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Research paper

Evaluation of osteoclastogenesis via NFκB decoy/mannosylated cationic liposome-mediated inhibition of proinflammatory cytokine production from primary cultured macrophages

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Running head: Delivering NFκB decoy to macrophages for inhibiting bone loss

ABSTRACT

Purpose: To explore the effect of NFκB activation in macrophages on osteoclastogenesis of bone marrow cells for potential application as a new type of therapy for preventing bone loss.

Methods: Primary cultured macrophages and bone marrow cells were prepared from mice. As macrophage-targeted carriers, Mannosylated cationic liposomes (Man-liposomes) were prepared and were allowed to form complexes with NFκB decoy (a double-stranded oligonucleotide). Cellular uptake, inhibition of NFκB activation, and cytokine production were evaluated using macrophages. Osteoclastogenesis was investigated using bone marrow cells, which were cultured in the conditioned medium prepared from macrophages with or without Man-liposome/NFκB decoy complexes treatment.

Results: Cellular accumulation of NFκB decoy was enhanced by Man-liposome. NFκB activation in macrophages and TNF-α production were suppressed in macrophages by Man-liposome/NFκB decoy complexes but not by the naked NFκB decoy, Gal-liposome/NFκB decoy complexes, or Man-liposome/random decoy complexes. Osteoclastogenesis of bone marrow cells was induced in the conditioned medium prepared from activated macrophages but not by activated macrophages treated with Man-liposome/NFκB decoy complexes.

Conclusion: Osteoclastogenesis induced by activated macrophages could be suppressed by the treatment macrophages with Man-liposome/NFκB decoy complexes. Macrophage-targeted delivery of NFκB decoys using Man-liposomes may be promising in its use for the remediation of bone loss.

KEYWORDS: Man-liposomes; NFκB decoy; Proinflammatory cytokines; osteoclastogenesis

INTRODUCTION

A large proportion of the world's population has rheumatoid arthritis (RA), which is a systemic autoimmune disease characterized by chronic inflammation of the synovium that spreads to the cartilage and bone, leading to bone destruction (1). Moreover, bone destruction is a severe problem in osteoporosis and periodontitis (2). In RA, there is a marked increase in the production of the proinflammatory cytokines tumor necrosis factor (TNF) and receptor activator NF-kappa B ligand (RANKL), which is members of the TNF family that are known to stimulate bone resorption by osteoclasts (3,4). In RA, the synergistic relationship between TNF-α and RANKL serves to enhance the osteoclastogenic properties of both proinflammatory cytokines (5,6). TNF-α functions in a manner similar to that of RANKL and not other osteoclast-stimulating factors; and it directly promotes osteoclast differentiation via its Type 1 or 55 receptors (7). Furthermore, severe arthritis model mice which have similar pathology to the human pathology could be established by overexpressing human TNF in mice (8). Therefore, TNF-α is likely to be one of the key cytokines in RA therapy. In fact, anti-TNF-α agents have significantly improved the symptoms and disabilities of patients with RA (9). However, some anti-TNF-α drugs have been recently found to interfere with innate immune responses such as phagolysosome maturation and monocyte apoptosis (10); hence, new therapeutic agents are required.

In macrophages, the production of TNF-α is centrally regulated by the transcription factor nuclear factor kappa B (NFκB) (11,12). Because a magnified activation of NFκB in macrophages occur at the site of inflammation, the act of suppressing NFκB activation by NFκB inhibitors has been demonstrated to be beneficial for use in inflammation therapy (13). Being an inhibitor of NFκB, the double-stranded oligonucleotide with the binding sequence to the

transcription factor NFκB, referred to as the NFκB decoy, is an attractive drug candidate because the NFκB decoy suppresses excess NFκB activation in a sequence-dependent manner (14). Therefore, NFκB decoy is expected to hardly affect innate immune response, and consequently to work with a small number of unexpected side effects. However, the use of NFκB decoy in vivo is limited due to its low cellular permeability, which is caused by large amount of anionic charge on the molecule (15,16). Therefore, it is necessary to develop suitable carriers for delivering NFκB decoy to the target cells.

To date, several delivery carriers for NFκB decoy have been developed; they include hemagglutinating virus of Japan-envelope liposomes (HVJ liposomes) (17), cationic liposomes (18,19), PLGA microspheres (20), folate liposome (21), and dendritic poly(l-lysine)(22). As far as the macrophage-selective targeting is concerned, we developed the mannosylated cationic liposomes (Man-liposomes) for use in plasmid DNA delivery (23) because mannose receptors are found in high numbers on macrophages. More recently, we have demonstrated that intravenously injected Man-liposomes/NFκB decoy complexes were efficiently taken up by Kupffer cells, liver macrophages, and liver endothelial cells via mannose receptors and functioned to suppress lipopolysaccharide(LPS)-induced cytokine production by macrophages in a liver failure model (24). In RA, macrophages in the articular cavity release cytokines, which promote severe inflammation and osteoclastogenesis. Therefore, targeted delivery of NFκB decoys by Man-liposomes would be a promising approach for treating RA. However, the mechanism of bone loss in RA is extremely complicated because various types of cells participate in evoking the bone loss in more closed space than systemic space. Currently, there is little information regarding the relationship between proinflammatory cytokine production from NFκB-activated macrophages and osteoclastogenesis of precursor cells. In oder to develop new

therapy for RA by targeted delivery of NFκB decoy using Man-liposomes, it is nesessary to investigate that osteoclastgenesis could be suppressed by the inhibition of cytokines production from macrophage in vitro because its contribution would be clearly evaluated in the simple system.

In this study, we prepared primary cultured macrophages isolated from mice and confirmed their time-course expression levels of mannose receptors. We then investigated the cellular uptake characteristics and inhibitory effects of Man-liposomes/NFκB decoy complexes on TNF-α production and NFκB activation. Finally, we evaluated osteoclastogenesis of precursor cells, bone marrow cells prepared from mice, by analyzing secretions from macrophages treated with Man-liposome/NFκB decoy complexes.

MATERIALS AND METHODS

Materials

Oligonucleotides (diester type) (NFκB decoy: 5′ -AGTTGAGGGGACTTTCCCAGGC-3′ /5 ′ -GCCT CCCAAAGTCCCCTCAACT-3 ′ ; random decoy: 5 ′ -TTGCCGTACCTGACTTAGC C-3′ /5′ -GGCT AAGTCAGGTACGGCAA-3′) and rhodamine labeled oligonucleotides (diseter type) were purchased from Hokkaido System Science Co., Ltd. (Hokkaido, Japan). Oligonucleotide (thioate type) were kindly provided by Anges MG Inc. (Osaka, Japan). *N*-(4-aminoethyl) carbamic acid *tert*-butyl ester was obtained from Tokyo Kasei Kogyo Co. Ltd. (Tokyo, Japan). Dioleoylphosphatidylethanolamine (DOPE) was obtained from Avanti Polar Lipids Inc. (Alabaster, AL, USA). Cholesteryl chloroformate was purchased from Sigma Chemicals Pvt. Ltd. (St Louis, MO, USA).

Synthesis of Man-C4-Chol

Man-C4-Chol was synthesized as previously reported (23). Briefly, cholesteryl chloroformate and *N*-(4-aminobutyl)carbamic acid *tert*-butyl ester were allowed to react in chloroform for 24 h at 4°C. A solution of trifluoroacetic acid and chloroform was added dropwise and the mixture was stirred for 2 h at 4°C. The solvent was then evaporated in order to obtain the *N*-(4-aminobutyl)-(cholesten-5-yloxyl)formamide, which was then combined with 2-imino-2-methoxyethyl-1-thiomannoside for 24 h at room temperature. After evaporation, the resulting precipitate was suspended in water, dialyzed against distilled water for 48 h, and finally lyophilized. Gal-C4-Chol was synthesized by the same protocol with Man-C4-Chol after preparation of 2-imino-2-methoxyethyl-1-thiogalactoside.

Preparation of Man-liposomes and Man-liposomes/NFκB decoy complexes

Man-C4-Chol was mixed with DOPE in chloroform at the molar ratio of 3:2. The mixture was then dried, vacuum desiccated, and the precipitate resuspended in sterile 5% dextrose. After hydration for 30 min at room temperature, the suspension was sonicated for 10 min in a bath sonicator and for 3 min with a tip sonicator in order to form Man-C4-Chol/DOPE liposomes (Man-liposomes). The Man-liposome/NFκB decoy complexes were prepared by using the method previously described (24). Equal volumes of NFκB decoy and stock Man-liposome solution was diluted by the addition of 5% dextrose at room temperature. Then, the NFκB decoy solution was rapidly added to the liposome solution. This mixture was agitated by pumping it up and down twice in the pipette tip. Finally, the mixture was left at room temperature for 30 min. NFκB decoy or random was complexed with Man- or Gal-liposomes at a charge ratio of 2.3 : 1.0 (+:-) following previous reports (19,24), because complexes with liposomes containig DOPE were stable at this mixing ratio in vivo (25). Particle sizes and zeta potentials of Man-liposomes and Man-liposome/NFκB decoy complexes were determined using a Zetasizer Nano ZS instrument (Malvern Instruments, Ltd., UK).

Preparation of primary cultured macrophages and bone marrow cells from mouse

Five-week-old female ICR mice and 6-week-old female C57BL6/crSlc mice were obtained from Shizuoka Agricultural Cooperative Association for Laboratory Animals (Shizuoka, Japan). All animal experiments were carried out in accordance with the Principles of Laboratory Animal Care as adopted and promulgated by the US National Institute of Health and the Guidelines for Animal Experiments of Kyoto University. Elicited peritoneal macrophages were prepared by

using the method previously described (26). Briefly, cells were harvested from ICR mice or C57BL6/crSlc mice 4 days after intraperitoneal injection of 1 ml 2.9% thioglycolate medium (Nissui Pharmaceutical, Tokyo, Japan). The washed cells were suspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Flow Laboratories, Irvine, UK), penicillin G (100 U/ml), and streptomycin (100 μg/ml) and the medium was then plated either on 6- or 24-well plates (Falcon, Becton Dickinson, Lincoln Park, NJ, USA) at a density of 1×10^6 cells/cm^2. After incubation for 2 h at 37°C in an atmosphere of 5% CO_2 and 95% air, non-adherent cells were washed off. The remaining cells were cultured for 24, 48, 72, or 96 h. Mouse bone marrow cells were collected according to the procedure described by Yagi K (27). In brief, bone marrow cells were isolated from mice by flushing the femurs with DMEM containing 10% FBS, 1000 U/ml penicillin G, 100 mg/l streptomycin, 100 μg/l amphotericin B, and 100 μg/l aprotinin using a 25-gauge needle. Isolated cells were seeded onto 24-well dishes at a density of 1.5×10^6 cells/cm^2 and cultured in DMEM at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Immunohistochemistry of mannose receptors on macrophages

After the macrophages were washed twice with PBS, cells were fixed with 4% paraformaldehyde in PBS for 10 min. Next, the cells were washed with 10 mM glycine in PBS in order to quench the aldehyde group, and then, they were incubated with 3% BSA in PBS for 30 min at room temperature to accomplish effective blocking. After washing the cells with 10 mM glycine in PBS, the cells were incubated with 10 μg/ml FITC labeled with anti-CD206 antibody (MCA2235FA) (AbD Serotec, Kidlington, UK) for 1 h at room temperature under shading. The cells were washed with 0.1% BSA in PBS 3 times and then incubated with 10 μg/ml DAPI

(4′,6-diamidine-2′-phenylindoledihydrochloride) for 1 min in order to stain the nuclei. After washing once with PBS, cover glasses were mounted on slide glasses with 50% glycerol-2.5% DABCO (1,4-diazabicyclo-[2,2,2]octane) (Sigma Chemical Co., Inc., St. Louis, MO, USA)- PBS. The samples were observed using fluorescent microscopy (Biozero, Keyence Corp., Osaka, Japan).

Uptake of NFκB decoy in macrophages

In order to allow observation by microscopy, macrophages were cultured on cover glasses. Macrophages were treated with rhodamine-NFκB decoy, Man-liposome/rhodamine-NFκB decoy complexes, or Gal-liposome/NFκB decoy complexes in OptiMEM® for 6 h before being challenged with 200 ng/ml LPS in OptiMEM® in 4 h at 37°C. Then, the cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 10 min. Cell nuclei were stained with 10 μg/ml DAPI for 1 min. After washing once with PBS, cover glasses were mounted on slide glasses and observed by fluorescent microscopy (Biozero, Keyence Corp., Osaka, Japan).

Measurement of cytokine and NFκB

For the measurement of cytokines and NFκB, primary cultured macrophages were cultured with naked NFκB decoy, Man-liposome/NFκB decoy complexes, Gal-liposome/NFκB decoy complexes, or Man-liposome/random decoy complexes with or without mannose in OptiMEM® for 6 h at 37°C. Then, the cells were cultured in 0.2 μg/ml LPS in OptiMEM® for 4 h at 37°C. When the effect of backbone of NFκB decoy was investigated, NFκB decoy and LPS were co-cultured for indicated time. After that, the supernatants were collected and used for the determination of TNF-α using an ELISA kit (eBioscience, San Diego, CA, USA). Nuclear

extracts of macrophages were prepared using the Nuclear/Cytosol fractionation kit (Pierce, USA). The amounts of activated NFκB in the nuclear extract of cells were measured using a Chemiluminescent electrophoretic mobility shift assay (EMSA) kit (Pierce Biotechnology, Inc., USA) according to the manufacturer's protocol. The competition reaction was measured by using a 2000-fold molar excess of unlabeled NFκB probe.

Osteoclastogenesis assay

After our macrophages were incubated with 0.2 μg/ml LPS in OptiMEM® for 4 h, supernatant were mixed with DMEM at a volume ratio of 1:3 and then MCSF and RANKL were added to the medium at the final concentration of 10 ng/ml for each (called conditioned medium). After replacing the medium, bone marrow cells were incubated in the conditioned medium for 72 h. The cells were washed and fixed with 10% formalin. Finally, the cells were stained with tartrate-resistant acid phosphatase (TRAP) and observed using a lightmicroscope (Biozero, Keyence Corp., Osaka, Japan).

Statistical analysis

Statistical comparisons were performed using a two-tail unpaired Student's *t* test for 2 groups. A value of $p < 0.05$ was considered statistically significant. For more than 3 groups, data were analyzed by ANOVA, and Bonferroni/Dunn procedure was used as a post hoc test.

RESULTS AND DISCUSSION

Particle size and zeta potential of Man-liposomes and their complexes with NFκB decoy

In our study, Man-liposomes were prepared using DOPE and Man-C4-Chol as carriers for NFκB decoy due to the molecule's ability to facilitate macrophage-targeted delivery. Firstly, the cationic charge on Man-C4-Chol would facilitate binding with the negative charge present on the NFκB decoy. Secondly, mannose molecules conjugated with the liposomes allowed specific binding to mannose receptors, which are present in large numbers on the surface of the macrophage. Finally, the helper lipid (DOPE) in Man-liposomes may accelerate the escape of NFκB decoy from the endosomes into the cytoplasm, thus avoiding lysosomal degradation. Then, NFκB decoy could be delivered to its active site in the cytosol and nuclei.

At first, we measured the mean diameter and zeta potential of Man-liposomes and Man-liposome/NFκB decoy complexes. The mean diameters of Man-liposome/NFκB decoy complexes were slightly higher than that of Man-liposome but still less than 100 nm. The Zeta potential of Man-liposomes/NFκB decoy complexes was slightly smaller than that of Man-liposomes (Table 1). This result suggests that the cationic charge was remaining on Man-liposome/NFκB decoy complexes. As a result, the cationic charge on the complexes could be used to aid in attachment to the surface of the cells.

Inhibitory effect of Man-liposomes/NFκB decoy complexes on TNF-α production in macrophages

The expression of mannose receptor on primary cultured macrophages isolated form mice was immunehistchemically stained. Although the expression of mannose receptor was not observed on the next day after cells were harvested, the expression of mannose receptor was

recovered on 3rd and 4th days (Fig. 1). To evaluate the suitability of Man-liposome/NFκB decoy complexes for use in the treatment of chronic inflammatory conditions, the decoy's inhibitory effect on TNF-α production was investigated in primary cultured macrophages. LPS-induced TNF-α production was significantly inhibited by Man-liposome/NFκB decoy complexes but not by naked NFκB decoy (Fig. 2A). It was noted that the strength of the inhibition of TNF-α production was directly proportional to the amount of NFκB present in the complex with Man-liposome (Fig. 2A). In the presence of excessive amounts of mannose, the inhibitory effect on TNF-α production by Man-liposome/NFκB decoy complexes was not observable. On the other hand, the inhibitory effect of Gal-liposome/ NFκB decoy complexes was not affected by the presence of excessive amounts of mannose (Fig. 2B). These results suggest that mannose receptors mediate the uptake of Man-liposome/NFκB decoy complexes by macrophages. Gal-liposome/NFκB decoy complexes also surpress TNF-α production to some extent. In orderto make complexes electrostatically with oligonucleotide, Man-C4-Chol and Gal-C4-Chol have positive charge on itself. Therefore, the data of zeta potential demonstrated thta Man- or Gal-liposome and its complexes with oligonucleotide have positive charge. The inhibitory effect of Gal-liposome/NFκB decoy complexes would be explained by the effect of the positive charge. However, in our previous reports, the effect of positive charge on the complexes is enough small for targeted delivery through the receptor mediated uptake in vivo (23, 24)

In order to evaluate the effect of the sequence of decoy on TNF-α production, random decoy, which did not include the NFκB-binding sequence was transfected to macrophage by Man-liposome. While Man-liposome/NFκB decoy complexes effectively suppressed LPS-induced TNF-α production from macrophages, Man-liposome/random decoy complexes did not inhibit

LPS-induced TNF-α production from macrophages, suggesting that the strong inhibitory effect of Man-liposome/NFκB decoy complexes was dependent on the sequence (Fig. 3A).

An oligonucleotide with a "natural" phosphodiester backbone is rapidly degraded by nucleaseses (28). In order to prevent degradation by nucleases, several types of chemically modified oligonucleotides were developed using phosphorothioate, methylphosphonate, and phosphoramidate (29,30). Of these chemically modified oligonucleotides, phosphorothioate-modified oligonucleotide advanced in clinical trials (30). NFκB decoy with phosphorothioate modification was also reported in vivo and in vitro (31). However, previous reports demonstrated that unexpected immunostimulation was caused by phosphorothioate modification in cultured murine splenic lymphocytes (32) and in cultured human B cells (33). In order to investigate the effect of phosphorothioate modification, we compared the inhibitory effect of NFκB decoy on TNF-α production with a NFκB decoy that had been modified with phosphorothioate. Both naked NFκB decoy with the natural phosphodiester backbone and that with phosphorothioate modification did not inhibit LPS-induced TNF-α production from macrophages (Fig. 3B). Man-liposome complexed with NFκB decoy having a phosphodiester backbone or thioate bond showed the same extent of inhibition on LPS-induced TNF-α production from macrophages (Fig. 3B). This result indicated that phosphorothioate modification does not influence the inhibitory effect of NFκB decoy in cultured macrophages in this extent of decoy's concentration.

Intracellular accumulation of Man-liposomes/NFκB decoy complexes in macrophages

In order to evaluate mannose receptor mediated uptake of Man-liposome/NFκB decoy complexes, intracellular accumulation of a rhodamine-NFκB decoy transfected by Man-liposome

or Gal-liposome were observed by using fluorescent microscopy. Intracellular accumulation of rhodamine-NFκB decoy was more enhanced by Man-liposome than by Gal-liposome (Fig. 4). Moreover, excessive amounts of mannose strongly inhibited the uptake of Man-liposome/rhodamine-NFκB decoy complexes but not Gal-liposome/rhodamine-NFκB decoy complexes (Fig. 4). These results correlated well with the result that the inhibitory effect of Man-liposome/NFκB decoy complexes on TNF-α production was suppressed by excessive amounts of mannose (Fig. 2A). These results support the hypothesis that mannose receptor mediates the uptake of Man-liposome/NFκB decoy complexes.

Previously, we have demonstrated that intravenously injected Man-liposome/NFκB decoy complexes accumulated in liver nonparenchymal cells consisting of Kupffer cells and endothelial cells, both of which express mannose receptors (24). Moreover, intratracheally injected Man-liposome/NFκB decoy complexes accumulated in macrophages isolated from lung tissue (34). In this study, mannose receptor-mediated uptake of Man-liposome/NFκB decoy complexes was directly demonstrated using primary cultured macrophages, for the first time, by the observed inhibitory effect of mannose. These data support the existence of a relationship between the recognition of mannose receptors and macrophage-targeted delivery of NFκB decoy by Man-liposome in mice.

Inhibitory effect of Man-liposomes/NFκB decoy complexes on NFκB activation in macrophages

NFκB is a transcription factor that regulates the expression of various cytokines in macrophages. In response to stimuli, IκB protein degraded and allowed NFκB to translocate into the nuclei. Activated NFκB was captured by the transfected NFκB decoy and as a result, the transcription of NFκB could be suppressed (13). To investigate NFκB activity, the amount of

NFκB in the nuclei was measured using EMSA. The amount of activated NFκB dramatically increased when macrophages were cultured with LPS (Fig. 5). When an excessive amount of non-labeled anti NFκB probe was applied to the nuclear extract of LPS-treated macrophages, the signal band became pale. This suggests that the signal band is actually the band of NFκB. This LPS-induced activation of NFκB could be effectively suppressed by Man-liposome/NFκB decoy complexes but not by naked NFκB decoy or Man-liposome/random decoy complexes (Fig. 5). This result scorrelated well with the inhibitory effect on TNF-α production (Fig. 2B, 3A).

Inhibitory effect of Man-liposomes/NFκB decoy complexes on osteoclastgenesis

In order to investigate whether the suppression of cytokine production in macrophages could prevent osteoclastogenesis, marrow cells were cultured in conditioned media in the presence of RANKL and M-CSF. The conditioned media was collected from LPS-treated macrophages and contained several cytokines such as TNF-α. The number of TRAP-positive cells was significantly larger than the bone marrow cells treated with RANKL and M-CSF, but not the cells cultured in media collected from LPS-treated macrophages. This suggests that secretions from macrophages could enhance osteoclastogenesis (Fig. 6). This increase in the number of TRAP-positive cells was not observed when bone marrow cells were cultured in the media that was collected from LPS-treated macrophages cultured with Man-liposome/NFκB decoy complexes (Fig. 6). On the other hand, the number of TRAP-positive cells, which were cultured in the media obtained from LPS-treated macrophages that had been cultured with naked NFκB decoy or Man-liposome/random decoy complexes, was smaller when compared with those cultured in conditioned media from LPS-treated macrophages that had been exposed to Man-liposome/NFκB decoy complexes. These results suggest that secretion under the regulatory control of NFκB in macrophages may promote osteoclastogenesis in bone marrow cells.

Therefore, inhibition of NFκB activation in macrophage might be sufficient to suppress osteoclastogenesis.

RANKL and TNF-α activate p50/RelA dimers in the canonical NFκB pathways in osteoclast precursors, which then promote osteoclast differentiation (13). Thus, NFκB in osteoclast precursors is believed to play a pivotal role in osteoclastogenesis. Shimizu et al. demonstrated that the transfection of naked NFκB into osteoclast precursor cells prevents osteoclastogenesis induced by 1,25-dihydroxyvitamin D3 or combinational stimulation of RANKL and M-CSF in culture cells. Moreover, when naked NFκB decoy is transfected in an ovariectomy rat model via the implantation of osmotic minipumps containing NFκB decoy, the increase in the number of TRAP-positive cells and the decrease of Ca in the bone was inhibited (35) Thus, transfection of NFκB decoy is likely to be an effective therapy for bone loss. However, the uptake of naked oligonucleotides is extremely low due to its negative charge. Moreover, the possible dose of NFκB decoy for intraarticular injection was limited by the narrow space of the articular cavity. Furthermore, although targeted delivery of NFκB decoy to osteoclast precursor cells is important, the multiplicities in the expression of cell surface protein on precursor cells serves as an obstacle in the development of a delivery system for osteoclast precursor cells. After having taken all of these factors into consideration, it is apparent that the inhibition of cytokine production from macrophages by Man-liposome/NFκB decoy complexes is a potential approach for preventing bone loss.

CONCLUSIONS

We demonstrated, for the first time, the enhanced uptake of NFκB decoy complexes and the inhibitory effect on NFκB activation and TNF-α production in LPS-treated macrophages by Man-liposomes/NFκB decoy complexes in primary cultured macrophages. Moreover, osteoclastogenesis was effectively induced by the conditioned medium prepared by using activated macrophages, but it was induced to a lesser extent by the conditioned medium prepared by activated macrophages treated with Man-liposomes/NFκB decoy complexes. Although further studies using bone loss model mouse are required, these results suggest that Man-liposomes/NFκB decoy complexes may be a promising strategy for preventing bone loss.

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NOTATIONS

ABBREVIATIONS

NFκB	nuclea factor kappa B
TNFα	tumor necrosis factor alpha
Man-liposome	mannosylated cationic liposome
RA	rheumatoid artitis
RANKL	receptor activator NF-kappaB ligand

Figure legends

Fig. 1 Immunohistochemical staining of mannose receptors on primary cultured macrophages. Macrophages isolated from ICR mice were plated for 1, 2, 3, and 4 days, and labeled with anti CD206 antibody (green) and DAPI (blue).

Fig. 2 Inhibitory effect on TNF-α production. (**A**) Effect of NFκB decoy dose. Primary cultured macrophages were cultured with naked NFκB decoy (open circle) or Man-liposome/NFκB decoy complexes (closed circle) for 6 h at concentrations of 0, 0.5, 1, and 2 μM. After the cells were washed with PBS, cells were cultured in OptiMEM containing LPS (200 ng/ml) for 4 h. Significant difference *** $P < 0.001$, v.s. (naked NFκB decoy). (**B**) Effect of excessive amounts of mannose. Primary cultured macrophages were cultured with Man-liposome/NFκB decoy complexes, or Gal-liposome/NFκB decoy complexes for 6 h at concentrations of 2 μM with (white) or without (black) 20 mM mannose. After cells were washed with PBS, cells were cultured in OptiMEM containing LPS (200 ng/ml) for 4 h.

Fig. 3 Inhibitory effect on TNF-α production. (**A**) Effect of sequence of NFκB decoy. Primary cultured macrophages were cultured with naked NFκB decoy, Man-liposome/NFκB decoy complexes, or Man-liposome/random decoy complexes for 6 h at a concentration of 2 μM. After the cells were washed with PBS, cells were cultured in OptiMEM containing LPS (200 ng/ml) for 4 h. Significant difference ### $P < 0.001$, v.s. (LPS), *** $P < 0.001$, v.s. (naked NFκB decoy), ††† $P < 0.001$ v.s. (Man-liposome/random decoy). (**B**) Effect of backbone in NFκB decoy. Primary cultured macrophages were cultured with naked NFκB decoy with a phosphodiester backbone (open triangle) or phosphorothioate modification (closed triangle), or

NFκB decoy with a phosphodiester backbone (open circle) or phosphorothioate modification (closed circle) complexed with Man-liposome for 1, 3, 6 and 12 h at a concentration of 2 μM under existence of LPS (200 ng/ml). As a control, cells (without NFκB decoy) were cultured with (closed square) or without (cross) LPS (200 ng/ml). Significant difference *** $P < 0.001$, v.s. (naked NFκB decoy with a phosphorothioate backbone), ††† $P < 0.001$ v.s. (naked NFκB decoy with a phosphodiester backbone).

Fig. 4 Intracellular accumulation of rhodamine-NFκB decoy, Man-liposome/rhodamine-NFκB decoy or Gal-liposome/rhodamine-NFκB decoy with or without excessive amounts of mannose. Primary cultured macrophages were cultured with (**A**) rhodamine-NFκB decoy (red), (**B**) Man-liposome/rhodamine-NFκB decoy (red), or (**C**) Gal-liposome/rhodamine-NFκB decoy (red) for 6 h at 2 μM with or without 20 mM mannose. After the cells were washed with PBS, they were cultured in OptiMEM containing LPS (200 ng/ml) for 4 h. Then, the nuclei were stained with DAPI (blue) after fixing with paraformaldehyde.

Fig. 5 Inhibitory effect on NFκB activation. Primary cultured macrophages were cultured with naked NFκB decoy, Man-liposome/NFκB decoy complexes, or Man-liposome/random decoy complexes for 6 h at 2 μM. After the cells were washed with PBS, they were cultured in OptiMEM containing LPS (200 ng/ml) for 4 h. Then, nuclear protein was extracted and evaluated using EMSA.

Fig. 6 Inhibitory effect of osteoclastogenesis. Primary cultured macrophages were cultured with naked NFκB decoy, Man-liposome/NFκB decoy complexes, or Man-liposome/random decoy

complexes for 6 h at 2 μM. After the cells were washed with PBS, they were cultured in OptiMEM containing LPS (200 ng/ml) for 4 h. Then, conditioned media was prepared with collected conditioned medium and DMEM. Bone marrow cells were cultured in the conditioned medium for 72 h. After fixing with 10% formalin, the cells were stained with TRAP.

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Table 1 Mean particle sizes and zeta potential of liposomes and complexes (Man-liposome; Man-C4-Chol :DOPE = 3: 2)

	Mean diameter (nm)	Zeta potential (mV)
Man-liposomes	63.8 ± 0.6	42.9 ± 0.7
Man-liposome/ NFκB decoy complexes	87.5 ± 5.0	36.1 ± 0.3

Figure 1

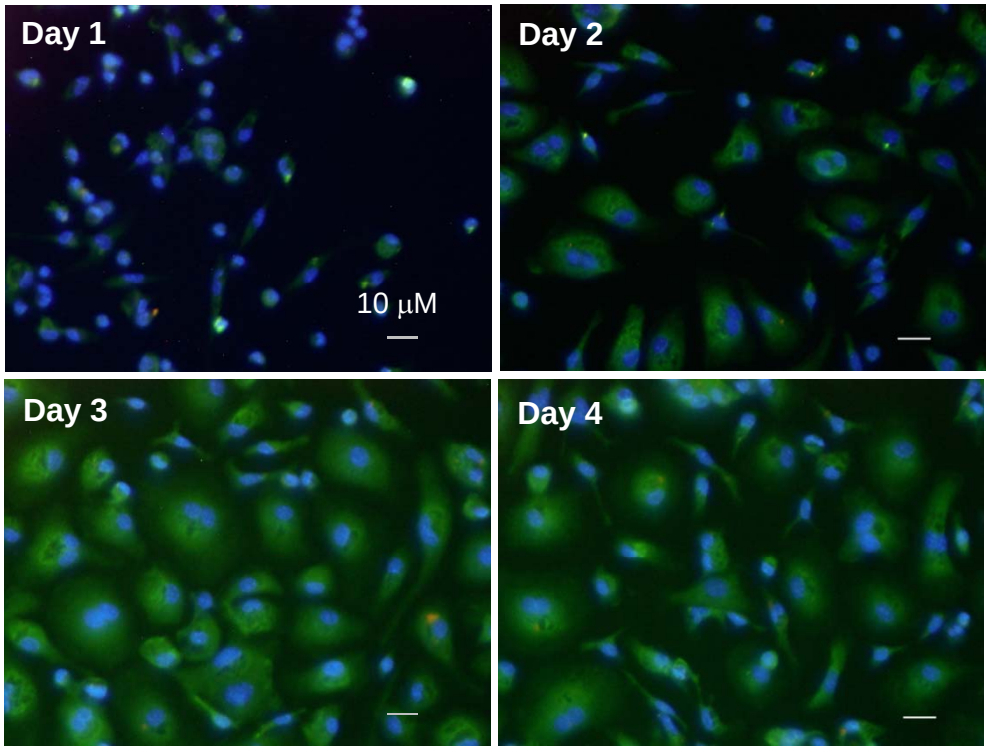
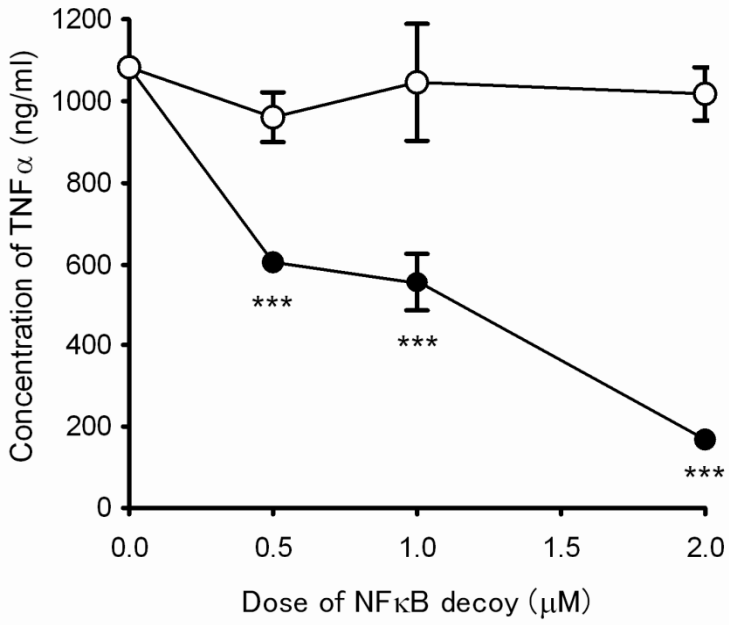


Figure 2

(A)


Concentration of TNFα (ng/ml)
1200
1000
800
600
400
200
0
***
***
***
0.0
0.5
1.0
1.5
2.0
Dose of NFκB decoy (μM)

(B)

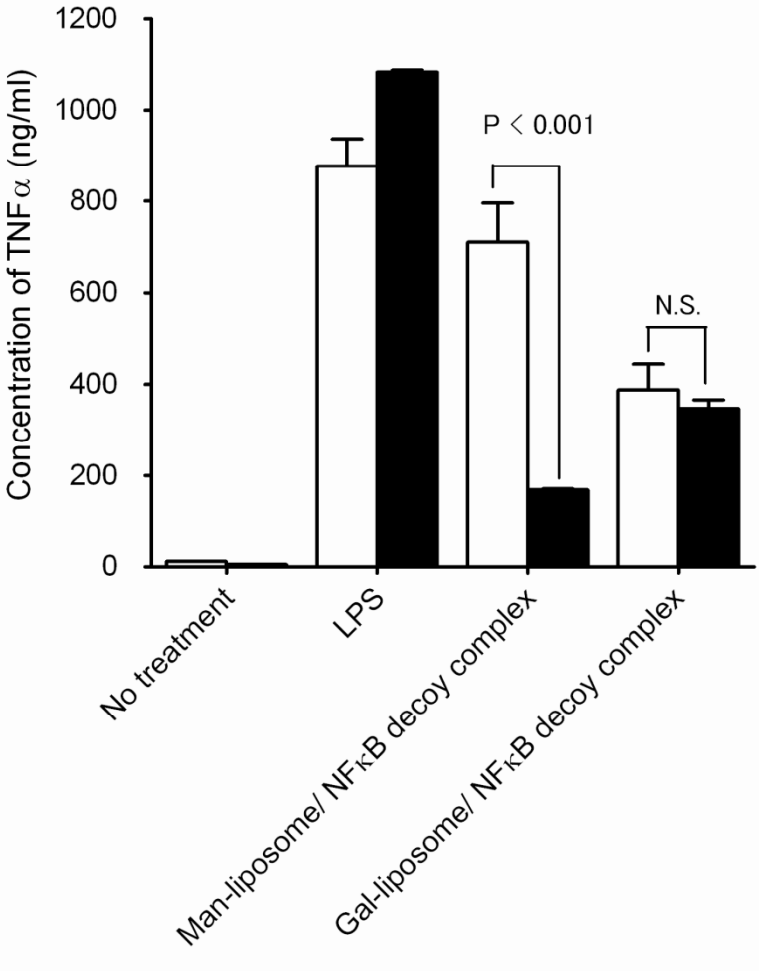

Concentration of TNFα (ng/ml)
1200
1000
800
600
400
200
0
P < 0.001
N.S.
No treatment
LPS
Man-liposome/ NFκB decoy complex
Gal-liposome/ NFκB decoy complex

Figure 3

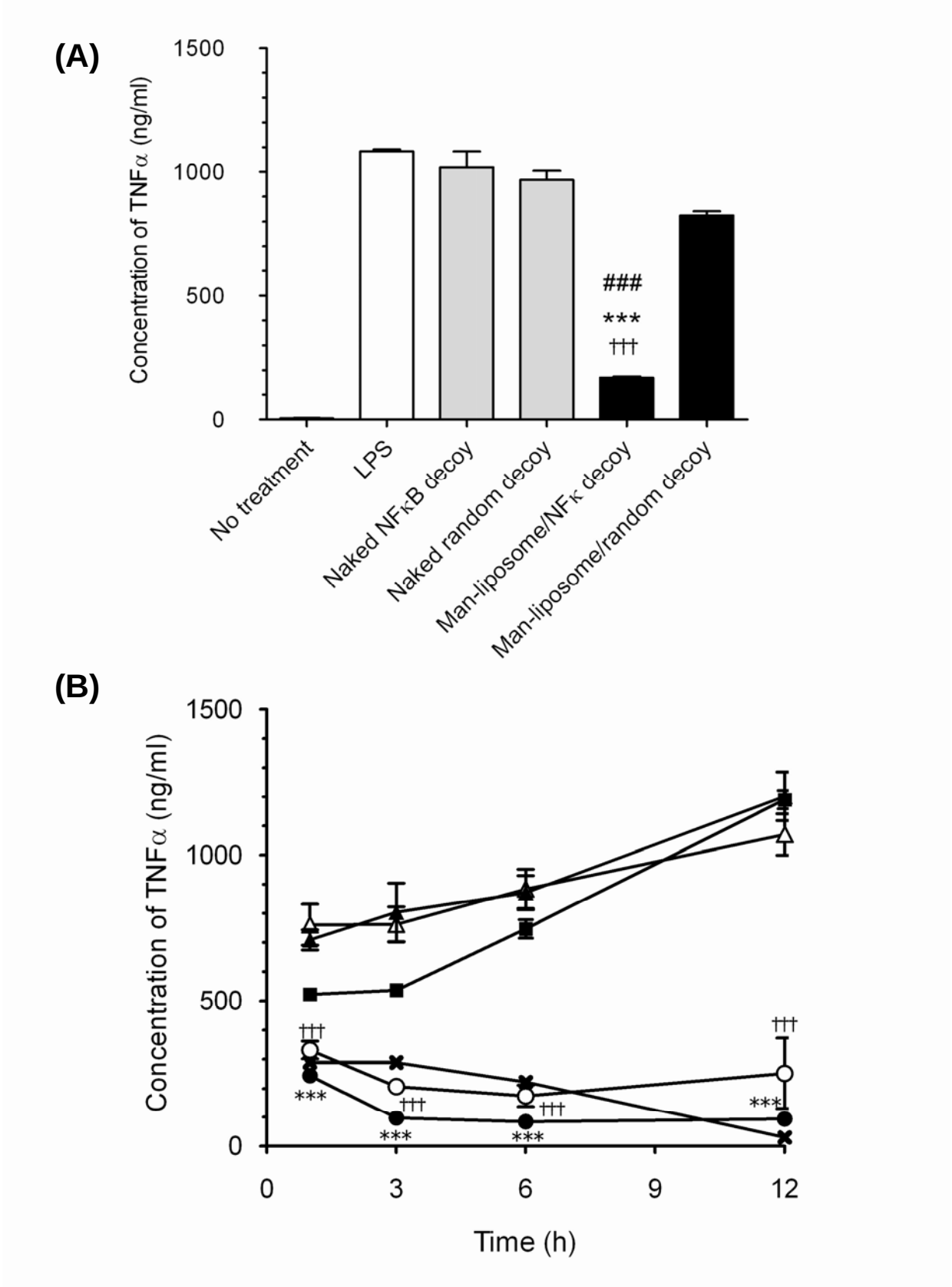
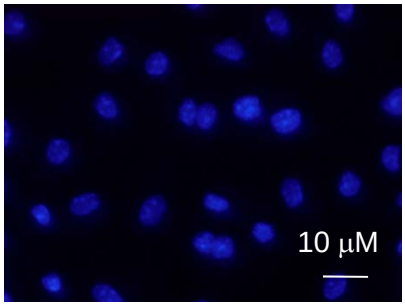
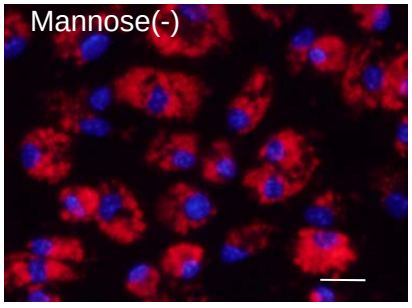

(A)
Concentration of TNFα (ng/ml)
1500
1000
500
0
###
***
†††
No treatment
LPS
Naked NFκB decoy
Naked random decoy
Man-liposome/NFκ decoy
Man-liposome/random decoy
(B)
Concentration of TNFα (ng/ml)
1500
1000
500
0
0
3
6
9
12
Time (h)
***
†††

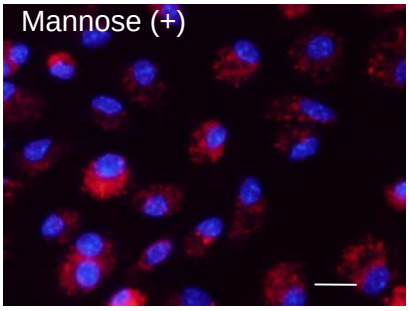
Figure 4

(A) Rhodamine-NFκB decoy

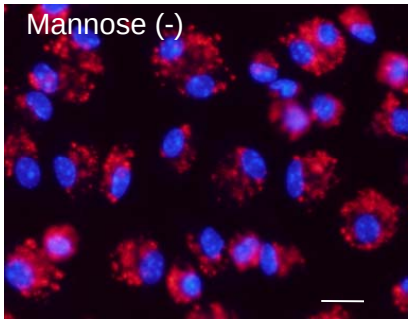


(B) Man-liposome/Rhodamine-NFκB decoy





(C) Gal-liposome/Rhodamine-NFκB decoy



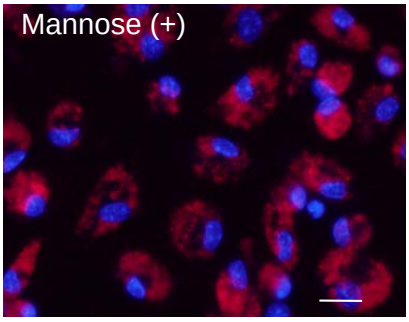


Figure 5

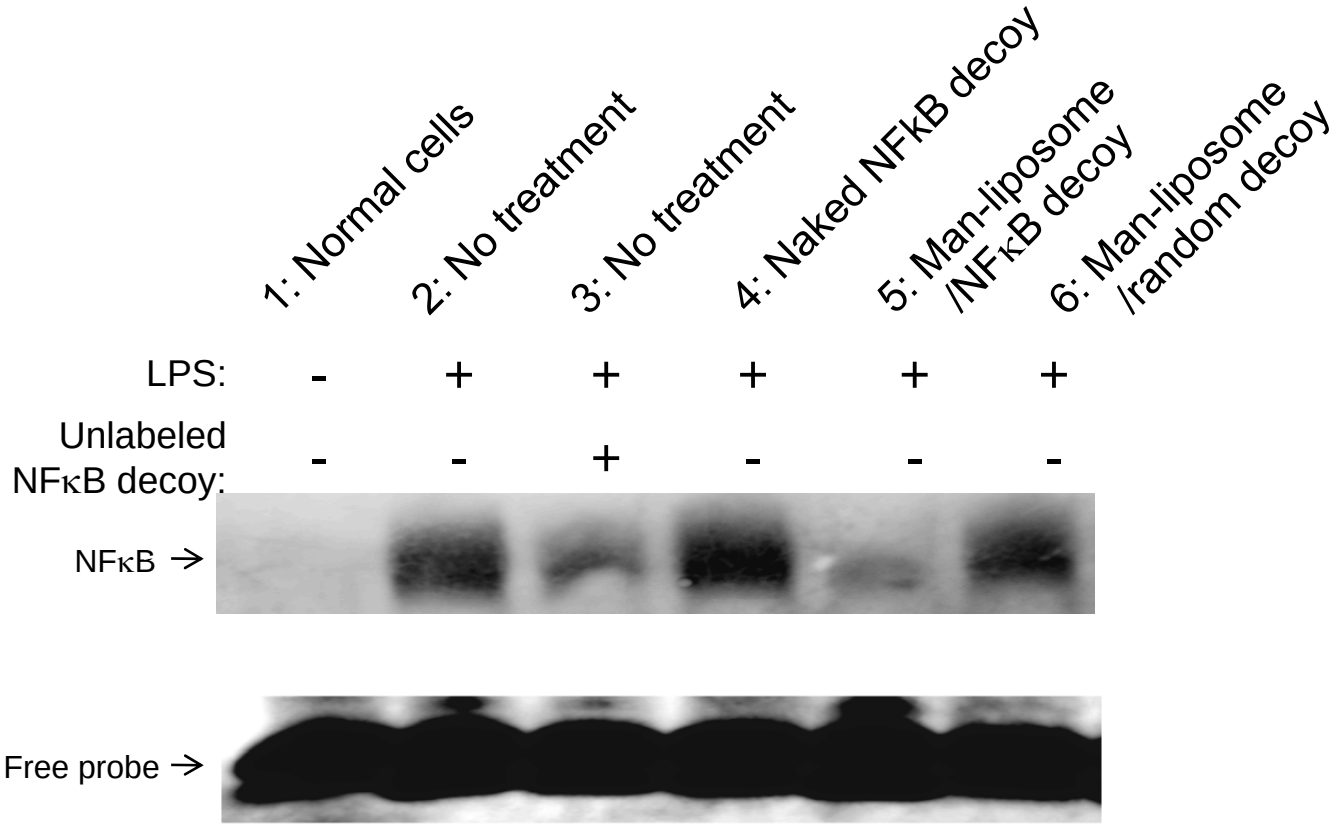
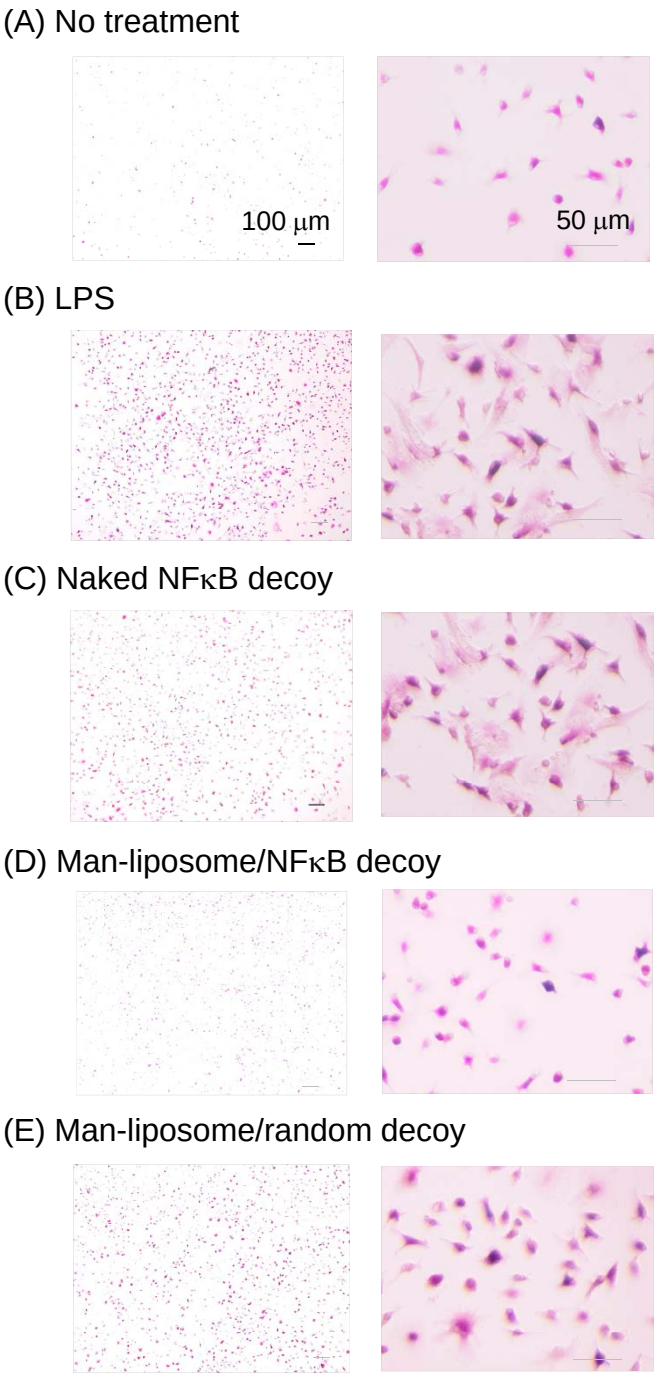

1: Normal cells
2: No treatment
3: No treatment
4: Naked NFkB decoy
5: Man-liposome /NFκB decoy
6: Man-liposome /random decoy
LPS: - + + + + +
Unlabeled NFκB decoy: - - + - - -
NFκB →
Free probe →

Figure 6


(A) No treatment
100 μm
50 μm
(B) LPS
(C) Naked NFκB decoy
(D) Man-liposome/NFκB decoy
(E) Man-liposome/random decoy