Biochemical and Biophysical Research Communications 456 (2015) 785–791

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Inhibition of osteoclastogenesis by osteoblast-like cells genetically engineered to produce interleukin-10

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ARTICLE INFO

Article history:
Received 2 December 2014
Available online 13 December 2014

Keywords:
Interleukin-10
Osteoblast
Inflammation
Rheumatoid arthritis
Gene therapy

ABSTRACT

Bone destruction at inflamed joints is an important complication associated with rheumatoid arthritis (RA). Interleukin-10 (IL-10) may suppress not only inflammation but also induction of osteoclasts that play key roles in the bone destruction. If IL-10-producing osteoblast-like cells are induced from patient somatic cells and transplanted back into the destructive bone lesion, such therapy may promote bone remodeling by the cooperative effects of IL-10 and osteoblasts. We transduced mouse fibroblasts with genes for IL-10 and Runx2 that is a crucial transcription factor for osteoblast differentiation. The IL-10-producing induced osteoblast-like cells (IL-10-iOBs) strongly expressed osteoblast-specific genes and massively produced bone matrix that were mineralized by calcium phosphate in vitro and in vivo. Culture supernatant of IL-10-iOBs significantly suppressed induction of osteoclast from RANKL-stimulated Raw264.7 cells as well as LPS-induced production of inflammatory cytokine by macrophages. The IL-10-iOBs may be applicable to novel cell-based therapy against bone destruction associated with RA.

1. Introduction

Rheumatoid arthritis (RA) is a chronic disorder characterized by systemic inflammation and multiple arthritis. In addition to synovial lesions, the bones at the inflamed joints are destructed by activated osteoclasts, resulting in severe pain, deformity and disability in patients. Although the pathogenesis of RA remains to be fully understood, crucial roles are played by proinflammatory cytokines such as IL-1β, IL-6, and TNF-α that are secreted from activated T cells and macrophages [1]. These cytokines also provoke synovial fibroblasts to produce RANKL, which subsequently induces osteoclasts to cause destruction of cartilage and bone [2]. Monoclonal antibodies and soluble receptors that block the pro-inflammatory cytokine signals have been used as biological agents and drastically improved the clinical outcome of RA; however, various adverse events may be associated with the therapies such as serious infection [3]. Meanwhile, any current medication has not succeeded in healing destructive bone, and surgical intervention is required to treat patients with severe joint destruction [4,5]. Therefore, it is necessary to develop a new therapeutic approach to suppress inflammation and archive repair of the destructed bones without causing undesirable adverse events.

IL-10 is a profound immunosuppressive cytokine produced by macrophages, T cells, and certain subsets of B cells and dendritic cells (DCs) [6]. Moreover, IL-10 remarkably prevents generation of osteoclasts through the inhibition of NFATc1 expression [7]. Therefore, IL-10 is regarded as a promising cytokine applicable to anti-rheumatic therapy, due to its powerful activities to suppress inflammation as well as osteoclastogenesis.

A number of reports indicated that systemic administration of IL-10 may provide significant therapeutic benefit to animal models of experimental arthritis [8–10]. Moreover, IL-10 suppresses production of inflammatory cytokines such as IL-1β and TNF-α by

Abbreviations: RA, rheumatoid arthritis; iOBs, induced osteoblast-like cells; IL-10-iOBs, IL-10-producing induced osteoblast-like cells; OCN, osteocalcin; OPN, osteopontin; BSP, bone sialoprotein; ALP, alkaline phosphatase.

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http://dx.doi.org/10.1016/j.bbrc.2014.12.040
0006-291X/

synovial fluid macrophages of RA patients [11]. In previous clinical trials, IL-10 administration was reported safe and well tolerated, but its therapeutic efficacy for RA was unsatisfactory [12,13]. This may be due to insufficiency of distribution of IL-10 at the joint lesions after systemic administration of the cytokine. Systemic administration of a higher dose of IL-10 could potentially cause cancer development, chronic infection, and Th2-dependent autoimmune disorders that represent lupus-like symptoms [14]. Therefore, local delivery of IL-10 to the inflammatory legions is desirable. Some studies have shown that the cells genetically modified to produce IL-10 were effective in treating experimental arthritis in animals [15,16].

Osteoblasts are crucially involved in bone formation and remodeling through production of calcified bone matrix. We hypothesized that if osteoblast-like cells are engineered to produce IL-10 and transplanted into the destructed bone tissue, such a procedure may offer a great deal of therapeutic benefits to RA patients due to remodeling of bone tissue as well as suppression of articular inflammation. Osteoblast-like cells can be induced from mouse fibroblasts by transducing the Runx2 gene that plays an essential role in osteoblast differentiation [17], and this sort of technology may enable production of patient-specific, induced osteoblast-like cells (iOBs) that are suitable for transplantation therapy.

However, effect of IL-10 on osteoblast differentiation remains controversial. IL-10 gene knockout mice showed loss of alveolar bone and osteopenia-like phenotypes including bone mass reduction [18–20]. In contrast, van Vlasselaer et al. reported that administration of exogenous IL-10 inhibited the osteoblast differentiation from mouse bone marrow cells through the inhibition of TGF-β1 [21,22].

In this context, we examined whether co-transduction of Runx2 and IL-10 genes successfully induced mouse fibroblasts into IL-10-producing osteoblast-like cells with capability to produce bone matrix. We also tested whether the genetically modified cells inhibited production of pro-inflammatory cytokines by activated macrophages as well as induction of osteoclasts.

2. Materials and methods

2.1. Cells

Mouse embryonic fibroblasts (MEFs) were obtained from Balb/c embryos at the gestational age of day 13.5 by digestion with collagenase (NB4G®; Serva, Heidelberg, Germany). MEFs were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 10 mM non-essential amino acids (standard medium). Raw264.7, A20, and SaOS2 were purchased from RIKEN cell bank (Tsukuba, Japan). Yac1 was maintained in our laboratory. SCC-7 was kindly gifted by Dr. Oya at the Department of Therapeutic Radiology and Oncology, Kyoto University, Japan.

2.2. Retrovirus vectors

The cDNA fragments encoding mouse Runx2 and IL-10 genes were obtained from pFLCI-mouse Runx2 and pFLCI-mouse IL-10 plasmids (Danaform, Kanagawa, Japan), respectively, and inserted into the pMX-puro with GeneArt Seamless Cloning and Assembly (Life Technologies, Carlsbad, CA). The resultant retrovirus vector plasmids, pMX-mRunx2.puro and pMX-mIL10.puro, were transfected into the Plat-E packaging cell line with X-treme Gene 9 (Roche Diagnostics, Basel, Switzerland). Twenty-four hours later, the culture medium was replaced by fresh one, and after incubation for another 24 h, culture supernatant containing retrovirus vectors was harvested.

2.3. Induction of osteoblasts

MEFs were seeded onto a 24-well plate at a density of 1.5×10^4 per well. On the next day, the retrovirus vector suspension was supplemented with 4 μg/mL polybrene (Nacalai Tesque, Kyoto, Japan) and added to the cells, which were subsequently cultured in the standard medium supplemented with 100 nM dexamethasone (Nacalai Tesque), 50 μg/mL L-ascorbic acid (Nacalai Tesque), and 10 mM β-glycerophosphate (Tokyo Chemical Industry, Tokyo, Japan)(osteogenic medium). The culture was continued for 2 to 4 weeks, while the medium was changed every 2 days.

2.4. Alizarin red S staining and staining by von Kossa's method

For Alizarin red S staining, cells were fixed with 95% ethanol for 10 min at room temperature. After washing with distilled water, cells were stained with Alizarin red S solution (Sigma–Aldrich, St. Louis, MO) for 30–45 min, followed by another washing with distilled water. Stained area was calculated with ImageJ [23]. For von Kossa staining, cells were fixed in 10% neutral buffered formalin for 10 min at room temperature. After 3 times washing with distilled water, 5% silver nitrate solution (ScyTek Laboratories, Logan, UT) was added to the cells, which were subsequently exposed to ultra violet for 30–60 min. Culture dishes were rinsed 3 times with distilled water, and incubated with 5% sodium thiosulfate solution (ScyTek Laboratories) for 2 min.

2.5. Alkaline phosphatase (ALP) staining

ALP activity was determined by ALP staining using a Leukocyte Alkaline Phosphatase Kit (Sigma–Aldrich) following the manufacture's instruction. Briefly, cells were fixed with 60% acetone/40% citrate. After washing with deionized water, cells were stained with a diazonium salt solution containing fast violet blue salt and 4% of naphthol AS-MX phosphate alkaline solution for 1 h under protection from light.

2.6. Immunofluorescence staining

Cells were fixed with 4% paraformaldehyde at 4 °C for 30 min, followed by washing with 0.02% Tween-20/PBS. After blocking, cells were washed and incubated with FITC-conjugated rat anti-mouse IL-10 (final concentration was 1:100) (eBioscience, San Diego, CA) and Cy5.5-conjugated rabbit anti-mouse osteocalcin (OCN) (final concentration was 1:100) (Bioss, Woburn, MA) antibodies. On the next day, cells were washed, and observed under a fluorescence microscope.

2.7. Real time RT-PCR

Cells were homogenized in Isogen 2 (Nippongene, Tokyo, Japan), and total RNA was harvested by the phenol guanidinium acid-based procedure. After reverse transcription using ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan), cDNA served as template for real time PCR using Applied Biosystems 7300 Real-Time PCR System. The primers and dye probe for osteocalcin (OCN) (Bglap: Mm03413826_mH), osteopontin (OPN) (SPP1: Mm00436767_m1), bone sialoprotein (BSP) (IBSP: Mm00492555_m1), alkaline phosphatase (Alpl: Mm00492555_m1), IL-1β (Il1b: Mm00434228_m1), telomerase reverse transcriptase (Tert: Mm00436931_m1) and β-actin (Mm00607939-m1) genes were purchased from Applied Biosystems (Carlsbad, CA). The primer sequences for TNF-α and IL-6 genes were as follows: TNF-α forward, 5′-tcttctcattcctgcttgtgg-3′; TNF-α reverse, 5′-ggtctgggccatagaactga-3′; IL-6 forward, 5′-gctaccaaactggatataatcagga-3′, IL-6 reverse, 5′-ccaggtagctatggtactccagaa-3′. Corresponding proves were purchased from Roche Applied

Science (Basel, Switzerland) (Universal Probe Library: TNF-α#49, IL-6#6). Samples were incubated at 95 °C for 10 min for an initial denaturation, followed by forty PCR cycles that consisted of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. β-actin gene was regarded as endogenous standard and normalization was calculated using RQ software. All experiments were performed in triplicate.

2.8. ELISA

The concentration of IL-10 was measured using Mouse IL-10 Ready SET Go ELISA kit (eBioscience) according to the manufacturer's instruction.

2.9. Induction of osteoclasts

Raw264.7 cells obtained from the RIKEN cell bank were plated in 12-well plates at a density of 1×10^4 cells/well, and cultured in a standard medium supplemented with or without 100 ng/mL of RANKL (PeproTech, Rocky Hill, NJ). In some wells, culture supernatants that had been harvested from MEFs, induced osteoblast-like cells (iOBs), and IL-10-producing iOBs (IL-10-iOBs) were added. The culture medium was replaced by a fresh one on day 3. Six days after the initiation of the culture, cells were subjected to tartrate-resistant acid phosphatase (TRAP) staining using the TRAP kit (Primary Cell, Sapporo, Japan). Briefly, the cells were fixed with 10% neutral buffered formalin for 5 min, followed by incubation at 37 °C for 1 h in 50 mmol/L tetrate buffer containing substrate. After washing with distilled water, the TRAP-positive multi-nuclear cells were regarded as mature osteoclasts.

2.10. Activation of peritoneal macrophages

6–8 week-old female Balb/c mice were intraperitoneally injected with 2 mL of thioglycolate medium. Three days later, mice were sacrificed and 5 mL of PBS was injected into the peritoneal cavity. After gentle massage, the peritoneal exudate cells were retrieved and seeded into 12-well plates at 1.5×10^6 cells per well. After 2 h of culture, the floating cells were removed, and residual adherent cells were cultured for 24 h in the presence of supernatants of MEFs, iOBs and IL-10-iOBs. Cells were then stimulated with LPS (100 ng/mL)(Invitrogen, Carlsbad, CA) for 2 h.

2.11. Contact inhibition

SaOS2 cells and MEFs that had been transduced with Runx2 and IL-10 genes were seeded in 60 mm culture dishes at a density of 1.5×10^5 cells/dish, and cultured for 15 days without trypsinization and reseeding. Cell morphology was observed under phase-contrast microscopy.

2.12. Transplantation

Twenty-four hours after infection with the Runx2 and/or IL-10 retrovirus vectors, MEFs, iOBs and IL-10-iOBs were seeded on hydrogel scaffold (MedGel® SP; MedGEL, Tokyo, Japan) at a density of 3.5×10^4 cells/scaffold. After pre-culture for 2 days in osteogenic medium, the cells were subcutaneously transplanted into the flank of 6-week-old female Balb/c mice. Four weeks later, the graft was excised and cryosectioned. Mineralization status of the specimens was estimated by Alizarin red staining and staining by the von Kossa's method as above. To examine tumor formation, IL-10-iOBs or MEFs that had been cultured in osteogenic medium for 2 days were subcutaneously inoculated into 7-week-old male SCID/NOD mice with or without hydrogel scaffold at a dose of either 3.5×10^4 or 3.0×10^6/mouse.

2.13. Statistical Analysis

All the data were analyzed by Student's unpaired *t*-test, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. IL-10 gene transduction did not prevent Runx2-mediated induction of osteoblast-like cells from fibroblasts

To generate IL-10-secreting osteoblast-like cells, we transduced primary mouse embryonic fibroblasts (MEFs) with Runx2 and IL-10 genes via retrovirus vectors, and analyzed the phenotypes of the resultant cells (IL-10-iOBs) in comparison with those of the osteoblast-like cells induced by transduction of Runx2 gene alone (iOBs). The ALP staining indicated that both IL-10-iOBs and iOBs showed high activities of ALP, an early stage marker of osteoblast differentiation, 10 days after the gene transduction (Fig. 1A, top). Alizarin red S staining unveiled that the IL-10-iOBs produced mineralized bone matrix as massively as iOBs on day 20, whereas un-transduced MEFs failed to show any significant staining (Fig. 1A, middle). Calculation of the Alizarin red S-stained areas also demonstrated comparable degrees of calcification in IL-10-iOBs and iOBs cultures (data not shown). Staining by the von Kossa's method also confirmed massive calcium deposition by IL-10-iOBs and iOBs, but not by MEFs (Fig. 1A, bottom).

To further confirm the osteoblast-like characteristics of the cells, we examined expression of osteoblast marker genes 15 days after the gene transfer (Fig. 1B). Quantitative RT-PCR analysis demonstrated that IL-10-iOBs expressed similar or even higher levels of mRNA for the osteocalcin (OCN), osteopontin (OPN), bone sialoprotein (BSP) and ALP genes as iOBs.

These data strongly suggested that IL-10 gene transfer did not hamper osteoblast-like phenotypic conversion of MEFs.

3.2. IL-10-iOBs produced IL-10 at a high level

To assess the IL-10 production by IL-10-iOBs, we performed quantitative RT-PCR 15 days after the initiation of the osteoblast induction. The cells expressed a high level of IL-10 mRNA, which was not the case with iOBs and MEFs (Fig. 2A). We also harvested the supernatants of the IL-10-iOBs and measured IL-10 concentrations by ELISA, which showed robust IL-10 secretion from the cells on days 1 and 2 (Fig. 2B).

Because IL-10-iOBs potentially contained heterogeneous populations, further experiments were performed to clarify whether the IL-10 was produced by the cells with osteoblast-like features. Immunofluorescence staining of IL-10-iOBs was performed to visualize OCN and IL-10 on day 14. As shown in Fig. 2C, OCN and IL-10 were co-localized in the same cells, indicating that IL-10 was produced by the osteoblast-like cells.

3.3. Conditioned medium of IL-10-iOBs suppressed induction of osteoclasts

To estimate whether the IL-10 secreted from IL-10-iOBs was capable of inhibiting osteoclastogenesis, we collected the culture supernatants of the IL-10-iOBs, iOBs and MEFs, and examined their effect on the mouse macrophage cell line, Raw264.7, that was induced to differentiate into osteoclasts by an addition of RANKL [24,7]. Six days after the induction, a number of TRAP-stained multinuclear cells (MNCs) appeared in the cultures in which the supernatant of MEFs or iOBs had been added (Fig. 3A). In contrast, the

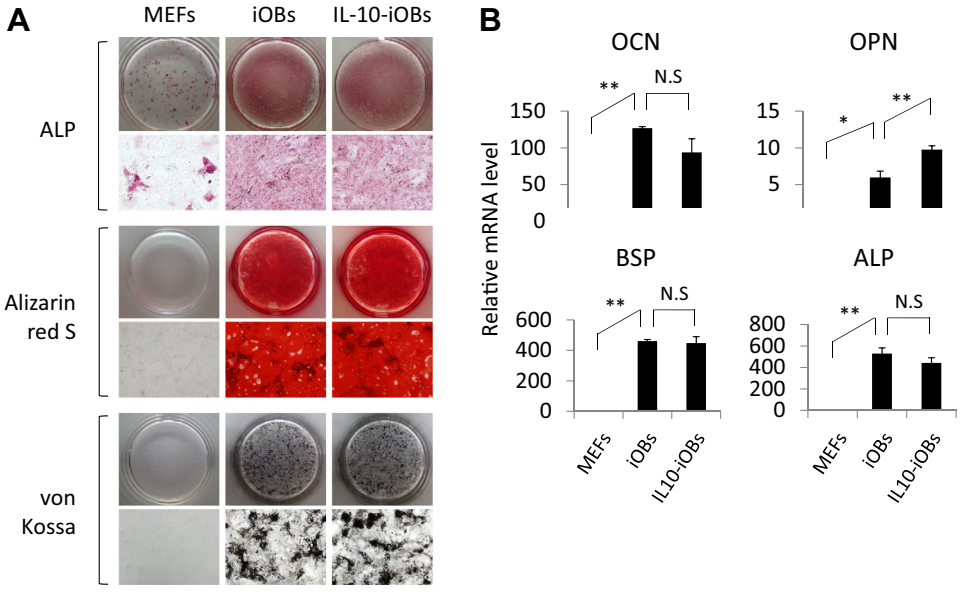

Fig. 1. Transduction of IL-10 gene did not inhibit induction of osteoblast-like phenotypes in MEFs. MEFs were infected with Runx2 (iOBs) and both Runx2 and IL-10 (IL-10-iOBs) retrovirus vectors. (A) Cells were cultured for 10 days and subjected to the ALP staining (top), while the other aliquots of cells were cultured for 20 days and subjected to the Alizarin red S staining (middle) or to staining by von Kossa's method (bottom). Gross appearance (upper) and microscopic images at magnification of ×100 (lower) are shown. (B) Fourteen days after gene transfer, RNA was extracted from the cells, and qRT-PCR analysis was performed to evaluate mRNA levels for osteocalcin (OCN), osteopontin (OPN), bone sialoprotein (BSP) and ALP genes. Data represent the means ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, N.S.: not significant.

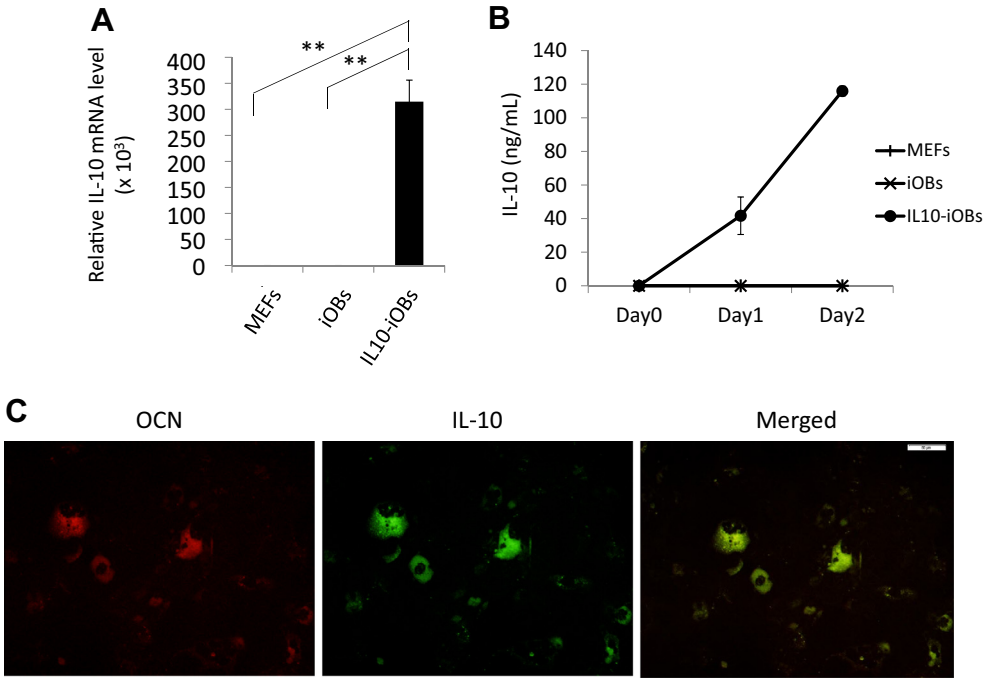

Fig. 2. IL-10-iOBs produced a large amount of IL-10. (A) RNA was extracted from MEFs, iOBs and IL-10-iOBs 14 days after gene transduction, and qRT-PCR analysis was performed to evaluate IL-10 mRNA. Data represent the means ± SD ($n = 3$). $^{**}p < 0.01$. (B) Culture supernatants of MEFs, iOBs, and IL-10-iOBs were harvested at the indicated days, and the concentrations of IL-10 were measured by ELISA. Data represent the means ± SD ($n = 3$). $^{**}p < 0.01$. (C) IL-10-iOBs were immunostained using FITC-conjugated anti-IL-10 and Cy5.5-conjugated anti-OCN antibodies 14 days after gene transfer. Scale bar shows 50 μm.

osteoclast-like cells were hardly detectable in the culture that had received the supernatant of the IL-10-iOBs. We counted the number of TRAP-positive MNCs in each group and confirmed that an addition of supernatant of IL-10-iOBs significantly reduced the number of osteoclast-like cells induced from Raw264.7 cells (Fig. 4B).

3.4. Conditioned medium of IL-10-iOBs suppressed production of inflammatory cytokines by activated macrophages

We examined the effect of IL-10-iOB supernatant on cytokine production by activated macrophages. The thioglycollate-elicited peritoneal macrophages were cultured with supernatant of IL-10-

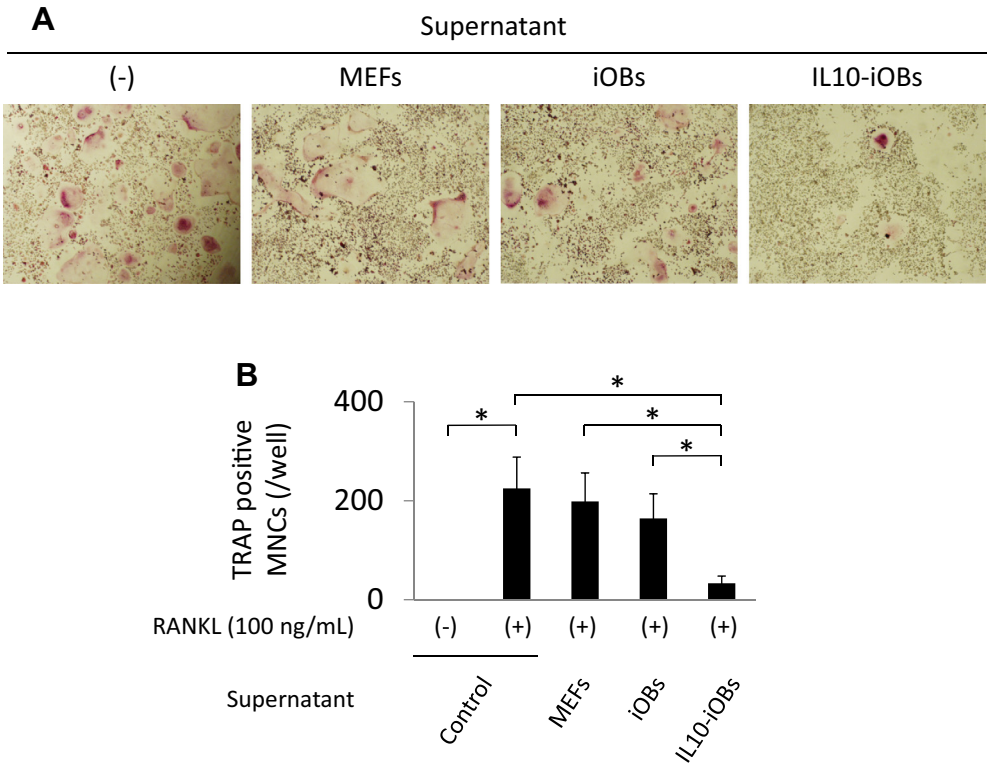

Fig. 3. Culture supernatant of IL-10-iOBs suppressed differentiation of RANKL-stimulated Raw264.7 cells into osteoclasts. Raw264.7 cells were cultured with RANKL and supernatant of the indicated cells. Six days later, cells were subjected to TRAP staining. (A) Microscopic images at magnification of ×100 are shown. (B) The numbers of TRAP-positive multi-nuclear cells (MNCs) per well are plotted. Data represent the means ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$.

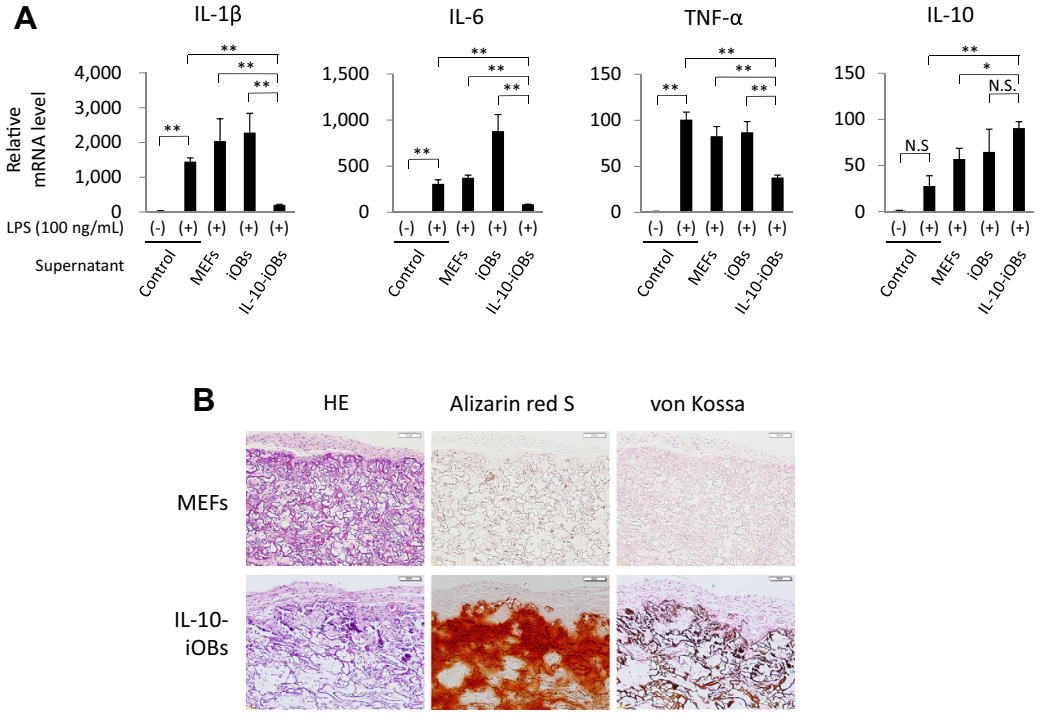

Fig. 4. Culture supernatant of IL-10-iOBs prevented LPS-stimulated macrophages from producing proinflammatory cytokines, while IL-10-iOBs produced mineralized bone matrix in vivo. (A) Peritoneal macrophages were cultured with the supernatants of the indicated cells, and stimulated with LPS as described in the materials and methods. Two hours later, RNA was extracted from the cells and mRNA for the indicated cytokines was evaluated by qRT-PCR. Data represent the means ± SD ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$, N.S., not significant. (B) MEFs were transduced with Runx2 and IL-10 genes and seeded on hydrogel scaffold. After culturing for 2 days, the cells and scaffold were transplanted into the flank mice. Graft was excised 4 weeks later, and tissue was subjected to the indicated staining (Scale bars indicate 100 μm).

iOBs, iOBs or MEFs, followed by stimulation with 100 ng/mL LPS. 2 h later, mRNA levels for IL-1β, IL-6, TNF-α and IL-10 were evaluated by real time RT-PCR. As shown in Fig. 4A, the supernatant of IL-10-iOBs significantly suppressed mRNA expression of IL-1-β, IL-6 and TNF-α. Neither MEF nor iOB supernatants significantly affected the expression of the pro-inflammatory cytokines, except that the supernatant of iOBs significantly enhanced IL-6 mRNA expression. In sharp contrast, the supernatant of IL-10-iOBs rather elevated the expression of IL-10 mRNA by the LPS-stimulated macrophages.

These results strongly suggested that IL-10 secreted from IL-10-iOBs suppressed production of the pro-inflammatory cytokines.

3.5. Significant bone formation in vivo by ectopically transplanted IL-10-iOBs

We tested whether the IL-10-iOBs are capable of forming bone in vivo. The IL-10-iOBs cultured on hydrogel scaffold were subcutaneously transplanted into the flank of the syngenic mice. After 4 weeks, histological assessment of the graft was performed, and the specimen was strongly stained with Alizarin red S (Fig. 4B, lower middle). The bone formation was also confirmed by the positive staining by the von Koss's method (Fig. 4B, lower right), while the HE staining did not show any sign of inflammatory cell infiltration (Fig. 4B, lower left). Meanwhile, bone formation was totally absent in the tissue into which MEFs had been transplanted as a control (Fig. 4B, upper).

Therefore, IL-10-iOBs remarkably produced a mineralized bone matrix in vivo after transplantation, suggesting applicability of the cells to novel cell therapy for RA.

3.6. IL-10-iOBs lacked tumor-like characteristics

Finally, we examined whether IL-10-iOBs exhibited tumor cell-like features. In culture, IL-10-iOBs proliferated until they form a confluent monolayer and subsequently underwent growth arrest (Supplementary Fig. 1A). This is in sharp contrast to SaOS2 osteosarcoma cells that showed loss of contact inhibition. The IL-10-iOBs expressed mRNA for the telomerase reverse transcriptase (TERT) gene at as low level as fibroblasts, whereas most tumor cells highly expressed TERT (Supplementary Fig. 1B) [25].

Tumorigenic potential of IL-10-iOBs was assessed by subcutaneously inoculation into immune-deficient mice. Tumor development was not seen in all the mice. Taken together, it was strongly suggesting that the IL-10-iOBs did not have tumorigenicity (Supplementary Table S1).

4. Discussion

In the present study, we proposed a novel therapeutic procedure for RA based on the transplantation of genetically modified osteoblast-like cells. By transducing MEFs with the genes for osteoblast differentiation factor Runx2 and potent anti-inflammatory cytokine IL-10, the established IL-10-iOBs expressed osteoblast marker genes at high levels (Fig. 1B) and massively produced mineralized bone matrix both in vitro (Fig. 1A) and in vivo (Fig. 4B), while IL-10 was also abundantly produced by the osteocalcin-positive cells (Fig. 2), strongly suggesting that IL-10 production did not interfere with Runx2-mediated induction of the osteoblast-like phenotypes. Previous reports indicated that administration of IL-10 suppressed differentiation of osteoblasts through inhibition of the TGF-β signaling [22], while TGF-β signaling contributes to osteoblast differentiation by inducing Runx2 expression [26]. Therefore, exogenous Runx2 gene may have substituted for the endogenous Runx2 that was suppressed by IL-10, resulting in restoration of the IL-10-mediated interference of osteoblast differentiation.

The supernatant of IL-10-iOBs significantly suppressed both RANKL-mediated induction of osteoclasts from Raw264.7 (Fig. 3) and production of inflammatory cytokines by LPS-activated macrophages (Fig. 4A). The anti-inflammatory and anti-osteoclast functions of the IL-10-iOBs may cooperatively ameliorate the inflammatory bone destruction at the joint lesions of RA patients, if transplantation therapy using these cells is clinically applicable to RA patients in the future. An extremely high production of IL-10 may cause some adverse events such as immunosuppression. To limit the serum level of IL-10 not to exceed the tolerable level, patients may be transplanted with a mixture of IL-10-iOBs and iOBs at an appropriate ratio.

The iOBs may be quite adequate for the cell-based therapy for RA, because a sufficient number of iOBs with high bone formation capacity may be induced from patients' own somatic cells. Fibroblasts may be particularly useful as the somatic cells to be induced into iOBs, because they can be obtained from RA patients in a minimally invasive fashion and allowed to proliferate to a large number in culture before induction into osteoblast-like cells. Other cells such as leukocytes may become an alternative for fibroblasts, but this point should be examined in further studies. Although the transduction of Runx2 gene alone fails to induce osteoblast-like phenotypic change in somatic cells of human origin, this problem may be overcome by using some combinations of genes instead of Runx2 gene alone, and we have already established such a procedure (Yamamoto et al., a manuscript submitted for publication).

Acknowledgments

This work was supported by grants from the Japan Science and Technology Agency, and the Japanese Ministry of Education, Culture, Sports, Science and Technology. All authors have declared there are no financial conflicts of interest in regards to this study.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.12.040.

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