoclastogenesis concomitantly elicited. CSs from *Brucella*-infected macrophages were pre-incubated with anti-IL-6 antibody or its isotype control, used to activate LT. *Brucella* activated LT, and co-cultured with BMMs in the presence of M-CSF, and osteoclastogenesis was determined by the generation of TRAP-positive cells. Neutralization of IL-6 dramatically reduced the production of IL-17 in LTCS and the concomitant osteoclastogenesis induced by LTs that were activated by CS from *Brucella*-infected macrophages at any of the MOIs used to infect macrophages. On the contrary, isotype control had no effect on the production of IL-17 by T cells or induced osteoclastogenesis (Figure 6, A and B). These results indicated that IL-6, generated by *Brucella* infection, induced the production of pro-osteoclastogenic IL-17 from LT.

IL-17 Indirectly Induces BMM Osteoclastogenesis Mainly through the Induction of TNF-α from Osteoclast Precursors

The understanding of osteoimmunological features indicated that IL-17 exerted its osteoclastogenic effects indirectly by chiefly stimulating the expression of RANKL, but also by inducing the pro-inflammatory triad of IL-1β, IL-6, and TNF-α from osteoclast precursors and osteoclast-supporting cells.[20,29] Although RANKL was introduced as the major cytokine that regulated osteoclast differentiation,[20] TNF-α was indicated as the most important osteoclastogenic molecule in pathological conditions.[38] To assess whether TNF-α had a role in IL-17–induced osteoclastogenesis, BMMs from TNFRp55 KO mice were cultured in the presence of M-CSF with activated T cells from wild-type mice that were stimulated with CS from *B. abortus*–infected peritoneal macrophages, and osteoclastogenesis was evaluated by the generation of TRAP-expressing cells. BMMs from C57BL/6 wild-type mice were used as a control. Although LTs that were stimulated with CS from *B. abortus*–infected macrophages secreted IL-17 (Figure 3B), these cells were unable to induce osteoclastogenesis on BMMs from TNFRp55 KO mice (when pretreated with CS of *Brucella*-infected macrophages of low MOI) or showed significantly ($P < 0.05$) reduced osteoclastogenesis compared with BMMs from wild-type mice (when pretreated with CS of *Brucella*-infected macrophages of MOI 100) (Figure 7, A versus B). Because BMMs from IL-17R KO mice were unable to secrete TNF-α (Figure 5B), IL-6, or IL-1β (Figure 7, C and D) when stimulated with wild-

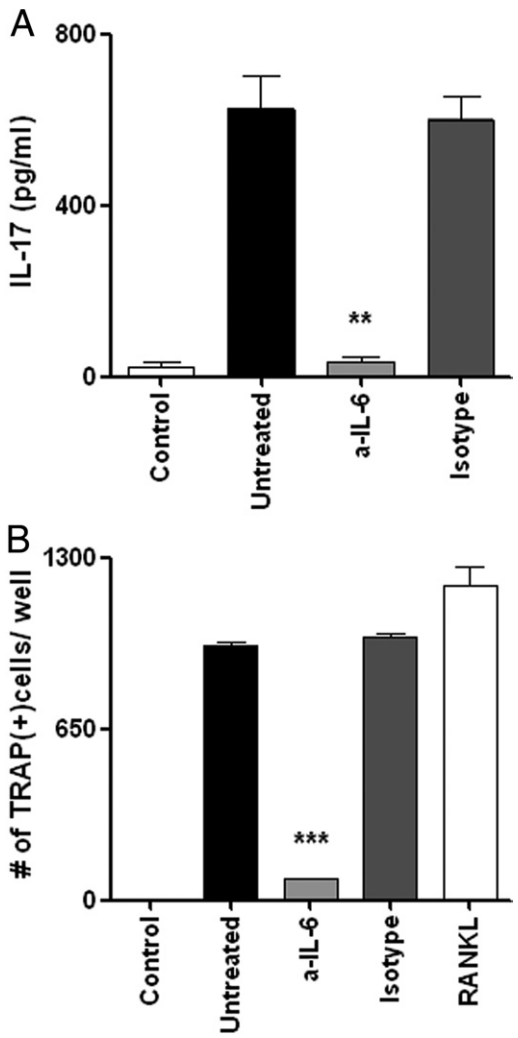

Figure 6. IL-6 secreted by *B. abortus*–infected macrophages determines IL-17 production by LT and concomitant osteoclastogenesis. CSs from *B. abortus*–infected (MOI 100) or uninfected (control) peritoneal macrophages are pre-incubated with anti-IL-6 (a-IL-6)–neutralizing antibody or its isotype control, used to activate LT. *Brucella* activated LT and co-cultured with BMM in the presence of M-CSF. IL-17 secretion (**A**) is determined in CSs by ELISA, and osteoclastogenesis (**B**) is evaluated by the generation of TRAP-expressing cells. Data express the mean ± SEM of duplicates. Data shown are from a representative of three experiments performed. $^{**}P < 0.01$, $^{***}P < 0.001$ versus control.

type LT, the remaining osteoclastogenesis observed in BMMs from TNFRp55 KO, when cultured with wild-type LT pretreated with CS from *Brucella*-infected macrophages at MOI 100, indicated that other pro-osteoclastogenic cytokines were induced by TNFRp55 KO BMMs under these experimental conditions. Indeed, BMMs from TNFRp55 KO mice secreted significant amounts of IL-6 and IL-1β when cultured with wild-type LTs treated with CSs of macrophages infected with an MOI of 100 (Figure 7, C and D). As expected, recombinant TNF-α was unable to induce TRAP-positive cells from TNFRp55 KO BMMs, as against RANKL (Figure 7B). Altogether, these results indicated that IL-17 induced the production of the pro-inflammatory triad of cytokines from osteoclast precursors, of which primarily TNF-α induced osteoclastogenesis.

Brucella-*Activated T Cells Induce Osteoclasts in the Tibiae of Mice*

Finally, to determine the *in vivo* relevance of our hypothesis, *Brucella*-activated T cells were injected in the knee joint of C57BL/6 wild-type mice. *E. coli* LPS and PBS were used as positive and negative controls, respectively. Five days later, animals were sacrificed, whole knee joints were removed, and histological sections of the proximal tibiae were stained for TRAP. Extensive and widespread osteoclastogenesis, defined as TRAP-positive multinucleated cells, was observed in the tibiae of all animals injected with LT that were treated with CS from *Brucella*-infected macrophages; however, in those inoculated with LT that were treated with CS from uninfected macrophages, osteoclastogenesis was not observed. *E. coli* LPS also induced extensive osteoclastogenesis, as against PBS (Figure 8). These results indicated that the presence of *B. abortus*–activated T cells within the bone

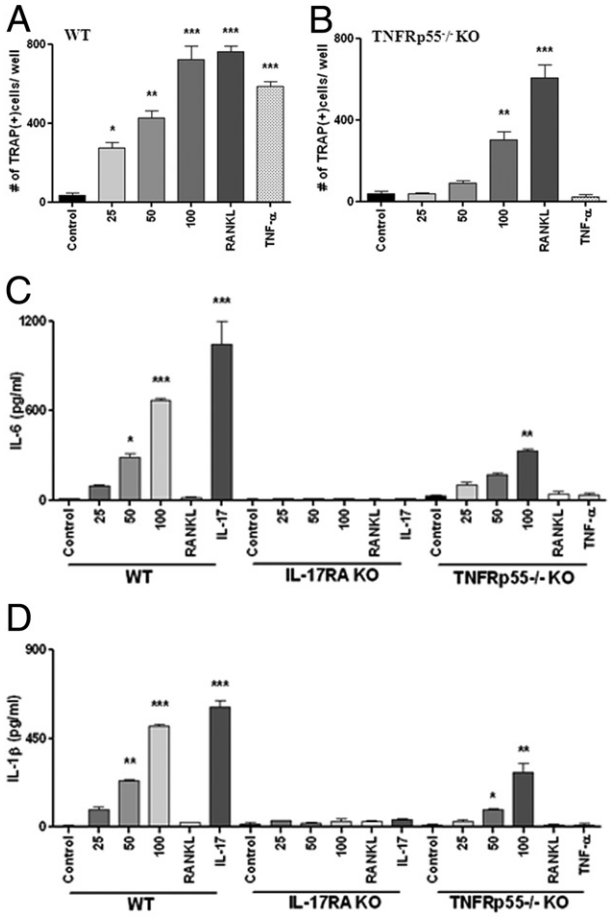

Figure 7. Osteoclastogenesis and cytokine production induced by *Brucella*-activated T cells on TNFRp55 KO and IL-17R KO BMMs. Wild-type (WT), TNFRp55 KO, or IL-17R KO BMMs are cultured with M-CSF and activated T cells that are stimulated by CS from *B. abortus*–infected or uninfected (control) peritoneal macrophages. Osteoclastogenesis is evaluated by the generation of TRAP-expressing cells (**A** and **B**). IL-6 and IL-1β secretion is determined in CSs by ELISA (**C** and **D**). RANKL and IL-17A are used as positive controls. Data express the mean ± SEM of duplicates. Data shown are from a representative of five experiments. Numbers underneath figures represent the MOI used to infect macrophages. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus control.

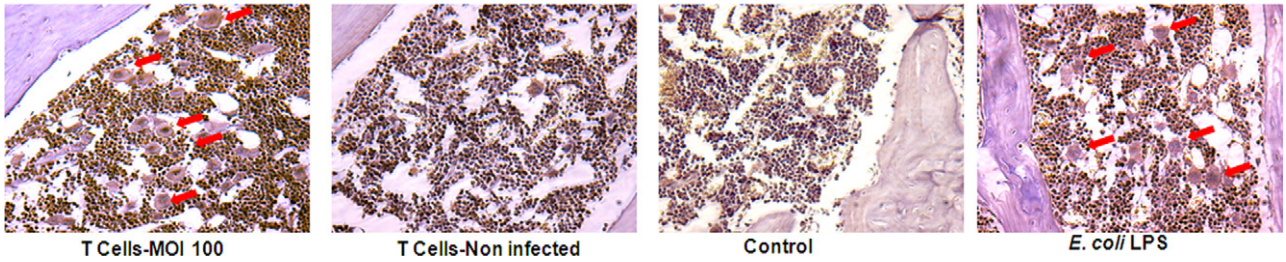

Figure 8. *Brucella*-activated T cells induce osteoclastogenesis *in vivo*. The ability of *Brucella*-activated T cells to induce *in vivo* osteoclastogenesis is determined by their ability to induce osteoclast-like cells in the tibiae of mice. Longitudinal sections of tibia from mice inoculated with *Brucella*-activated T cells are removed after 5 days and are stained for TRAP. TRAP-positive multinucleated cells are observed (**arrows**) in the tibiae of all animals injected with *Brucella*-activated T cells or *E. coli* LPS. LTs treated with CS from uninfected (control) peritoneal macrophages did not display TRAP staining. Data shown are from a representative of two experiments performed.

tissue promoted an inflammatory response that led to the induction of multinucleated TRAP-positive osteoclast cells.

Discussion

Several years of investigation have revealed a close relationship between the immune and skeletal systems.[20] In particular, the interaction between immune cells and osteoclasts is a major topic of critical interest in the field of osteoimmunology. Hitherto, the macrophage-osteoclast interaction has long been studied,[39,40] and the T cell–osteoclast interaction also has attracted much attention in the study of arthritis[20] and in other inflammatory conditions.[18,19] Because infiltration of T cells into the bones and joints is a hallmark pathological finding of osteoarticular brucellosis,[3,25] it is essential to address whether and how T cells are linked to enhanced osteoclastic bone resorption in this form of brucellosis.

We describe an immune mechanism by which pre-activated T cells, influenced by the inflammatory milieu elicited by *B. abortus*–infected macrophages, promote the generation of osteoclasts. Osteoclast development was corroborated phenotypically by the formation of TRAP-, CR-, and vitronectin receptor–positive multinucleated cells with MMP-9 activity, and functionally by the ability of these cells to induce dentine resorption. Moreover, the presence of *B. abortus*–stimulated T cells within the tibiae of mice induced the formation of TRAP-positive multinucleated osteoclasts, underlining *in vivo* the importance of LTs in promoting osteoclastogenesis on *B. abortus* infection.

A more detailed analysis indicated that the LTs that induced osteoclastogenesis were cells of the helper lineage. Although committed on pre-activation with anti-CD3 to secrete IFN-γ, inflammatory mediators from *B. abortus*–infected macrophages tilt this phenotype and provide these $CD4^+$ T cells the capacity to secrete the pro-osteoclastogenic cytokines: RANKL and IL-17. Although RANKL has been postulated as the major cytokine that regulates osteoclast differentiation,[29] our result using blocking anti-IL-17 antibodies or osteoclast precursors from IL-17R KO mice undoubtedly demonstrates that IL-17 drives osteoclastogenesis induced by *Brucella*-activated T cells. The reasons for why RANKL produced by *B. abortus*–activated T cells is unable to induce osteoclastogenesis when IL-17 is blocked merit discussion. A possible explanation, although Th17 cells are able to secrete RANKL, has been proposed that T-cell–induced RANKL is not mainly responsible for inducing osteoclastogenesis.[21] By contrast, it seems that RANKL, expressed by mesenchymal cells, has a predominant role in eliciting the development of osteoclasts.[41,42] More important, together with IL-17 and RANK, *Brucella*-activated T cells produced IL-10, a cytokine that has inhibited the osteoclastogenesis induced by the RANKL/RANK interaction,[43] and also enhanced collagen-induced arthritis, favoring the recruitment of IL-17–producing T cells into the arthritic joints.[44] The production of IL-10, together with pro-inflammatory cytokines, is a common feature of murine and human LTs on infection with *B. abortus*.[25,45,46] Also, IL-17 production has been induced by *B. abortus* infection.[47]

Whether IL-17 acts directly or indirectly to promote osteoclastogenesis is controversial[29]; however, it is particularly important for understanding the pathogenesis of bone loss induced by *B. abortus*–activated T cells. Only a few studies have demonstrated a direct contribution of Th17 cells to osteoclastogenesis.[48] On the contrary, the fact that Th17 cells do not induce osteoclastogenesis in the absence of mesenchymal supporting cells (synovial fibroblasts and osteoblasts)[21] would sustain the contention that IL-17 indirectly induces osteoclastogenesis. Our results indicate that IL-17 induces the production of the pro-inflammatory cytokines from osteoclast precursors, of which primarily TNF-α induces osteoclastogenesis. The pro-inflammatory triad of cytokines (TNF-α, IL-1β, and IL-6) and prostaglandins play a fundamental role in osteoclastogenesis and bone resorption. All these molecules indirectly promote osteoclastogenesis by increasing the expression of RANKL and M-CSF by stromal cells and T cells, and also by acting directly on osteoclast precursors to synergize with RANKL in driving osteoclastogenesis.[49,50] Among these molecules, TNF-α is the most important osteoclastogenic molecule in pathological conditions driving osteoclastogenesis through several different mechanisms, such as increasing the number of osteoclast precursors and enhancing the expression of RANKL in osteoclastogenic-supporting cells.[38]

Of all of the pro-inflammatory mediators produced by *B. abortus* infection,[6,25,30–36] IL-6 induced the production of pro-osteoclastogenic IL-17 from LT. This agrees with

the contention that IL-6, together with transforming growth factor-β, is fundamental for the differentiation of the Th17 lineage.[51] Our IL-6 neutralization experiments, together with our previous results indicating that *B. abortus*–infected macrophages do not secrete transforming growth factor-β,[6] clearly demonstrated that IL-6 is a key component of the induction of pro-osteoclastogenic IL-17. Yet, because transforming growth factor-β is present in fetal bovine serum, we cannot definitively exclude its role as a co-adjuvant differentiation cytokine of the Th17 lineage induced by *B. abortus*–infected macrophages.

The understanding of *Brucella* immunity dictates that infection activates the immune system, leading to a pro-inflammatory response that favors the differentiation of T-cell responses toward a Th1 profile.[52] This response, which mainly involves IFN-γ–producing T cells, is considered important to restrain and/or abrogate infection.[34,35,53] However, the way in which *Brucella*-induced T-cell responses contribute to immunopathological characteristics is not known. Our results shed light on how T cells could be involved in osteoclastogenic bone loss during *B. abortus* infection. They indicate that *B. abortus*–induced osteoclastogenesis is driven by Th17 cells. Because there is increased recognition of plasticity within the helper lineage,[54] we consider that our findings are complementary to the previous observations indicating a significant role of IFN-γ in *Brucella* infection.[55] We envision a scenario in which infiltrating *Brucella*-specific IFN-γ–producing Th1 cells would become pathogenic Th17 cells under the influence of the local inflammatory milieu generated by the bacterium. A better understanding of the factors that control stability and plasticity of *Brucella*-specific $CD4^+$ T cells will have important therapeutic applications to combat infection and to control the pathogenic manifestation of osteoarticular brucellosis.

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