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Adrenergic receptor β2 activation by stress promotes breast cancer progression through macrophages M2 polarization in tumor microenvironment

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Stress and its related hormones epinephrine (E) and norepinephrine (NE) play a crucial role in tumor progression. Macrophages in the tumor microenvironment (TME) polarized to M2 is also a vital pathway for tumor deterioration. Here, we explore the underlying role of macrophages in the effect of stress and E promoting breast cancer growth. It was found that the weight and volume of tumor in tumor bearing mice were increased, and dramatically accompanied with the rising E level after chronic stress using social isolation. What is most noteworthy, the number of M2 macrophages inside tumor was up-regulated with it. The effects of E treatment appear to be directly related to the change of M2 phenotype is reproduced *in vitro*. Moreover, E receptor ADRβ2 involved in E promoting M2 polarization was comprehended simultaneously. Our results imply psychological stress is influential on specific immune system, more essential for the comprehensive treatment against tumors. [BMB Reports 2015; 48(5): 295-300]

INTRODUCTION

Psychological stress by a mental assault is a risk factor for breast cancer (1, 2). Usually, the expression of norepinephrine (NE) and epinephrine (E) will increase significantly when the body is in acute or chronic stress state (3). The research enunciated recently that the adrenergic system played pivotal roles in the initiation and promotion of tumor caused by stress. Adrenergic receptors (ADRs) were expressed on multiple kinds of cancers such as pancreatic cancer, oral squamous cell cancer, colon cancer, ovarian cancer and breast cancer (4-7). Stress hormones NE and E could affect the proliferation and migration of breast cancer cells and enhance VEGF expression (8, 9). ADRα2 was detected in a variety of human breast cancer cell lines. Its expression level is positively related with the proliferation ability of breast cancer cells and its function could be inhibited by the selective ADRα2 antagonist (10, 11). ADRβ was also detected to be activated in many carcinomas. Khan S (12) demonstrated that lipid-mobilizing factor (LMF) released by carcinoma cells could promote lipolysis and give rise to cachexia and meanwhile promote tumor angiogenesis and VEGF, MMP-2 and MMP-9 expression through ADRβ-cAMP-PKA signaling pathway, thus create a vital microenvironment which suits for the metastasis and settling of carcinoma cells. So for the states, we infer that stress related hormones and adrenergic receptors can propel the tumor progression.

The macrophages were divided into two categories according to the secretion of IL-12 and IL-10; classical activated M1 macrophages (IL-12^{high}) and alternative activated M2 macrophages (IL-10^{high}) (13). Tumor associated macrophages (TAM) belong to M2 phenotype (14). Clinical studies showed that in 80% of the reported breast cancer cases, high density of M2 macrophages usually indicated the poor prognosis (15). Tumor progression will be effectively restrained as the number of M2 phenotype macrophages lowered in tumor matrix (16).

Human stress experiments showed that stress could suppress the immune function (17). Moreover, that activated NE and ADR subtypes (ADRα2, ADRβ1) could regulate TNFα and NO release in macrophages through different signaling pathways was revealed (18-20). These suggest that researchers have begun to focus on how stress affects the function of macrophages, though the relationship between stress or related hormones and macrophage phenotype transformation is still largely unknown.

On the basis of our previous studies (21, 22), we hypothesized that stress and stress hormones (E) may boost the macrophage phenotype transformation from M1 to M2, and ADRs activation may be required for the transformation. Herein, we presented evidence to confirm this hypothesis. Moreover, fur-

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ther evidence of inhibition of ADRβ2 leading to a decrease in the proliferation of cancer cells will be initially presented.

RESULTS

In vivo social isolation stress induced breast cancer growth

To know if *in vivo* chroic stress model using social isolation induces breast cancer growth or not, one mouse was individually housed in one cage as described in Materials and methods (Fig. 1A). Our results showed that tumor grow faster after chronic stress and the tumor weight increased by 1.40 ± 0.13 g and the size 665 ± 35 mm^3 ($P < 0.05$, Fig. 1B). In particular, after dissected we found the size of the adrenal gland have no difference by the observation of naked eyes (data not shown), but the stress hormone E level in plasma of chronic stress group is visibly higher than the control group (523 ± 18 ng/ml vs.124 ± 12 ng/ml, $P < 0.05$, Fig. 1B). Paraffin section dying showed that $CD206^+$ cells (marker for M2 macrophages) intratumor of chronic stress group were obviously more than that of control group (Fig. 1C). Together, these above results strongly suggested that E could effectively create larger numbers of M2 macrophages in tumor of stressed mice.

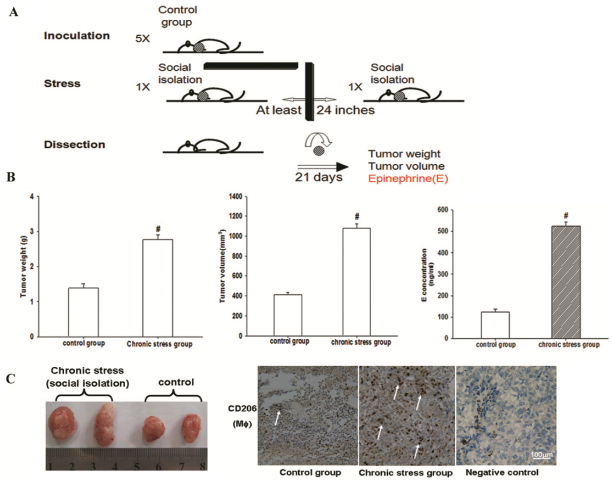

Fig. 1. Effect of chronic stress on breast cancer growth in mice that were injected with 4T1 breast cancer cells. (A) Schematic illustrating *in vivo* animal models. (B) Quantification of tumor weight, tumor volume and E (epinephrine) concentration in plasma in control mice and in chronic stress mice. (C) CD206 (marker for Mϕ) expression in tumor dissected from control mice and chronic stress mice. n = 6 per group. $^{\#}P < 0.05$.

In vitro stress hormone E increased wound-healing and migration ability of 4T1 cells

Next, to clarify the impact of E on the macrophages phenotype transformation and then resulting in tumor metastasis, we performed functional wound healing and Transwell migration assay, and it showed consistent results by both of the methods. After being gashed, the 4T1 cells were cultured with supernatant from RAW 264.7 cells that pretreated with different doses of E. As shown in Fig. 2A, we found that 1 μM and 5 μM E had a little effect in wound healing ability. 10 μM E could induce the most significant wound closure by 21% ± 2.14% ($P < 0.05$, Fig. 2B). Therefore, 10 μM was chosen for continued study. Then, we explored the effects of E treated macrophages on the migratory behavior of 4T1 cells with Transwell assay. It was found that 4T1 cells in TSN (the supernatant of 4T1 cells) treated-RAW 264.7 groups had much more migration cells than that in untreated-RAW 264.7 alone. While, the number of 4T1 cells in TSN treated-RAW264.7 stimulated with E group (10 μM) was the highest among the three groups. The number of migrated 4T1 cells of E + TSN treatment group increased 28% ± 1% compared with TSN treatment group ($P < 0.05$, Fig. 2C). These findings revealed that treatment of E enhanced the vitality of cancer cells.

E promoted macrophages transformed to M2 phenotype

These initial results indicated that stress hormones may be able to affect the certain features of the macrophages. To explore the role of E in macrophage phenotype transformation, we used flow cytometry, real-time PCR and ELISA to detect the ex-

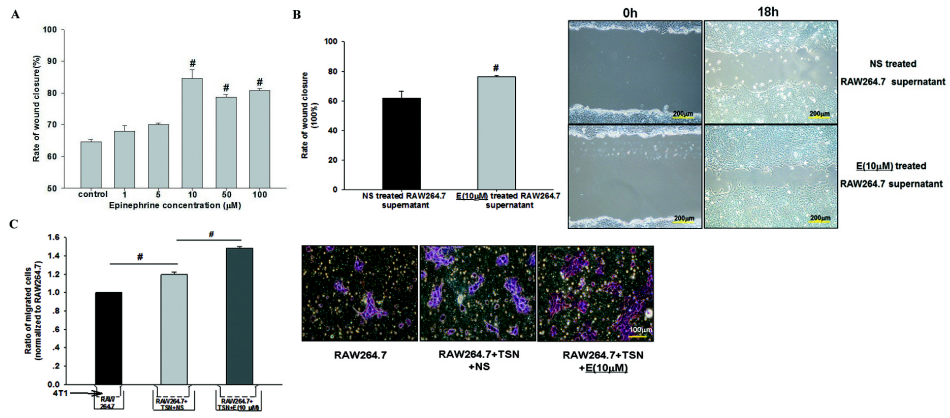

Fig. 2. Supernatant from RAW 264.7 treated with E increased 4T1 migration ability. (A) Dose responses of epinephrine on 4T1 cells wound healing ability. (B) and (C) Representative images and measurement of E (10 μM) promoting 4T1 migration ability by wounding closure assay (B) and Transwell assay (C). NS: normal saline; TSN: the supernatant of the 4T1 cells. In Transwell assay, 4T1 cells were added into the upper chamber and incubated for 18 h. The migrated cells were quantified in 10 random fields. $^{\#}P < 0.05$.

pression of M1 and M2 phenotype molecules. Comparing with the control group, We found that the ratio of F4/80$^+$/CD206$^+$ double positive RAW 264.7 cells was strongly increased by 3.94% and 7.14% by E treatment for 24 h and 48 h, respectively (Fig. 3A). Moreover, using real-time PCR assay, we showed that E obviously promoted transformation from M1 (iNOS and TNFα) to M2 (IL-10 and Arg-1) phenotype (Fig. 3B); Furthermore, we detected CCL22 (M2 phenotype molecule) concentration in the supernatant from different group cells. After cultured 12, 24 and 48 h, CCL22 concentration of E +TSN treatment group cells increased by 210.3 ± 33, 294 ± 54 and 296 ± 67 compared with TSN treatment group cells, respectively ($P < 0.05$, Fig. 3C). These results implied us that E was likely to facilitate macrophages M2 polarization.

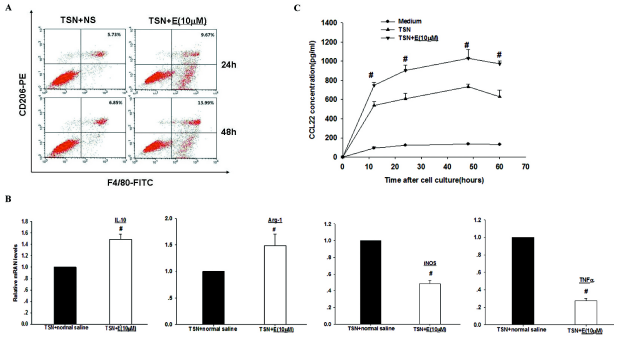

Fig. 3. E (10 μM) increased macrophage M2 polarization. (A) F4/80$^+$/CD206$^+$ cells were determined by Flow cytometry in TSN-treated RAW 264.7 after E addition for 24 h or 48 h. (B) Real-time PCR analysis of M2 macrophage phenotype molecules (IL-10, Arg-1) and M1 macrophage phenotype molecules (iNOS and TNFα). (C) Measurement of CCL22 concentration by ELISA. $^{\#}P < 0.05$.

Adrenergic receptor β2 involved in the effect of E promoting macrophages M2 polarization

Epinephrine induced its physiological effect need to activate adrenergic receptors. So we speculated that there was adrenaline receptors expressed on the macrophages. We examined the expression of ADR subtypes ADRα1a, ADRα2a, ADRβ1 and ADRβ2 in primary macrophages from murine ascites, RAW264.7 and U937 by RT-PCR and Western blotting. We found that both the mRNA and protein expression of ADRα1a, ADRα2a and ADRβ2 were detected, but that of ADRβ1 was impressively undetectable. After E treatment, ADRβ2 mRNA expression was increased (Fig. 4A).

We next sought to which receptor subtype was involved in this process by different inhibitors. The ADRα antagonist phentolamine (1 μM) or the ADRβ antagonist propranolol (10 μM) was added to the medium 3 h before E treatment and then detected the number changes of F4/80$^+$/CD206$^+$ cells. As shown in Fig. 4B, ADRβ antagonist propranolol could sharply reduce the number of M2 macrophages (10.2 ± 0.4 vs. 3.1 ± 0.4, $P < 0.05$), while phentolamine induced slight effect (10.2 ± 0.4 vs. 9.6 ± 0.9, $P > 0.05$). Therefore, this is a reminder that E is functional through influencing β2 receptor activity.

To ravel out whether β2 receptor truly works, we employed two ADRβ2 siRNAs and assessed their silence efficiency using real-time PCR. Then, the effect of E on M2 transformation of macrophages in which ADRβ2 knockdown was measured by Flow cytometry. As shown in Fig. 4C, the transfection efficiency was 14% ± 0.01 and 95% ± 0.01 by ADRβ2 (1) siRNA and ADRβ2 siRNA, respectively. We added E to RAW264.7 cells in which ADRβ2 silenced by ADRβ2 siRNA and found that, the ratio of F4/80$^+$/CD206$^+$ cells probably maintained normal ratio. The cell ratio of E + control siRNA group, E + ADRβ2 siRNA group and control siRNA group were 9.5 ± 0.8, 3.0 ± 0.4 and 3.6 ± 0.7 (E+ control siRNA

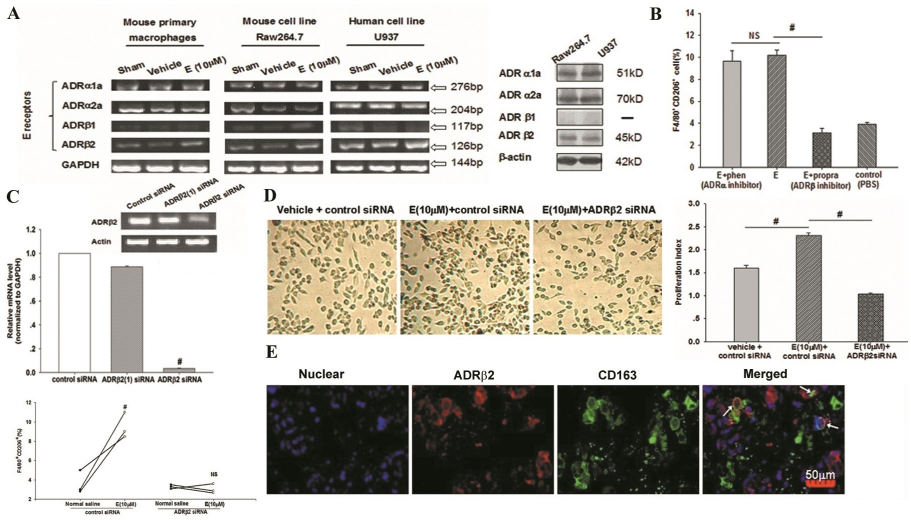

Fig. 4. ADRβ2 was selectively involved in the effect of E (10 μM) promoting macrophages M2 polarization and then impeded breast cancer progression. (A) ADRα1a, ADRα2a and ADRβ2 mRNA and protein was expressed on the primary macrophages from ascites, murine macrophages RAW 264.7 and human macrophages U937. (B) ADRβ antagonist propranolol (10 μM) could inhibit the effect of E, but ADRα antagonist phentolamine (1 μM) had no influence. The antagonists were added to the medium 3 h before E treatment. (C) Transfection efficiency of siRNAs in RAW264.7 cells (upper); the number of F4/80$^+$/CD206$^+$ cells was reduced after ADRβ2 silence (lower). (D) ADRβ2 silence suppressed 4T1 proliferation. (E) The expression of ADRβ2 (red) and CD163 (marker for M2 phenotype in human tissues, green) on tissues from breast cancer patients. Arrow indicates the superimposed staining. NS: no significance; $^{\#}P < 0.05$.

group vs.E+ADRβ2 siRNA group, $P < 0.05$, Fig. 4C). As we all know, M2 phenotype macrophages could promote the proliferation of tumor cells. Thus, we investigated the influence of ADRβ2 silence of macrophages on the proliferation ability of tumor cells. The proliferation index of 4T1 cells treated with E and the supernatant from ADRβ2 silenced macrophages was obviously lower than that of control siRNA group (Fig. 4D). The results further suggested that the activated ADRβ2 expressed on macrophages could promote the progression of tumor by accelerating macrophage M2 polarization.

Lastly, we confirmed the relationships between ADRβ2 and M2 phenotype macrophages in human tumor tissues. ADRβ2 and the human M2 macrophages marker CD163 were detected on tissues obtained from breast cancer patients. Our data revealed that the ADRβ2 and CD163 positive particles co-expressed on carcinoma tissues (Fig. 4E). Collectively, these above results implied us that in breast cancer patients interdicting the activity of ADRβ2 might inhibit tumor progression by reversing M2 macrophage polarization.

DISCUSSION

Stress and its related hormones E can provide help for tumor progression (23-25). Also ADR subtypes expressed on breast cancer cells, and NE or E may promote breast cancer proliferation and migration through activating different ADR subtypes (26). Our studies showed that E level in plasma increased significantly and tumor grew faster obviously after tumor-bearing mice suffered chronic stress. These results remind us that if cancer patients are in stress states, the deterioration and progression of tumor will be more serious, and then the prognosis will be relatively poor.

Our important finding was that E could promote the transformation of macrophages which stimulated with TSN to M2 phenotype and after chronic stress we detected the up regulation of CD206 positive cells in the tumor of mice. This result demonstrated that E could affect the malignant transformation of macrophages in TME and then promote tumor progression. In the early 1990s, there were studies about the sympathetic nervous system involved in the immune response, but the influence of stress hormone on the macrophage phenotype transformation in the TME was unclear. In 2012, a group found that β1 receptor autoantibodies from peripheral blood serum of 18 heart failure patients enhanced the TNF-α secretion in RAW 264.7 cells *in vitro*, but β1 receptor agonist isoprenaline could not induce the same effect under the same conditions in this study(19). Jiang CL. *et al*. reported that NE promoted TNFα production by LPS-stimulated macrophages (20). Other studies have revealed that carvedilol, a unique vasodilating β-adrenergic antagonist, and endogenous adrenergic agonists (adrenalin and noradrenalin) inhibited NO production and iNOS protein expression in LPS-stimulated RAW 264.7 cells (18). A new study suggested that E could induce M2 polarization through ADRβ2 then exert anti-inflammatory effect during endotoxemia and acute lung injury (27). In this study after TSN stimulation and E treatment, the expression of TNF-α and iNOS were obviously reduced in RAW 264.7 cells in TME. These studies indicated that adrenergic system did have an impact on the function of macrophages, but the results might not completely be the same under different conditions. Theoretically, macrophages could differentiate to M1 after LPS stimulation (28) and to M2 after TSN stimulation (21), so we speculated that the influence of stress-related hormones on iNOS and TNFα expression in macrophages would be different under different stimulating conditions.

We used specific blockers and siRNA to inhibit the function of receptors and found that stress hormones affecting on the transformation of macrophages to M2 mainly or at least in part induced by β2 receptor. Zinyama R. B. found that E inhibited macrophage NO production through ADRβ (29) and ADRβ2 stimulation could inhibited NO generations by mycobacterium avium infected macrophages (30). These results were consistent with our studies. Another discovery of our study was that after chronic stress we detected the M2 type TAM significantly increased in mice tumors *in vivo* experiments. This was indeed the first time to confirm the influence of stress on macrophage phenotype transformation in TME on animal model, and this results proved our hypothesis. We further tested human breast cancer tissues and found that the expression of ADRβ2 receptor and type M2 macrophages (CD206+) obviously increased in cancer tissue compared with para-carcinoma tissue, and they were positively correlated with each other. These results showed that stress could increase the transformation of macrophages to M2 by activating adrenaline receptors in TME, and then promote cancer progression.

In conclusion, our study found E could promote the transformation of macrophages from M1 to M2 and promote the progress of cancer by influencing the TME. This effect was mediated by β2 receptor. Negative stress is the 'most popular' kind of stress. It's a stress that causes negative implication such as anxiety, fatigue and illness. Considering the published reports, our study suggested that negative stress may not only influence the cancer progress directly (31), but also influence the TME including macrophages. These dual functions would result in poor prognosis of the cancer patients. In future, we will keep on studying the downstream signal pathways of ADRs and completely elucidate the underlying mechanisms of stress affecting the macrophages transformation in TME. No doubt, the molecular level research further analyzed the potential role of stress in tumorigenesis and progression. It would provide a more profound reference for antitumor drug screening from the angle of antianxiety, antidepressant or antianxiety-associated hormones.

MATERIALS AND METHODS

Animal models

Female Balb/c mice, 6 to 8 week-old, were purchased from

Vital River Lab Animal Technology Co., Ltd. All experimental procedures were approved by the Medical Ethics Committee, Nankai University. After inoculating subcutaneously to the left breast gland of the mice with $1*10^5$ 4T1 cells, the animals (Female Balb/c mice, 6 to 8 week-old,) were divided randomly to two groups: control group and chronic stress group. In control group, 5 mice were group housed in one cage. While the mice in chronic stress group were implemented with social isolation stress referred to Anil K Sood (23), and in this group one mouse was individually housed in one cage, and there was a wall and at least a 24-inch distance between cages. The mice were sacrificed by dislocation on 21st day and 0.5 ml blood was drawn from the heart. And then the tumor was weight, measured and frozen at -70^{o}C.

Cell culture

Murine breast cancer cell lines 4T1, murine macrophages RAW 264.7 and human macrophages U937 were purchased from ATCC. Primary Mφs were obtained from ascites of BALB/c mice. The cell culture has been described by QS Wang (19). Briefly, RAW 264.7 cells were co-cultured with the medium containing 40% TSN (RPMI 1640: TSN=1.5:1) to mimic the TME *in vitro*.

Measurement of total E and CCL22 secretion

The concentration of E in mice serum and CCL22 in the supernatants from the cells was measured through a mouse E (Bogoo Biotechnology, China) and CCL22-specific (R&D Systems) ELISA kit. The results are from three independent experiments performed in triplicate.

Wound closure assay

Monolayer 4T1 cell were scraped using 10 μl pipette tips. Incubating with the supernatants collected from TSN co-cultured RAW 264.7 cell in the presence or absence of E for 18 h. Photographs were taken and analyzed using Image J software to measure the wound closure area.

Transwell migration assay

4T1 migration ability was detected in Transwell chambers (Corning) with 8.0 μm pore size polycarbonate membrane. 1×10^5 4T1cell were resuspended in 300 μl RPMI 1640 with 2%FBS and seeded in the upper chamber. The lower chamber was added with the supernatant from RAW 264.7 cells, or TSN treated RAW 264.7 with E or not which incubated in RPMI 1640 with 10% FBS. After 18 h of incubation, the undersurface cells were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet.

Flow cytometry

1×10^5 RAW 264.7cells treated with TSN in the absence or presence of E (10 μM) were trypsinized and stained with FITC conjugated anti-mouse F4/80 (Santa Cruz Biotechnology, Inc.) and PE conjugated CD206 (BD Biosciences) and analyzed on BD FACS Canto II. In the experiments with ADR inhibitors, the ADRα inhibitor phentolamine (1 μM) and the ADRβ inhibitor propranolol (10 μM) was added 3 h before E treatment.

siRNA

Control siRNA, ADRβ2 siRNA (target sequence 5′-CAGAGTGGATATCACGTGGAA-3′) and ADRβ2 (1) siRNA (target sequence 5′-CCGATAGCAGGTGAACTCGAA-3′) (QIAGEN) were transfected into RAW 264.7 with lipofectamine 2000.

RT-PCR and Real-time PCR

RT-PCR was used to detect the expression of ADRs subtypes including ADRα1a, ADRα2a, ADRβ1 and ADRβ2. Real-time PCR with CFX96 Touch™ (Bio-rad) was performed to semi-quantify IL-10, Arg-1, TNFα, iNOS and ADRβ2. Briefly, total cellular RNA (1 μg) was isolated from cultured cells and reverse-transcribed using oligo (dT) and M-MLV reverse transcriptase (Promega). Then real-time PCR amplifications were done with SyberGreen Mix (ABI). Gene expression was derived according to the equation2^ -ddCt.

Immunoblotting

Rabbit anti-ADRα1a, ADRα2a, ADRβ1, ADRβ2 (1:800) and anti-β-actin antibodies were obtained from Abcam. Finally, labeled proteins were revealed by ECL plus (Merck Millipore).

Cell proliferation

1×10^5 4T1 cells incubated with the supernatant RAW 264.7 cells transfected with control siRNA or ADRβ2 siRNA in the presence of E. After adding CCK-8, the absorbance was read 450 nm.

Immunohistochemistry and Immunofluorescence analyses

For immunohistochemistry, the tumor tissue paraffin specimen from tumor bearing mice was stained with anti-CD206 Ab, and immunoactivity was detected with diaminobenzidine (DAB). Samples of breast cancers were obtained from Tianjin Cancer Hospital with local ethical guidelines. 4-μm paraffin sections were processed for immunofluorescence analysis with anti-ADRβ2 and CD163 monoclonal Ab (Abcam). Immunoreactive proteins were detected by incubating with TRITC-conjugated goat anti-rabbit IgG, FITC-conjugated goat anti-mouse IgG. The nuclear stained with DAPI. Images were assessed with a confocal microscope (Olympus, FV1000).

Statistics

Data were presented as means ± sem. Statistical differences were determined by ANOVA or Student's t test by Sigmastat3.5 software. Significance was accepted at P values < 0.05.

ACKNOWLEDGEMENTS

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