Roxatidine inhibits fibrosis by inhibiting NF-κB and MAPK signaling in macrophages sensing breast implant surface materials

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Abstract. Capsular contracture is an important complication after silicone mammary implant surgery. Fibroblasts and macrophages play critical roles in the pathogenesis of capsular contracture, making these two cell types therapeutic targets. It has been reported that inhibiting histamine receptors results attenuates fibrosis, but the role of roxatidine (a histamine receptor 2 inhibitor) in preventing fibrosis caused by breast implant materials remains unknown. The aim of the present study was to assess the hypothesis that roxatidine might have a prophylactic effect in capsular contracture induced by implant material. Inflammation induced by breast implant materials was mimicked by co-culturing macrophages or fibroblasts with these materials *in vitro*. Capsular contracture was modeled in mice by planting breast implant materials in a subcutaneous pocket. Roxatidine was added in the culture medium or administered to mice bearing breast implant materials. By co-culturing macrophages or fibroblasts with common breast implant materials (micro-textured or smooth breast implants), the present study demonstrated that macrophages respond to these materials by producing pro-inflammatory cytokines, a process that was abolished by addition of roxatidine to the culture medium. Although fibroblasts did not respond to implant surface materials in the same way as macrophages, the conditioned media of macrophages induced proliferation of fibroblasts. Mechanistically, administration of roxatidine inhibited activation of NF-κB and p38/mitogen-activated protein kinase (MAPK) signaling in macrophages. Furthermore, treatment with roxatidine in implant-bearing mice reduced serum concentrations of transforming growth factor-β and the abundance of fibroblasts around the implant. The present study concluded that roxatidine plays an important role in preventing fibrosis by inhibiting activation of NF-κB and p38/MAPK signaling in macrophages.

Introduction

Silicone mammary implants (SMI) have been widely used for breast augmentation worldwide, making these materials the most commonly consumed in aesthetic surgery (1-3). It has been reported that approximately two million breast augmentation procedures are performed annually worldwide (4). Systemic and local complications after SMI breast augmentation surgery are often observed in patients, among which capsular contracture is the most common complication, with an incidence of >20% (5,6).

Capsular contracture is a serious complication and may resulted in distorted breast shape, pain and other symptoms (5,6). Based on clinical presentation, capsular contracture after breast augmentation is classified into 4 stages by the Baker grading system (7). Although a number of strategies have been proposed to prevent capsular contracture, such as different implant choices and improved surgical technique, the prevalence of this complication has not yet decreased (8). One strategy has been to preferentially choose textured materials over smooth breast implants (9,10). However, a fraction of patients would eventually undergo re-operation for capsular contraction, especially those with Baker-IV capsules, despite application of textured materials (9,10). As capsular contracture is a consequence of inflammation induced by the implanted silicone material surface (5,6), it is important to understand the mechanism of capsular contracture formation and the corresponding prophylaxis.

Fibroblasts and macrophages are major components in fibrotic capsules related to breast implants (10). Fibroblasts play a critical role in the pathogenesis of fibrotic capsules. For example, fibroblasts are enriched at the 'contact zone' of the implant and at the capsule responsible for initial capsule formation, by producing collagen fibres (11-14). The abundance of fibroblasts is positively associated with the severity of capsular contracture measured by the Baker grade (15). On the other hand, macrophages are key players in initiating tissue repair and remodelling (16). As it has been reported that

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macrophages enter the wound before fibroblasts do (17,18), it is possible that macrophages may have an influence on fibroblast functions. However, the crosstalk between macrophages and fibroblasts has not been explored.

Histamine was recognized to promote collagen formation more than four decades ago (19). Although it has been reported that blocking of histamine receptor (HR) 1 and 2 has inhibitory roles in collagen formation, these studies primarily focused on mast cells (15,20). Therefore, whether other cell types are involved in histamine-mediated collagen formation remains unknown. The aim of the present study was to assess the hypothesis that the HR2 inhibitor roxatidine can prevent capsular contracture after implantation. In this study, the effect of roxatidine on macrophages and fibroblasts during the pathogenesis of capsule formation was investigated.

Materials and methods

Ethics consideration. The ethics committee of the Fourth Affiliated Hospital of Harbin Medical University approved and supervised the research proposal (approval number, 170023).

Silicone surface particles. Spherical particles with a diameter of 6 mm were prepared from silicone implant envelopes under sterile conditions. The two most common materials in clinical practice were utilised, Mentor® Perthese™ micro-textured (MT) and smooth (SM) breast implants (21). Both implant materials were purchased from Johnson & Johnson, Inc.

Treatment. Roxatidine acetate (hydrochloride) was purchased from Sihuan Pharmaceutical Co., Ltd. For *in vitro* studies, roxatidine was dissolved in 0.05% DMSO (diluted in PBS). For *in vivo* studies, roxatidine was mixed with autoclaved tap water in a bolus of 100 μl.

Cell lines and culture. The murine macrophage cell line RAW 264.7 (ATCC) and the fibroblast cell line L929 (ATCC) were utilized in the present study. Both cell lines were cultured in α-Modified Eagle's Medium (α-MEM; Thermo Fisher Scientific, Inc.) supplemented with L-glutamine (Thermo Fisher Scientific, Inc.) and 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2. For pre-treatment with roxatidine, roxatidine (25 μM) was added to the culture medium for 1 h at 37˚C (22). For controls, vehicle (0.05% DMSO) was added to the control wells for 1 h at 37˚C. Subsequently, RAW 264.7 macrophages (1×10^4) were co-cultured with different silicone surface particles for 24 h at 37˚C, and then the conditioned media was collected for future L929 stimulation. Stimulated RAW 264.7 cells were then cultured in serum-free medium for another 24 h at 37˚C, by the end of which the cells and media were used for reverse transcription-quantitative PCR (RT-qPCR) and ELISA analyses, respectively. Cells in the wells without addition of silicone implant surface materials served as controls. Cells were collected by straining culture media through a 100 μm cell strainer. Following centrifugation at 400 x g for 10 min at 4˚C, cell-free culture media were used for ELISA analyses, whereas cells were lysed for RT-qPCR analyses. Following the 1 h culture in the presence or absence of roxatidine, L929 fibroblast cells (1×10^4) were co-cultured with different

silicone surface particles or the macrophage-conditioned media for 24 h at 37˚C. For culture of L929 cells using macrophage-conditioned media, at the end of the 24 h culture the media was replaced with serum-free media for another 24 h at 37˚C, to perform ELISA and RT-qPCR analyses. For L929 proliferation analyses, implant materials were added to the wells, and fresh complete α-MEM was changed every 24 h at 37˚C. To neutralize effects of TGFβ, TGFβ neutralizing antibodies (10 μg/ml, R&D Systems, Inc., cat. no. AB-100-NA) were applied to the culture medium when the breast implant materials were added or the conditional medium was used for L929 cells; whereas isotype control antibodies (Rabbit IgG, 10 μg/ml, R&D Systems, Inc., cat. no. AB-105-C) were used as control. Experiments (n=6 wells/group) were conducted in triplicate.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from RAW 264.7 and L929 cells using a commercial kit (RNeasy Mini kit; Qiagen GmbH) following the manufacturer's instructions. All RNA samples were stored at -80˚C until analysis. Total RNA (2 μg) was reverse-transcribed to cDNA using an RT kit (High-Capacity cDNA Reverse Transcription kit, Thermo Fisher Scientific, Inc.), according to the manufacturer's manual. qPCR for target genes and the housekeeping gene GAPDH were performed using the SYBR® Green Master mix (Thermo Fisher Scientific, Inc.) following the manufacturer's instructions. Relative mRNA expression values were normalized according to levels of GAPDH. The fold change of mRNA expression was calculated using the formula: $2^{-\Delta\Delta Cq}$ (23). The sequences of primers used are listed in Table I.

ELISA. ELISA was performed using R&D Systems DuoSet® kits equipped with corresponding mouse antibodies (R&D Systems, Inc., cat. nos.: IL1β, DY407; IL6, DY406; TNFα, DY470; and TGFβ, DY1679), according to the manufacturer's instructions. Briefly, a 96-well ELISA plate was incubated with the capture antibody (based on the target protein) overnight at room temperature. Following thorough washes using the Wash Buffer (R&D Systems, Inc., cat. no. WA126) the plate was blocked using 10% FBS at room temperature for 1 h. Cell culture media and standard samples were then added to the plate, followed by incubation at room temperature for 2 h. Biotinylated detection antibodies (provided with the DuoSet®kits) were then applied to the plate. Subsequently, the streptavidin-horseradish peroxidase solution was added and incubated for 20 min at room temperature, followed by the substrate solution for 20 min at room temperature. After adding the stop solution, the optical density of each well was detected by a microtiter plate reader at a wavelength of 450 nm.

Flow cytometry. At the end of culture, cells were collected from cell culture plates using 0.5 mM EDTA (Thermo Fisher Scientific, Inc.). Cells originating from tissue surrounding breast implant materials in mice were obtained using mechanical dissociation and enzymatic digestion, according to a previous paper (24). Cells were subsequently stained with antibodies listed in Table II for 30 min at 4˚C, at the designated dilutions. To analyze proliferation, cells were stained with anti-Ki67 antibodies. To analyze signalling, RAW 264.7

Table I. Primers used for RT-qPCR.

Target gene	Primers (5'→3')	
	Forward	Reverse
IL1β	AACCTGCTGGTGTGTGACGTTC	CAGCACGAGGCTTTTTGTTGT
IL6	GACAAAGCCAGAGTCCTTCAGAGAG	CTAGGTTTGCCGAGTAGATCTC
TNFα	GCCTCTTCTCATTCCTGCTTG	CTGATGAGAGGGAGGCCATT
TGFβ	GCTAATGGTGGACCGCAACAACG	CTTGCTGTACTGTGTGTCCAGGC
GAPDH	TTCTTGTGCAGTGCCAGCCTCGTC	TAGGAACACGGAAGGCCATGCCAG

IL, interleukin; TNF, tumor necrosis factor; TGFβ, transforming growth factor β.

Table II. Antibodies used for flow cytometry.

Antibody	Manufacturer	Cat. no.	Clone	Dilution
Ki67-PE	Abcam	Ab16667 (self-conjugated)	Polyclonal	1:100
p-p65-PE	eBioscience; Thermo Fisher Scientific, Inc.	MA5-15160 (self-conjugated)	T.849.2	1:100
p-p38-PE	eBioscience; Thermo Fisher Scientific, Inc.	MA5-15218 (self-conjugated)	C.7.8	1:100
αSMA-PE	Abcam	Ab7817 (self-conjugated)	1A4	1:100

PE, phycoerythrin; p, phosphorylated; SMA, smooth muscle actin.

macrophages were collected. Briefly, RAW 264.7 macrophages were cultured in serum-free media at 37˚C for 24 h. Roxatidine (25 μM) was subsequently added to the media 1 h prior to stimulation with silicone surface materials. Macrophages were collected for analysis of phosphorylation of NF-κB subunit p65 and p38 MAPK by flow cytometry 15 minutes after adding silicone surface materials to culture media. The primary gate of cells was set for viable cells according to the plots of forward scatter side scatter. Corresponding isotype control antibodies were used for setting the negative population. Cells were analyzed using an Attune NxT flow cytometer (Thermo Fisher Scientific, Inc.) and Kaluza software (version 2.1; Beckman Coulter, Inc.).

Mouse breast implant studies. Prepared breast implant surface materials were seeded into 8-week old Balb/c female mice (weight ~20 g, Jackson Laboratory), according to a published paper (25). Mice were maintained in the specific-pathogen-free environment (control at constant 23˚C and 40-60% humidity) with a 12-h light/dark cycle. Normal chow and water were allowed *ad libitum*. General anesthesia was administered by intraperitoneal injection of one dose of ketamine hydrochloride (100 mg/kg) and xylazine hydrochloride (10 mg/kg; Sihuan Pharmaceutical Co., Ltd.). Animals were maintained on a heated blanket at 37˚C. Each mouse received one piece of implant material (up to 6 mm in diameter) in a subcutaneous pocket surgically created in the left flank and the incision was closed using 6-0 Vicryl sutures (Ethicon, Inc.). No animals died during the experimental period. Starting the day after surgery for 14 days, implant-bearing mice were treated daily by oral gavage with roxatidine (15.62 mg/kg) or PBS (control) (26). At day 90 post-surgery, mice were sacrificed under anesthesia

and the fat tissue surrounding the implant material (5 mm distance from the implant surface) was collected for flow cytometry analysis of fibroblast abundance. Experiments (n=9 mice/group) were performed in duplicate.

Statistical analysis. Data are presented as the mean ± SD. Data were compared using the Student's t-test for two groups or one-way ANOVA followed by a Bonferroni post hoc test for more than two groups. Data with >2 groups at different time points were analyzed using two-way ANOVA with Tukey post hoc tests. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analyses were performed using GraphPad Prism software (version 7; GraphPad Software, Inc.).

Results

Responses of macrophages cultured with silicone implant surface materials in the presence or absence of roxatidine. RAW 264.7 murine macrophages (1×10^4 cells/well) were maintained in 6-well plates containing each of the aforementioned materials for 24 h at 37˚C. Proinflammatory cytokines, interleukin (IL) 1β, IL6, tumor necrosis factor α (TNFα) and transforming growth factor β (TGFβ), have been reported to have critical roles in fibrosis generation after breast implant surgery (4,27). Therefore, the levels of these cytokines were assessed. As shown in Fig. 1A, culture of RAW 264.7 cells with different silicone implant surface materials increased mRNA levels of the proinflammatory cytokines assessed. Similar results were also observed at the protein level (Fig. 1B).

As it has been reported that administration of roxatidine inhibits macrophage activation upon stimulation (22),
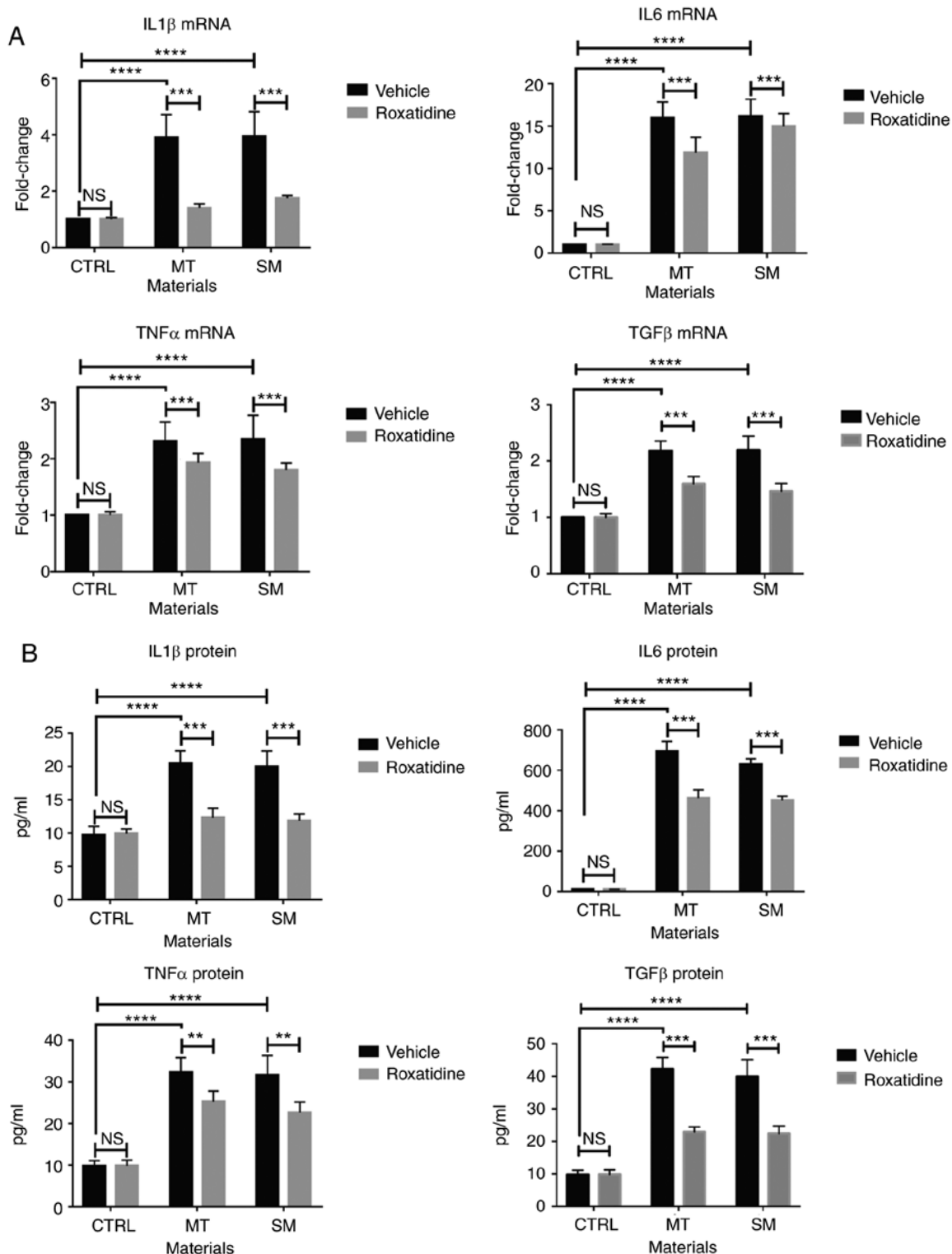
Figure 1. Pretreatment of roxatidine in the culture medium of macrophages stimulated with silicone surface materials reduced the production of proinflammatory cytokines. Cells were pretreated with roxatidine (25 μM) or vehicle for 1 h at 37˚C before adding implant materials. At the end of culture (24 h after co-culture with implant materials at 37˚C), cells were collected for another 24 h culture in serum-free medium at 37˚C for subsequent RT-qPCR and ELISA. (A) Administration of roxatidine reduced proinflammatory cytokine production by macrophages stimulated with breast implant materials at the mRNA level. (B) Roxatidine decreased secretion of proinflammatory cytokines by stimulated macrophages. n=6 wells/group, from one of triplicated experiments. n.s., not significant, **P<0.01, ***P<0.001 and ****P<0.0001, as indicated. Data were analyzed using one-way ANOVA followed by a Bonferroni post hoc test. RT-qPCR, reverse transcription-quantitative PCR; IL, interleukin; TNF, tumor necrosis factor; TGF, transforming growth factor; CTRL, control; MT, micro-textured; SM, smooth.

the subsequent investigation assessed whether roxatidine suppressed secretion of these proinflammatory cytokines by macrophages in a co-culture system with silicone implant surface materials. As shown in Fig. 1A and B, addition of roxatidine significantly reduced both the mRNA and protein levels of proinflammatory cytokines (P<0.01), indicating that roxatidine inhibited silicone implant material induced activation of macrophages.
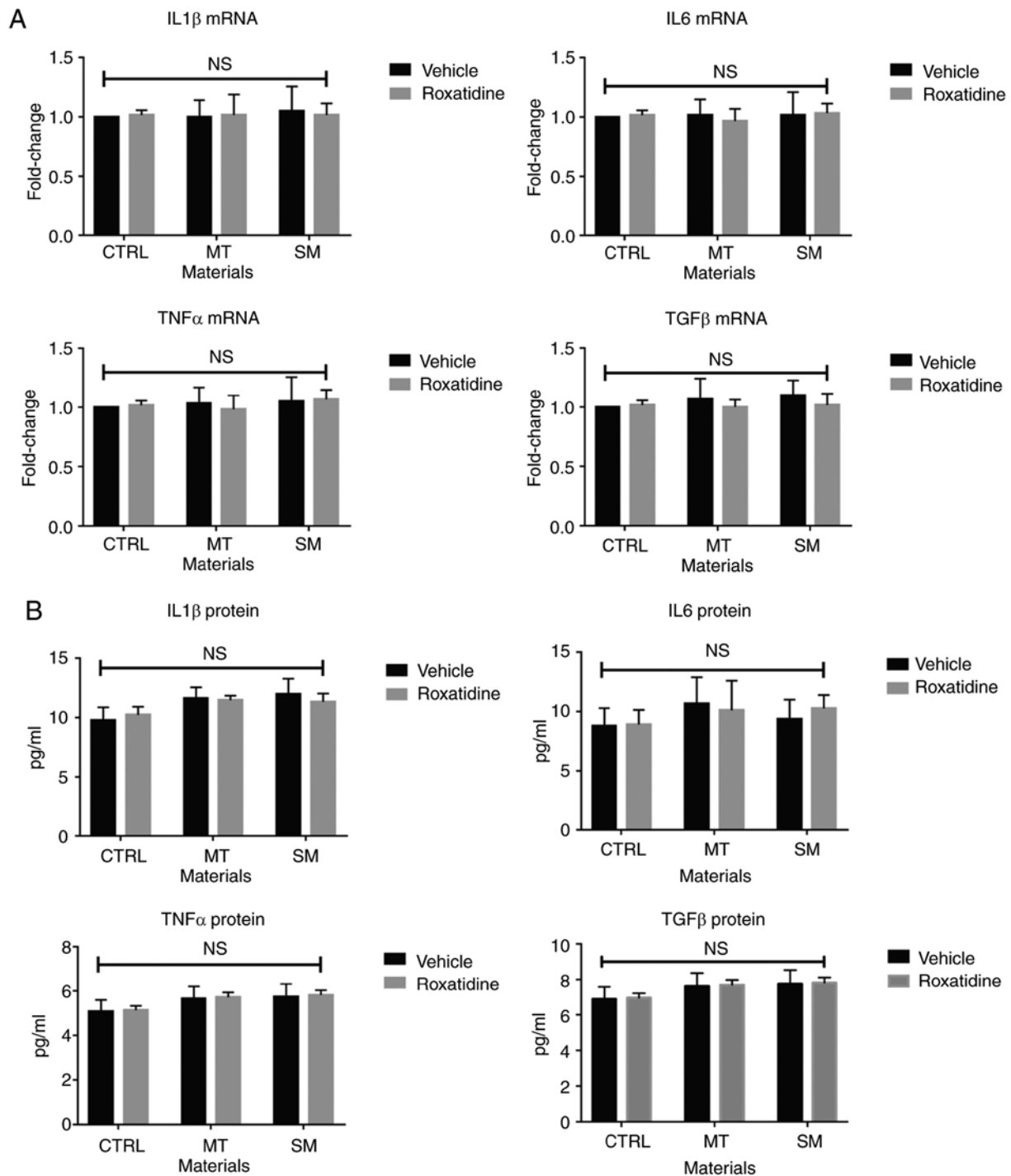
Figure 2. Pretreatment of roxatidine in the culture medium of fibroblasts stimulated with silicone surface materials did not affect production of proinflammatory cytokines. Cells were pretreated with roxatidine (25 μM) or vehicle for 1 h at 37˚C before adding implant materials. At the end of culture (24 h after co-culture with implant materials at 37˚C), cells were collected for another 24 h culture in serum-free medium at 37˚C for RT-qPCR and ELISA. (A) mRNA levels of proinflammatory cytokines. (B) Protein levels of proinflammatory cytokines. n=6 wells/group, from one of triplicated experiments. n.s., not significant, by one-way ANOVA among all groups. Data were analyzed by one-way ANOVA. RT-qPCR, reverse transcription-quantitative PCR; IL, interleukin; TNF, tumor necrosis factor; TGF, transforming growth factor; CTRL, control; MT, micro-textured; SM, smooth.

Roxatidine has no effect on cytokine production or proliferation of cultured L929 cells in the presence of silicone surface materials. Subsequently, the effects of roxatidine on cultured L929 fibroblast cells in the presence or absence of silicone surface materials were investigated. As shown in Fig. 2A and B, addition of silicone surface materials did not affect the production of TGFβ at either the mRNA or protein

level. Moreover, roxatidine treatment did not influence production of TGFβ by L929 fibroblast cells. Similarly, the levels of other proinflammatory cytokines did not change when L929 fibroblast were stimulated with or without the presence of roxatidine. The influence of roxatidine on L929 proliferation was then investigated, as excessive fibrosis caused by fibroblast proliferation is the main cause of implant failure (28). As
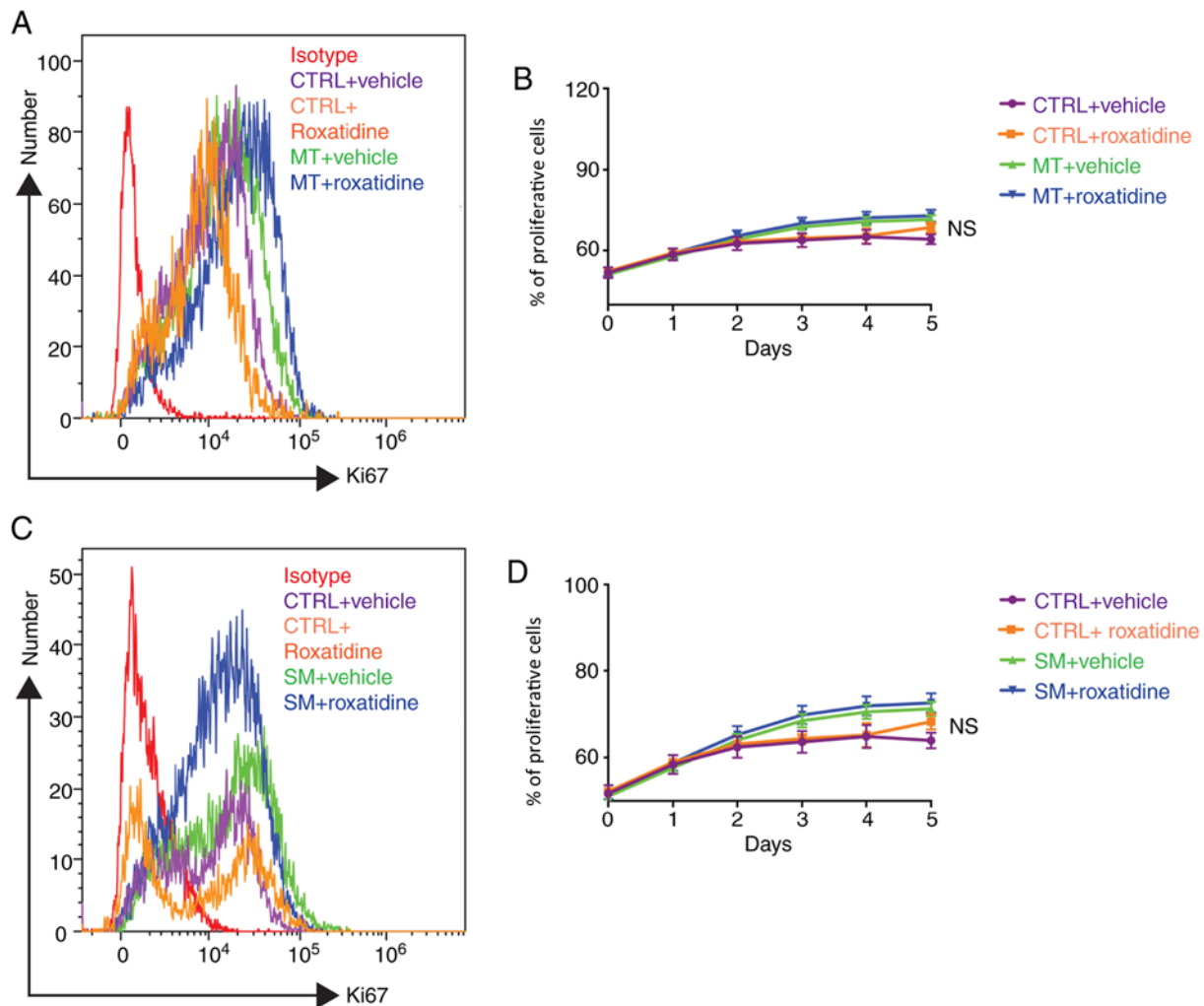
Figure 3. Addition of roxatidine to the culture medium of fibroblasts stimulated with silicone surface materials did not affect proliferation, as measured by Ki67 expression using flow cytometry. Cells were pretreated with roxatidine (25 μM) or vehicle for 1 h at 37˚C before adding implant materials. Fresh complete media was replaced every 24 h for up to 5 days. The primary gate of cells was set for viable cells according to the plots of forward scatter side scatter. (A) Representative histogram curves of Ki67 staining in fibroblasts stimulated with MT in the presence or absence of roxatidine at day 5. (B) Dynamic change in the percentage of Ki67-positive fibroblasts stimulated with MT in the presence or absence of roxatidine at day 5. (C) Representative histogram curves of Ki67 staining in fibroblasts stimulated with SM in the presence or absence of roxatidine at day 5. (D) Dynamic change in the percentage of Ki67-positive fibroblasts stimulated with SM in the presence or absence of roxatidine at day 5. n=6 wells per group, from one of triplicated experiments. n.s., not significant among all groups. Data were analyzed by two-way ANOVA. MT, micro-textured; SM, smooth; CTRL, control.

shown in Fig. 3, silicone surface materials had no effect on L929 proliferation over time, measured by Ki67 expression, and neither did roxatidine.

Macrophage-conditioned media stimulated with silicone implant materials increases L929 cell proliferation and TGFβ production. The following investigation assessed whether conditioned media collected from macrophages co-cultured with silicone implant materials would have an effect on L929 functions in terms of proinflammatory cytokine production. As illustrated in Fig. 4A, although silicone implant materials had no effect on L929 proliferation, media collected from macrophages stimulated with MT or SM increased proliferation of L929, measured by flow cytometry. Moreover, production of TGFβ, a key player in fibrosis (28), was also increased in L929 cultured in MT or SM stimulated macrophage-conditioned media (Fig. 4B). However, when roxatidine was added to the culture media

of macrophages in the presence of silicone implant materials, the levels of L929 proliferation and TGFβ production decreased to baseline (Fig. 4A and B). This result indicated that roxatidine-induced macrophage hypoactivation inhibited L929 proliferation and TGFβ secretion. Furthermore, when TGFβ neutralizing antibodies were added to the MT or SM stimulated macrophage-conditioned media for L929 culture, increased proliferation and TGFβ secretion were not observed (Fig. 4C). This indicated that the TGFβ-induced TGFβ production (a forward feedback loop) was essential for L929 proliferation.

Roxatidine inhibits macrophage activation by suppressing NF-κB and p38/MAPK signaling. It has been reported that roxatidine displays anti-inflammatory roles in other cell types by inhibiting activation of the NF-κB and p38/MAPK signaling pathways (22,29). Based on this, it was asked whether this would be the same case in macrophages stimulated with
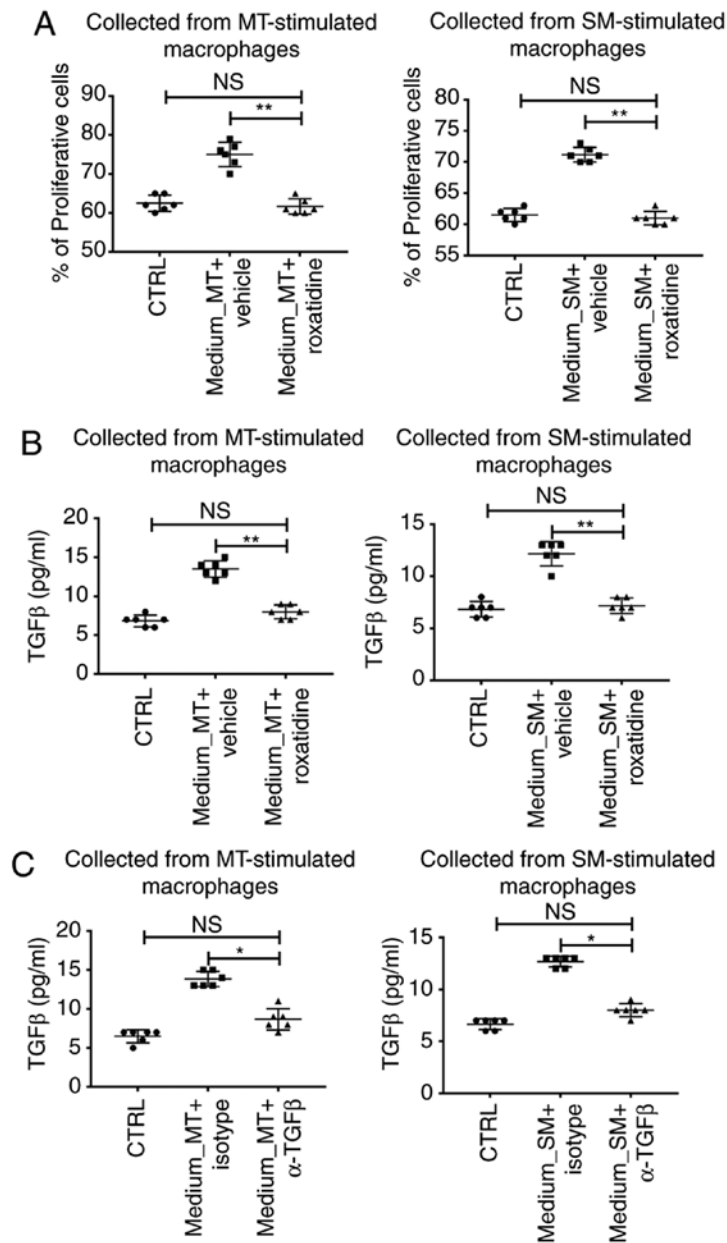
Figure 4. Conditioned media collected from stimulated macrophages increased L929 cell proliferation and TGFβ production. Cell-free conditioned media, collected from the co-culture system of macrophages and silicone material implants with or without pretreatment with roxatidine, were added to L929 culture for 24 h at 37˚C, and then the medium was switched to the serum-free medium for another 24 h at 37˚C. By the end of L929 culture, cells and media were harvested for analyses. (A) Presence of roxatidine during macrophage culture inhibited macrophage-conditioned media-induced enhanced proliferation in L929 cells. (B) The presence of roxatidine during macrophage culture decreased TGFβ production by L929 cultured in macrophage-conditioned media. (C) Presence of TGFβ neutralizing antibodies during fibroblast culture suppressed production of TGFβ by L929 cultured in macrophage-conditioned media. Left panels, media collected from MT-stimulated macrophages. Right panels, media collected from SM-stimulated macrophages. n=6 wells/group, from one of triplicated experiments. n.s., no significance; *P<0.05 and **P<0.01, as indicated. Data were analyzed by one-way ANOVA followed by the Bonferroni post hoc test. TGF, transforming growth factor; MT, micro-textured; SM, smooth; CTRL, control.

silicone implant materials. As shown in Fig. 5, administration of roxatidine to macrophages stimulated with silicone implanted materials inhibited activation of both signaling pathways. This suggested the possibility that inhibition of certain proinflammatory pathways might be the mechanism of roxatidine-mediated antifibrosis effects.

Administration of roxatidine in silicone implant bearing mice reduces fibrosis. Finally, the effects of roxatidine in silicone implant bearing mice were examined. Silicone implant surface materials were surgically seeded into the

subcutaneous pocket of mice. At three months post-surgery, the surrounding tissue of the implants was removed and the amount of fibroblasts present was assessed by flow cytometry. As shown in Fig. 6A, administration of roxatidine reduced serum levels of TGFβ in both MT- and SM- bearing mice at day 90 post-surgery, suggesting that roxatidine decreased systemic TGFβ levels in mice with silicone implant materials. Furthermore, at three months post-surgery, there were fewer fibroblasts in the surrounding tissues of implant materials (Fig. 6B and C), indicating that roxatidine protected against fibrosis.
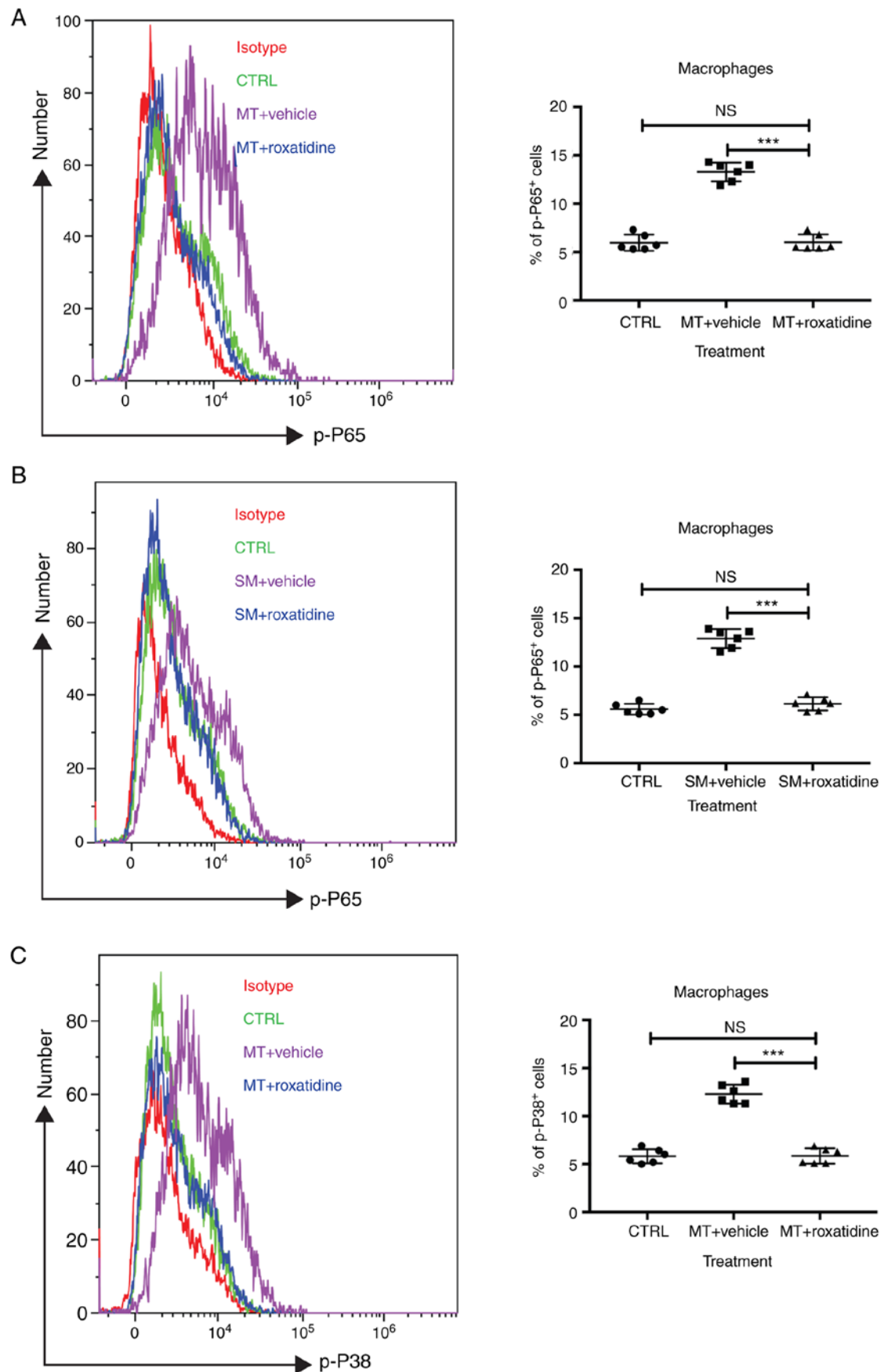
Figure 5. Addition of roxatidine to the culture medium of RAW 264.7 macrophages inhibited the activation of the NF-κB and MAPK signaling pathways. RAW 264.7 macrophages were cultured in serum-free media at 37°C. Then, 24 h after cells were seeded, roxatidine (25 μM) was added to the media 1 h prior to stimulation with silicone surface materials. Cells were collected for analysis of phosphorylation of NF-κB subunit p65 and p38 MAPK by flow cytometry 15 minutes after adding silicone surface materials to culture media. Administration of roxatidine suppressed p65 activation stimulated by (A) MT and (B) SM, as well as p38 phosphorylation stimulated by (C) MT.
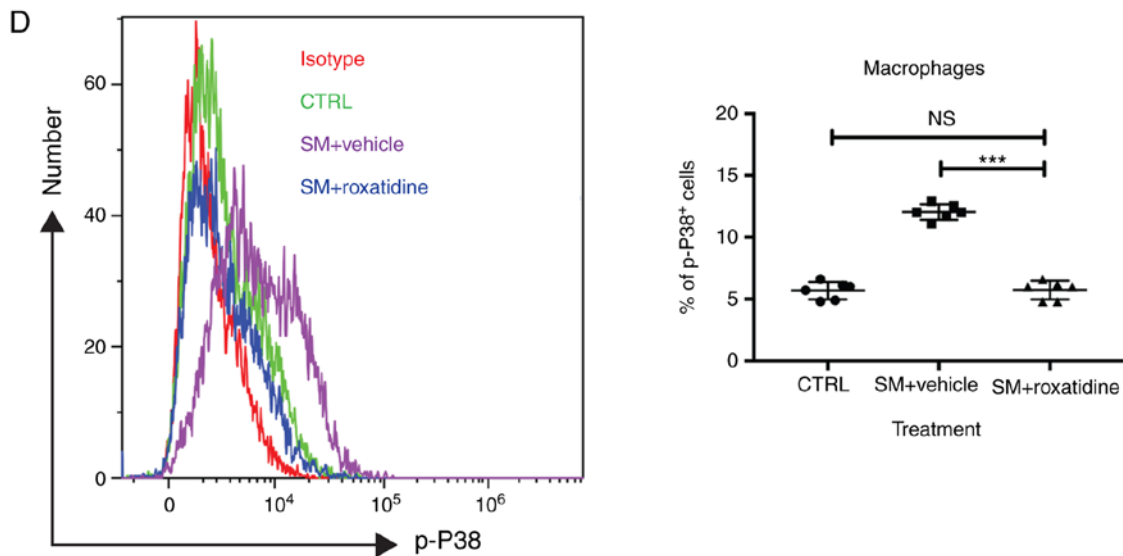
Figure 5. Continued. p38 phosphorylation stimulated by (D) SM. n=6 wells/group, from one of triplicated experiments. n.s., not significant and ***P<0.001, as indicated. Data were analyzed by one-way ANOVA followed by the Bonferroni post hoc test. MAPK, mitogen-activated protein kinase; MT, micro-textured; SM, smooth; CTRL, control.

Discussion

The present study demonstrated that roxatidine inhibited proinflammatory cytokine production in macrophages stimulated with breast implant surface materials. Although roxatidine seemed to have no effect on fibroblast function, the inhibitory effect of roxatidine on macrophages indirectly resulted in reduced fibroblast proliferation, potentially via the TGFβ-dependent forward feedback loop. *In vivo* studies using implant-bearing mice also showed that roxatidine provided protection against fibroblast hyperplasia and TGFβ production.

Inflammation, especially early inflammation during post-surgery wound healing, plays an important role in fibrosis (30,31). It has been widely accepted that aberrant inflammation causes excessive fibrosis and subsequent capsular contracture in patients with breast implants (5,6). Based on this speculation, researchers have been seeking to control inflammation after breast augmentation procedures in order to prevent capsular contracture (12,32-34). Applications of anti-inflammatory strategies have been well reviewed elsewhere (35). Results from numerous experiments support the idea that limiting inflammation during the phase of wound healing inhibits fibrosis (12,32-34). In agreement with the aforementioned results (5,6), the present study also highlighted the important roles of inflammation in fibrosis as well as the crosstalk between macrophages and fibroblasts. Moreover, the present study showed that application of roxatidine inhibited excessive inflammation, as well as the NF-κB and MAPK signalling pathways.

The present study demonstrated the different roles of macrophages and fibroblasts during fibrosis. Here, it is proposed that these two cell types have varied functions in different phases. Macrophages, as sentinel cells, sensed the presence of foreign objects, including implant materials, via activation of the NF-κB and MAPK signaling pathways. Activated macrophages produced and released proinflammatory cytokines to the surrounding environment. On the other hand, fibroblasts failed to sense implant materials, but could be stimulated by the proinflammatory cytokines produced by activated macrophages, especially TGFβ. By acting with TGFβ and other proinflammatory cytokines, fibroblasts displayed increased levels of proliferation and TGFβ production, which could accelerate fibroblast proliferation and TGFβ secretion induced by TGFβ. Furthermore, roxatidine could block activation of macrophages by inhibiting NF-κB and MAPK signaling pathways to reduce proinflammatory cytokine production, fibroblast proliferation and fibrosis *in vitro* and *in vivo*.

The present study builds on the understanding of the cell types that roxatidine has effects on. It has been well documented that roxatidine influences mast cells during inflammation (22). However, the current study revealed that roxatidine could also affect macrophages by blocking macrophage activation by inhibiting NF-κB and MAPK signaling pathways. It was hypothesized that roxatidine blocks NF-κB and MAPK signaling pathways in both mast cells and macrophages, which are both essential cell types in inflammatory responses such as allergy and infection (36). Therefore, it is reasonable to speculate that inhibition of HR2 by roxatidine might have a wide anti-inflammatory role in a number of conditions including fibrosis and allergies, which could be explored in further investigations.

This study also provided more evidence for the applicability of pharmaceutical therapy for capsular contracture after breast implant surgery. Previous results from small scale trials indicate the potential benefit in softening and improving the appearance of the breast in patients treated with leukotriene receptor antagonists such as montelukast (37) or zafirlukast (38). Veras-Castillo *et al* (39) indicated that pirfenidone was superior to capsulectomy in patients, displaying a higher improvement rate and a lower relapse rate. It is worth noting that the aforementioned trials involved small cohorts, and further investigations are required.

The major pitfall of the present study is the lack of exploration into the roles of histamine on macrophages in fibrosis
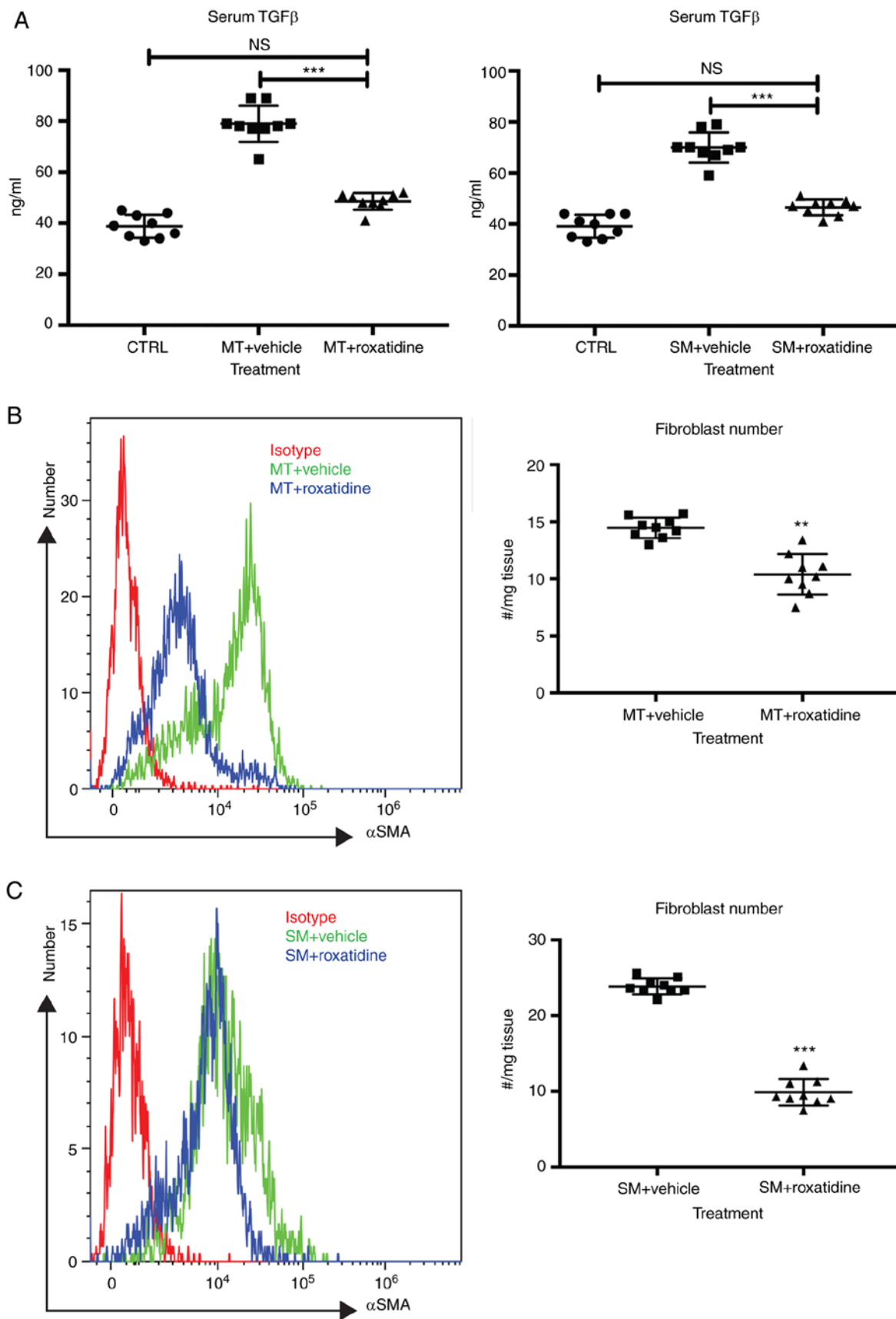
Figure 6. Treatment with roxatidine in implant-bearing mice reduced the severity of fibrosis. Implant materials (5 mm in diameter) were surgically seeded in a subcutaneous fat pocket in mice as described. Mice were treated daily by oral gavage with roxatidine (15.62 mg/kg) or PBS (control) for 14 days. At day 90 post-surgery, serum samples and fibrosis around implant materials were collected. (A) Administration of roxatidine reduced levels of TGFβ in the serum. n=9 mice/group, from one of duplicated experiments. n.s., not significant and ***P<0.001, as indicated. Data were analyzed by one-way ANOVA followed by the Bonferroni post hoc test. Administration of roxatidine reduced the number of fibroblasts in surrounding tissue of (B) MT and (C) SM in mice. Fibroblasts were defined as αSMA-positive cells by flow cytometry. n=9 mice/group, from one of duplicated experiments. **P<0.01 and ***P<0.001. Data were analyzed by the two-tailed Student's t-test. TGF, transforming growth factor; MT, micro-textured; SM, smooth; SMA, smooth muscle actin; CTRL, control.

generation when breast implant materials are present. The data from the present study suggested that blocking HR2 by roxatidine had no effect on fibroblasts, but these results highlight important roles of HR2 on macrophages. However, how the HR2 is activated and what the cellular sources of histamine are remain untouched in the current study and in the literature. The present study presented two theories; however further exploration is required for clarification. The first theory is that macrophages sense foreign objects in the tissue and produce histamine. Secreted histamine acts on macrophages to enhance macrophage activation. The second is that other cells (for instance, basophils and mast cells) produce histamine to stimulate macrophages. Moreover, the protective effects of roxatidine when breast implant materials are seeded in the submuscular position were not assessed in the present study. It has been reported that the rate of capsular contracture is lower when breast implant material is surgically placed in the submuscular position compared to the subglandular plate (40-42). Therefore, it is reasonable to presume that the combination of roxatidine administration and insertion into the submuscular position should have a synergistic effect in preventing fibrosis generation. However, this combination requires further investigation.

In conclusion, the HR2 antagonist roxatidine blocked activation of macrophages by inhibiting the NF-κB and MAPK signaling pathways to prevent fibrosis and subsequent capsular contracture. These results raise the possibility that administration of roxatidine could aid in preventing capsular contracture in clinical practice.

Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions

LJ performed most of the experiments including cell culture, flow cytometry, and animal surgery. TW performed data analysis and composed the first draft of the manuscript, as well as revisions. LT and HS performed statistical analysis and figure illustration. MG designed the present study and supervised the whole process. All authors have agreed the final version for publication.

Ethics approval and consent to participate

The ethics committee of the Fourth Affiliated Hospital of Harbin Medical University approved and supervised the research proposal (approval no.F 170023).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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