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Anti-inflammatory Effects of "Sanliangsan" on Gefitinib Induced Skin Rash *via* Modulation of Macrophages*

WAN Liang-Qin[1,2)**], SONG Chen-Chen[1)**], TAN Yan[1)], HE Fang[1)], ZHANG Ya-Li[1)], WANG Ya-Lei[1)], CHEN Zi-Wei[1)], ZHANG Ce[1)], GU Ruo-Xi[1)], ZHANG Ding-Yang[1)], WANG Xu[1)], HUA Qian[1)***]

([1)]*Beijing University of Chinese Medicine, Beijing* 100029, China;
[2)]*Beijing Gulou Hospital of traditional Chinese Medicine, Beijing* 100009, China)

Abstract Gefitinib induced rash is a common sequellae during cancer treatment. The mechanism and treatment of these rashes are unclear, therefore we investigated the anti-inflammatory effect of Sanliangsan on gefitinib induced rashes. Brown Norway (BN) rats were randomly selected and divided into five groups: control group, gefitinib rash model control group(model group), Sanliangsan model groups of low-dose, medium-dose, and high-dose(low,medium and high dose). Gifitinib was administered in the morning and Sanliangsan in the afternoon on the same day for 4 weeks .The BN model rats in the low-dose, medium-dose and high-dose groups were given 2 mg/kg/day, 4 mg/kg/day and 8 mg/kg/day of Sanliangsan respectively. The control group was given pure water. Macrophages were classified by flow cytometry. Protein expression was detected by immunohistochemistry. Protein chip array was used to detect the signaling pathways and inflammatory factors associated with inflammation. The results showed that the expressions of macrophage inflammatory protein (MIP)-1, MIP-2, myelocyte triggered receptor-1 (TREM-1) and IL-17A were significantly increased in the gefitinib rash model control group compared with the control group. The expressions of MIP-1, MIP-2, TREM-1 and IL-17A were significantly reduced in the Sanliangsan groups compared with the gefitinib rash model control group. It was also discovered that the anti-inflammatory effect of Sanliangsan on gefitinib-induced rash was closely related to the signaling pathway of IL-17A.

Key words Sanliangsan, gefitinib, skin rash, macrophage, interleukin 17A (IL-17A), inflammation
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Lung cancer, a malignant disease and the second most common cancer is presenting a significant health concern worldwide. In China alone, it is estimated that 1 million new cases and 62 600 deaths will occur in 2025[1]. New biologically targeted therapies that interfere with specific molecular pathways to disrupt the progression of cancer have become an indispensable options for many patients[2]. Gefitinib, an epidermal growth factor receptor (EGFR) inhibitor is one such biologically targeted therapy drugs. EGFR inhibitors are used to treat advanced or metastatic non-small cell lung cancer patients with EGFR mutations and have demonstrated significant therapeutic benefits[3-4]. However, because of the role of EGFR in skin biology, gefitinib is associated with multiple dermatological reactions, which include acneiform (papulopustular) rash, pruritus, xerosis, hair loss and eyelash trichomegaly[5]. Among these dermatological side effects skin rash has the biggest negative impact on the quality of patient life. Some patients have a more severe response to their skin rash, *e.g.* insomnia and depression. These conditions can lead to them

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** These authors contributed equally to this work.
*** Corresponding author.
Tel: 86-10-64286192
E-mail:hqianz@aliyun.com,huaq@bucm.edu.cn
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electing to withdraw from gefitinib treatment.

Clinical and laboratory studies have shown that the main mechanism of EGFR-induced rash is caused by inflammation. These studies show that immune cells such as macrophages and inflammatory factors play a significant role[6-8]. In epithelial cells treated with EGFR inhibitors *in vitro* the expression of related inflammatory factors including CCL-2, CCL-5 and CXCL-10 increased significantly[9]. The expression of the chemokines CCL-18, CXCL-1, CXCL-9 and CCL-3, which are associated with T cell recruitment also increased significantly[10]. Using knockout mice with EGFR and EGFR inhibitors to induce rash *in vivo* researchers were able to simulate the rash caused by targeted drugs in clinical cancer patients[11]. The macrophage count in the inflamed skin of these mice increased significantly. At the same time a collection of clinical cases with patients taking gefitinib who did or did not develop skin rash was compared for differences in various skin proteins[6]. Gefitinib was found to induce the expression of inflammatory protein-1β (MIP-1β/CINC-2β) in skin-associated macrophages in patients with skin rash. This suggests that macrophages play a major role in skin inflammation[6].

Interleukin-17A (IL-17A) signaling pathway is one of the key signaling pathways in inflammatory response cascades of skin T cells, B cells, macrophages et cetera. The main target cells of IL-17A related signaling pathways in the human body include epithelial cells, keratinocytes, macrophages, T cells and fibroblasts which activate the downstream signaling pathway mainly through the interaction of receptors and ligands. Inhibiting the expression of IL-17A can reduce the occurrence of inflammatory reactions in the skin[12-14]. In a study using macrophage-specific markers to assess the effects of an IL-17A inhibitor and a macrophage activating agent, it was found that IL-17A mainly exerted its effects by inducing the transformation of macrophages from the M2 to M1 type, which in turn aggravated inflammatory changes in the skin[15].

The traditional Chinese medicine formula known as "Sanliangsan" consists of Astragali radix 30 g, Lonicera japonica 30 g, Angelica sinensis 30 g, Glycyrrhiza glabra rhizome 10 g and Centipede 1 g. In traditional Chinese medicine it is believed that the combination of Astragali radix and Lonicera japonica can enhance body immunity, Angelica sinensis and Lonicera japonica can keep blood vessels smooth and increase blood flow, Centipede can dredge the meridians and Glycyrrhiza glabra rhizome has detoxifying therapeutic effects. Published cases have showed that Sanliangsan treatment can alleviate EGFR inhibitor-related skin rash and increase the patients quality of life[16]. Further these publications confirm the potential of Sanliangsan to improve the therapeutic efficacy of gefitinib in clinical practice. Clinical research has shown that "Sanliangsan" can treat interstitial pulmonary fibrosis[17]. However, to our knowledge there have been no studies on the mechanism of Sanliangsan in the treatment of skin rash induced by gefitinib treatment.

In this study we aimed to explore the anti-inflammatory action and mechanism of Sanliangsan on gefitinib-induced skin rash by examining the modulation of macrophages.

1 Materials and methods

1.1 Ethical approval

All experimental procedures conducted on animals were approved by the Animal Ethics Committee of Beijing University of Chinese Medicine (approval number BUCM-2016103101-1008).

1.2 Animal Models

Animal studies were conducted according to standard operating procedures using female Brown Norway (BN) rats (130−150 g body weight) from Beijing Vital River Laboratory Animal Technology Co., Ltd., and were approved by the Ethics Committee for the Treatment of Laboratory Animals.

The methods and dosages used to induce the animal skin rash model were described previously (The results were not published). Rats were randomly divided into five groups: Control group (no treatment), gefitinib rash model control group(model group), and three Sanliangsan model groups (low, medium and high dose). The skin rash animal model was established by gavage with gefitinib (25 mg/kg/day). The decoction group rats received oral administration of Sanliangsan of 2 mg/g/day (low), 4 mg/g/day (medium) or 8 mg/g/day (high) for 4 weeks (Figure 1).

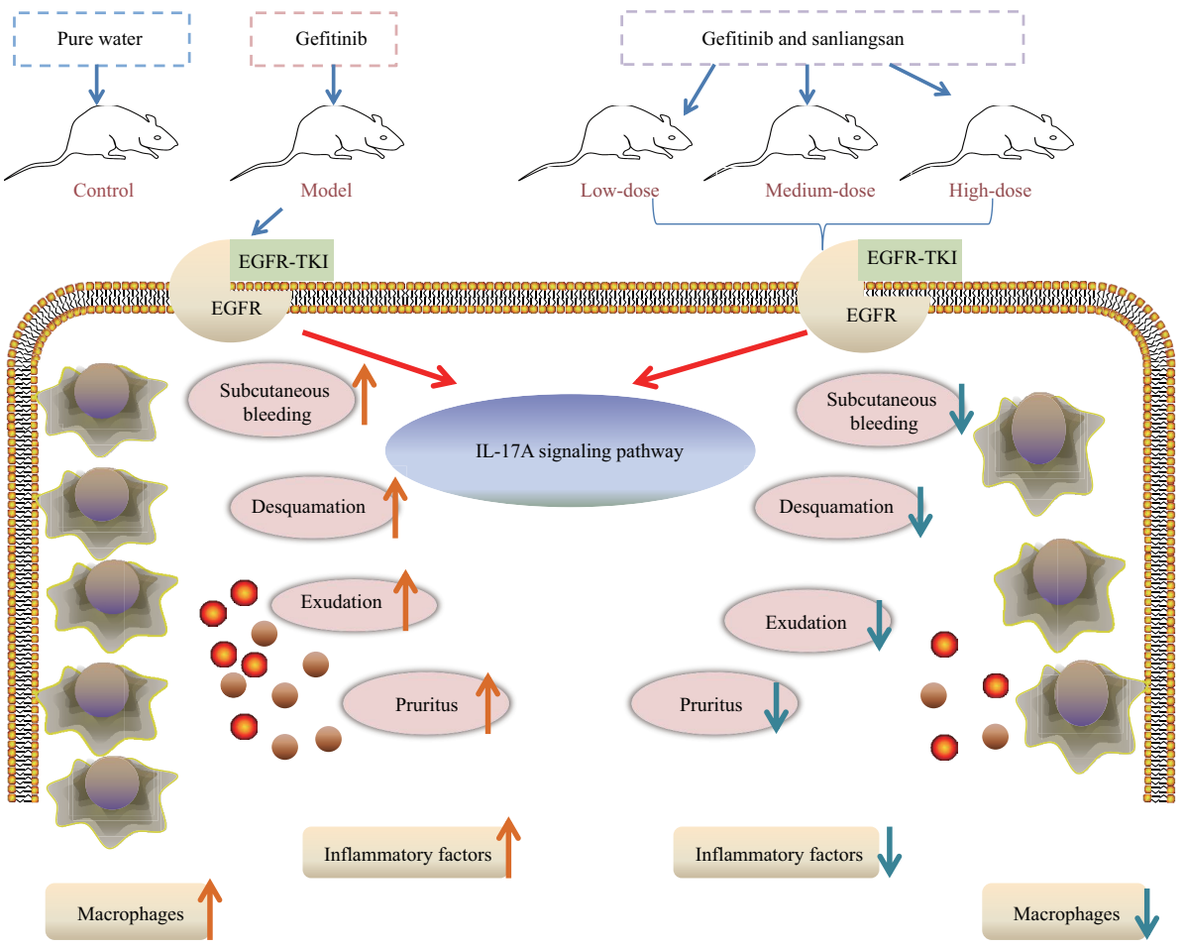

Fig. 1　Study rats receiving gefitinib and varying dosages of "Sanliangsan" for 4 weeks show reduction in adverse skin reaction, numbers of macrophages and inflammatory factors related to the IL−17A signaling pathway

4 weeks into the study, under anesthesia the hairs on the back of the rats were removed to fully expose their skin. Randomly selected rats from each group got their exposed skin photographed with a hand-held low-power microscope (10×). Assessment of scratching behavior was undertaken in rats allowed autonomous movement, with scoring based on the international Four-Item Itch Questionnaire scale.

1.3　Drugs and Reagents

Sanliangsan was purchased from the Beijing Tong Ren Tang Herbal Pharmacy (Beijing, China). Gefitinib (Iressa) was kindly supplied by the Cancer Hospital at the Chinese Academy of Medical Sciences (Chao-yang District, Beijing). Anti-rat CD68 antibody (ED1) (gtx43915) was supplied by GeneTex (Irvine, CA, USA). IL-17A (ab136668) was purchased from Abcam (Cambridge, UK). The DAB chromogenic kit (ZLI-9018) and immunohistochemistry detection kit (PV-9002) were provided by Zhongshan Jinqiao Biotechnology Co., Ltd (Beijing, China). The protein chip kit (GSR-CAA-67) was purchased from RayBiotech Co., Ltd (Atlanta, GA, USA).

2　Methods

2.1　Immunohistochemical analysis

After the rats were anesthetized and euthanized, an appropriate area of skin (3 cm × 3 cm) from the back was excised using a medical blade and following embedded in paraffin. All sections used for the experiments were 6 μm in thickness.

Antigen retrieval for ED1 and IL-17A was performed using Target Retrieval Solution (citrate pH 6, ZSJQ, Beijing, China). Slides containing skin sections were treated with 3% hydrogen peroxide for 1 hour and blocked for a minimum of 1 h with Serum-

Free Protein Block (immunohistochemistry detection kit, ZSJQ, Beijing, China). The skin sections were incubated overnight at 4℃ with primary antibodies, they were diluted in antibody dilution with goat serum sealing solution as described：ED1 (1：100) and IL-17A (1：200). Slides were treated for 1 h at room temperature with their respectively corresponding biotinylated secondary antibodies. Slides were then incubated for 1 h at 37℃. Following the slides were incubated with primary antibodies for 1 h at room temperature and washed once with PBS. DAB was used for staining development. Counterstaining was performed with hematoxylin. The tissue samples were dehydrated and following had sealing treatment. We prepared skin slides of three rats for each time point for analysis. Positive cells were on average measured in 10 independent fields per slide/rat.

2.2 Flow cytometry analysis

Inflamed singe-cell skin samples from the back of euthanized rats were suspended and treated with 0.25% trypsin (3−5 ml) in serum-free media for 20−30 min at 37℃. Enzyme activity was inhibited by adding 2−3 ml fetal bovine serum. The tissues were then mechanically disrupted and filtered through a 300 μm strainer to obtain single-cell suspension. For intracellular cytokine staining, cells were marked with ED1 antibody.

2.3 Protein chip detection

Glass slide arrays were removed and sealed in individual plastic bags at room temperature for 20−30 min to equilibrate their temperature. Following they were removed from their bags and the cover films peeled off allowing the slides to air dry for another 1−2 h. Sample diluent buffer (100 μl) was added and following incubated at room temperature for 30 min for blocking. We then decanted the solution into their respective container of 100 μl and incubated the arrays at room temperature for 1−2 h. Following we washed each sample 5 times (5 min per wash) with 150 μl 1×Wash Buffer I at room temperature with gentle shaking. The glass slides were completely covered with 1×Wash Buffer I and washed at room temperature with gentle shaking for an additional 20 min, then decanted and washed twice (5 min each wash) with 150 μl 1×Wash Buffer II at room temperature with gentle shaking. 1.4 ml of Aliquots (80 μl) detection antibody reconstituted diluent was added to each sample and incubated at room temperature for an additional 1−2 h. Samples were then decanted and washed 5 times (5 min each wash) with 150 μl 1×Wash Buffer I, then washed twice with 150 μl 1×Wash Buffer II at room temperature with gentle shaking. Aliquots (80 μl) of Cy3 equivalent dye-conjugated streptavidin reconstituted in 1.4 ml Sample Diluent were then added to each sample, covered with aluminum foil to avoid exposure to light and further incubated at room temperature for 1 h. Samples were decanted, washed 5 times (5 min each wash) with 150 μl 1×Wash Buffer I at room temperature with gentle shaking, and finally used for fluorescence detection.

2.4 Statistical analysis

All of the experiments were repeated three times or more. One-way analysis of variance and Student's *t*-test were used for comparisons. A P value less than 0.05 was considered statistically significant.

3 Results

3.1 Sanliangsan shows reduction of skin inflammation and can alleviate skin pruritus in BN rats

Low-power microscope imaging of skin samples of the model group revealed obvious desquamation, subcutaneous bleeding and even exudation. Treatment with Sanliangsan led to dose-dependent reduction in inflammatory symptoms of the skin, with significant reductions in desquamation and subcutaneous bleeding. In the high-dose group there was no obvious subcutaneous bleeding or exudation (Figure 2a). Additionally, scoring of skin inflammation using the related indexes[18], showed a significantly higher standard score of skin inflammation in the model group compared with the control group. Post-administration of Sanliangsan the Standard scores of skin inflammation in the medium- and high-dose groups were significantly lower compared with the model group (Figure 2b). We were able to score and quantitatively analyze the severity of pruritus in each group by observing the rats scratching behavior. While the degree of pruritus was more severe in the model group compared with the control group, the intervention of Sanliangsan significantly relieved pruritus in the medium- and high-dose groups (Figure 2c).

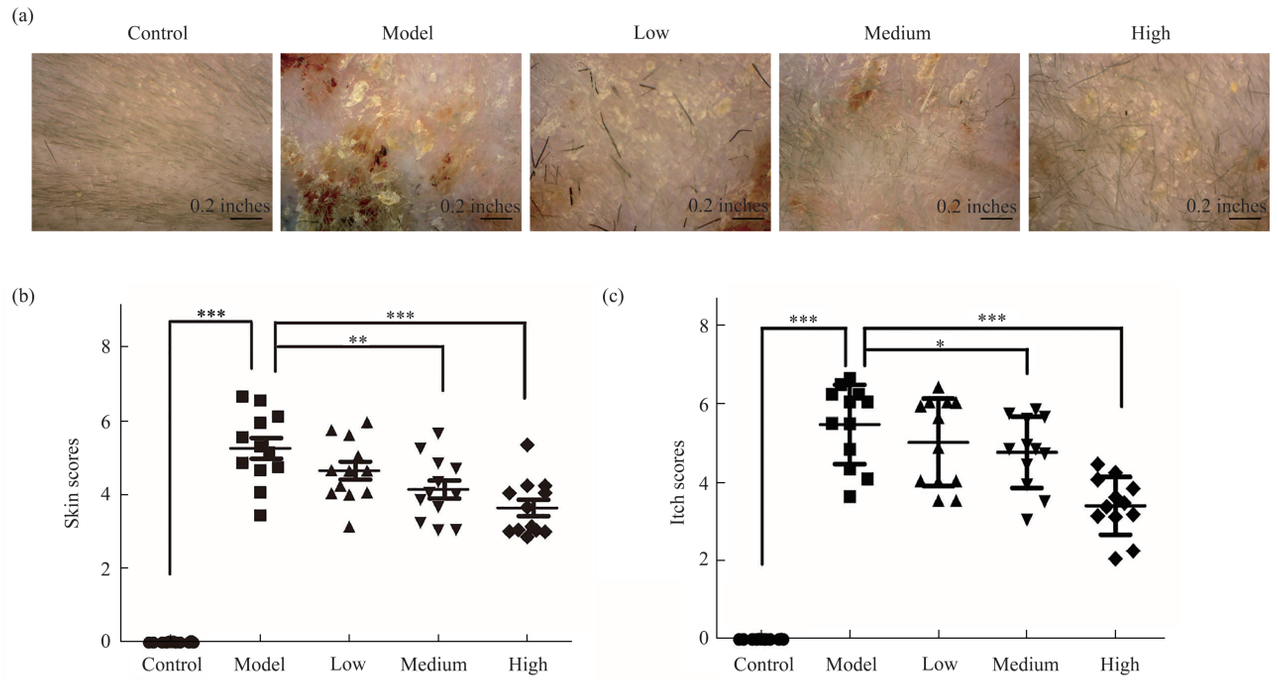

Fig. 2　Effect of Sanliangsan treatment on the degree of inflammation of skin

(a) Representative images of skin samples in each group after 4 weeks of treatment with Sanliangsan. (b) Inflammatory Scoring and counting results of 3 cm × 6 cm skin samples from each group 4 weeks post-administration. (c) Scoring of pruritus based on the scratching behavior of rats at 4 weeks post-administration. **P*<0.05, ***P*<0.01 and ****P*<0.001 compared with the model group.

3.2　Effect of Sanliangsan on the infiltration of inflammation−related cells

Skin inflammation involves the recruitment of immune cells such as macrophages to carry out phagocytosis and cause anti-inflammatory changes. We used ED1 expression as a marker of macrophages. The proportion of ED1-positive cells was lower in the control group than the model group (Figure 3a). Treatment with Sanliangsan significantly decreased the infiltration of ED1-positive cells in the middle and high dose groups (Figure 3a, b). To verify the extent of macrophage infiltration, flow cytometry analysis was performed to detect the post-gefitinib expression of ED1 in rat skin samples (Figure 3c, d). Significant dose-dependent decreases of ED1-positive cells were observed at the area of skin inflammation in all three Sanliangsan treatment groups compared with the model rats (Figure 3d).

3.3　Sanliangsan can down−regulate macrophage related inflammatory factors

Inflammation-related protein chip was used on BN rats to quantitatively analyze the expression of 67 chemokine and inflammation-related factors in skin samples. Sanliangsan treatment down-regulated the expression of three inflammatory factors that are mainly associated with the secretion by macrophages: CINC-2 (macrophage inflammatory protein [MIP]-1), CINC-3 (MIP-2) and TREM-1 (myeloid cell trigger receptor-1) (Figure 4a−c). Quantitative statistics showed that the levels of these three inflammatory factors were significantly higher in the model group compared with the control group and the three groups treated with Sanliangsan combined (Figure 4d).

3.4　The anti−inflammatory effect of Sanliangsan is closely related to IL−17A signaling pathway

We investigated the putative signaling pathways involved in the effects of Sanliangsan. By analyzing the differential expression of inflammatory factors on the protein chip arrays of the study groups we screened for those factors that correlate with known drug regulatory factors. Depicture the model group and control group. It shows that the top three mechanisms of skin inflammation in these groups were Legionella infection, IL-17 signaling and tumor necrosis factor- signaling pathways (Figure 5a). Depicture the model group and Sanliangsan groups

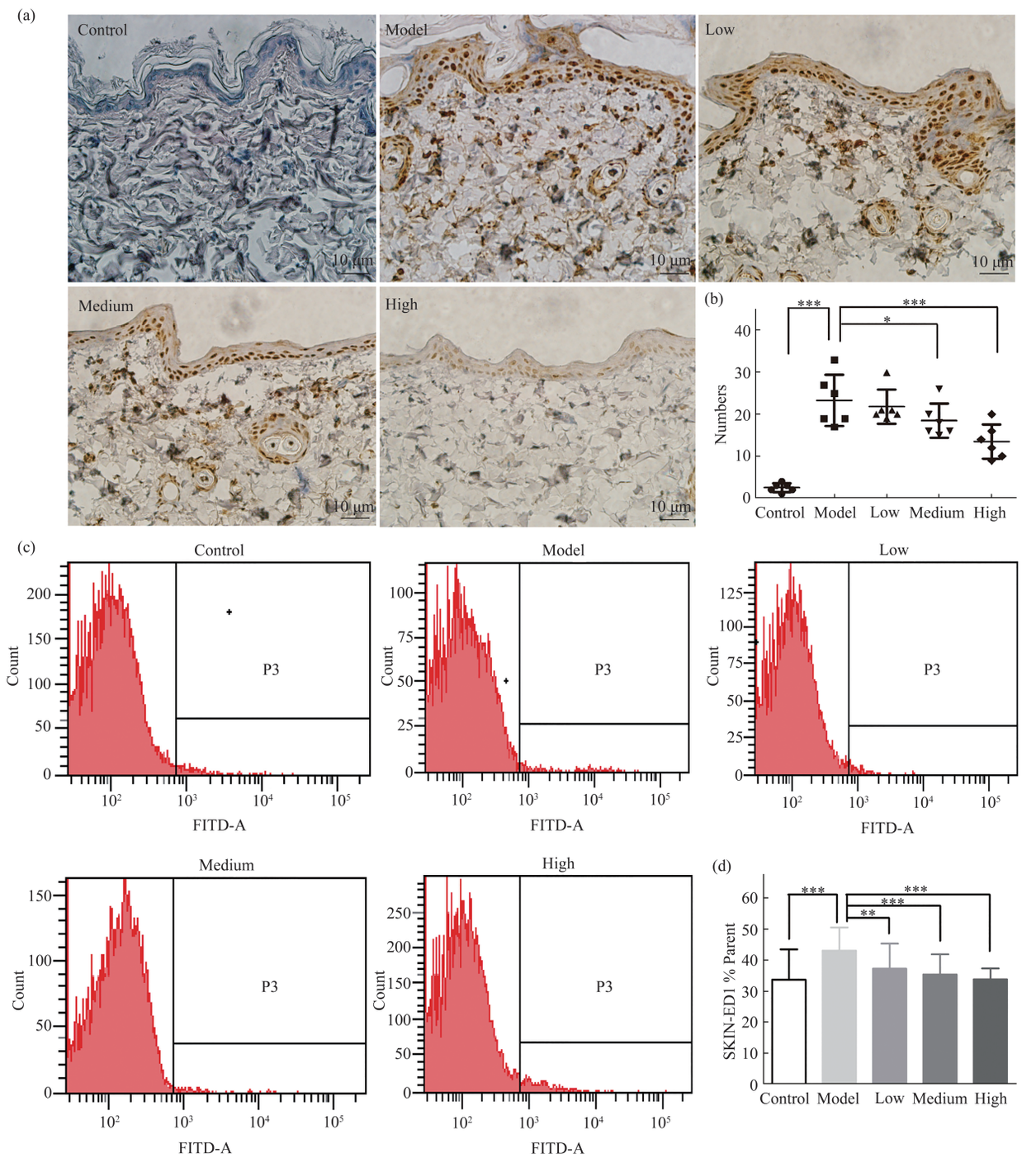

Fig. 3　Effect of Sanliangsan on the proportion of macrophages in skin tissue

(a) Immunohistochemical staining showed that the proportions of ED-1 positive cells were significantly increased (brown color) in the model group compared with the control group. At the same time it showed that ED-1 positive cells were significantly decreased in the model group post Sanliangsan administration. (b) Depicture the ED-1 positive cell count in the epidermis of BN rats measured by Quantitative flow cytometry. (c) The percentage of P3 gate in the total number of cells used to determine the proportion of macrophages in each group. (d) Quantitative analysis of the specific percentage of macrophages in each group. **P*<0.05, ***P*<0.01, ****P*<0.001.

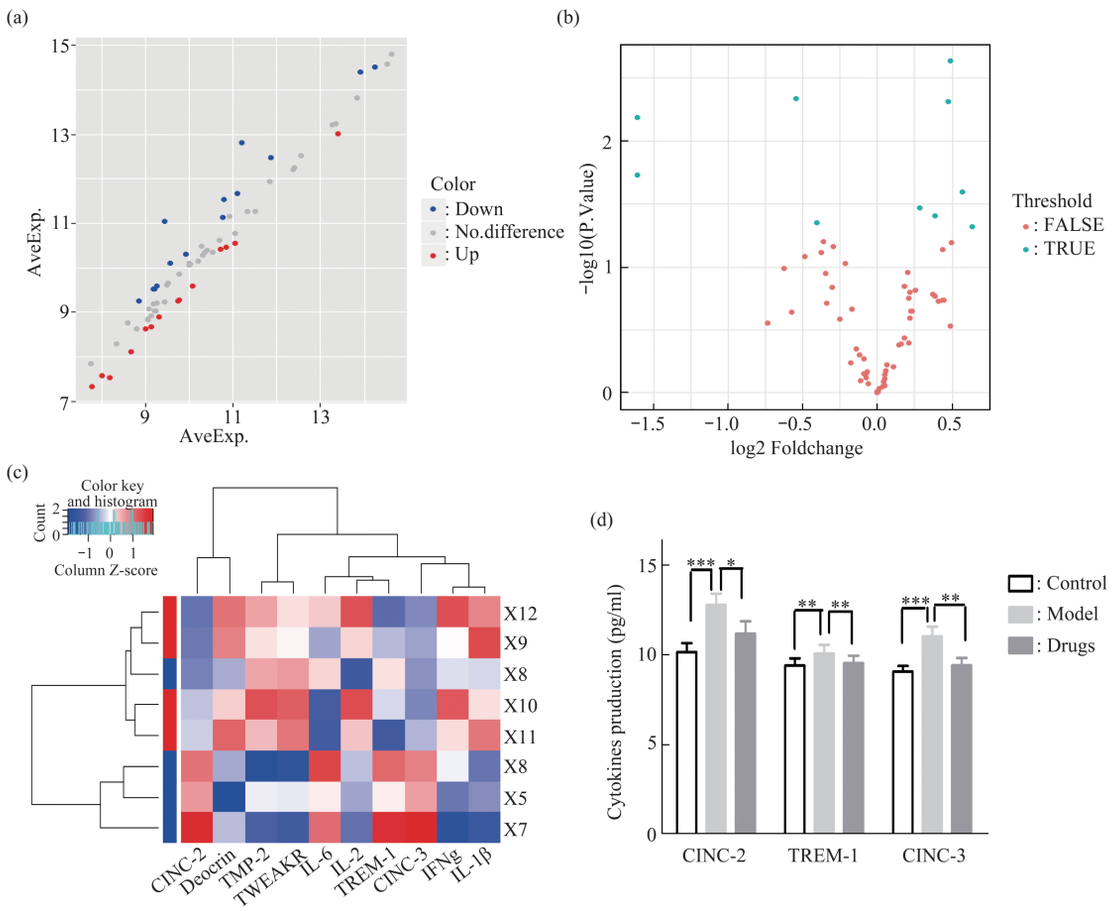

Fig. 4　Effects of Sanliangsan administration on protein expression levels of 67 inflammation−related factors

Using inflammation-related protein chip we performed quantitative and statistical measures of the 67 inflammatory factors and analyzed their expressed patterns in skin samples from rats in each respective group. (a) Scatter plot of the factors in rat skin of the Sanliangsan treatment groups: blue dots = down-regulation; gray dots = no change; red dots = up-regulation. (b) Volcanic diagram of the 67 factors: red dots = no statistical difference; blue dots = statistical differences. (c) Statistic heat map distribution of the inflammation-related factors that showed significant changes in expression: blue = down-regulated; red = up-regulated. (d) Expressed levels of three inflammatory factors in each respective group. **P*<0.05, ***P*<0.01, ****P*<0.001.

post administration. It shows that the top three mechanisms of skin inflammation in these groups were cytokine receptor interaction, *Salmonella* infection and the IL-17 signaling pathway (Figure 5b). We propose that the common factor of these two sets of comparative data — *i.e.* the IL-17 signal pathway — may be the pathway through which Sanliangsan exerts its anti-inflammatory effects on targeted drug related rashes. To further investigate this possibility we used immunohistochemical staining to detect the expression of IL-17A in the skin samples of the study groups. Significantly stronger staining of IL-17A-positive cells were observed in the skin of the model group compared with the control group (Figure 5c). Treatment with Sanliangsan led to significantly reduced IL-17A-positive cells, especially in the mediumiddle- and high-dose groups (Figure 5d).

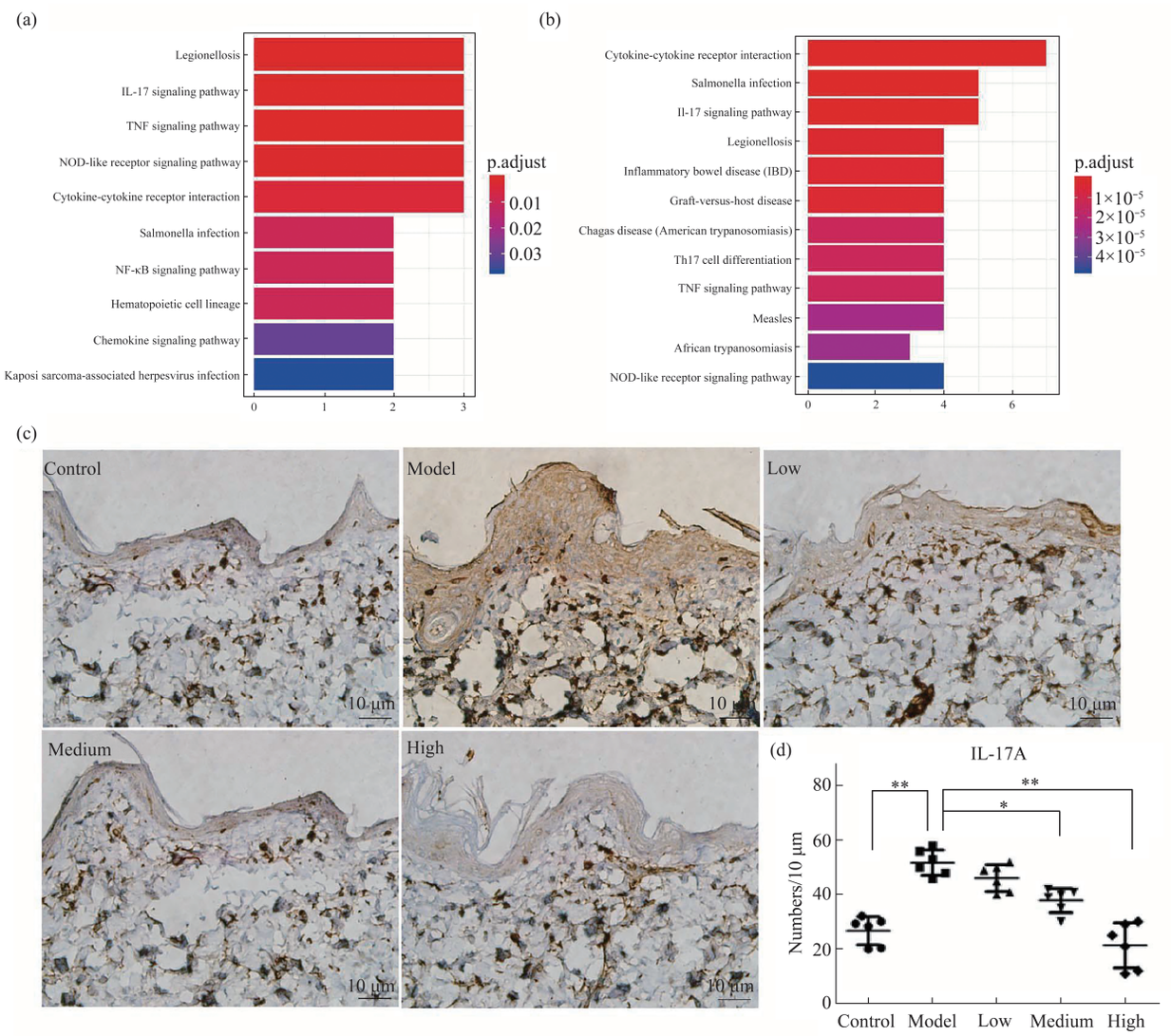

Fig. 5　Correlation analysis of gefitinib and Sanliangsan on inflammation−related protein expression levels and the identification of IL−17A as a key pathway

Correlation analysis of inflammation-related signaling pathways in rat skin was made from comparisons between (a) the control group compared with the model group and (b) the model group compared with the Sanliangsan groups. This was done to statistically rank the different signaling pathways. (c) Immunohistochemistry of IL-17A protein expression in rat skin of each study group. (d) Quantitative analysis showing a significantly higher number of IL-17A-positive cells in the model group compared with the control group and further by dose-dependent reductions of IL-17A-positive cells in the Sanliangsan treatment groups. *P<0.05, **P<0.01, ***P<0.001.

4　Discussion

The anti-inflammatory effect of Sanliangsan on gefitinib-induced skin rash remains somewhat enigmatic. Our results have shown that the administration of Sanliangsan can relieve skin itch in rats, reducing secondary injury and inflammatory reaction caused by skin bacteria. In traditional Chinese medicine theory, the loss of skin moisture and thus dryness is mainly caused by blood deficiency and wind-damp, making the skin prone to pruritus.

Sanliangsan consists of *Astragali radix*, *Lonicera japonica*, *Angelica sinensis*, *Glycyrrhiza glabra rhizome* and *Centipede*, all of which are associated

with detoxification, particular in cell types associated with inflammation. The available experiments support the role of Astragaloside, an active component of *Astragali radix*, in reducing macrophage infiltration[19-20], possibly *via* regulation of the macrophage-mediated inflammatory response[21]. *Lonicera japonica* is considered to clear heat and remove toxicity, while studies have shown that it can also reduce macrophage infiltration and lower the inflammatory response[22-23]. Ferulic acid reduces the level of reactive oxygen species and improves the inflammatory response of animal model with variant asthma[24]. Ferulic acid is one of the main active components of both *Lonicera japonica* and *Angelica sinensis*. Glycyrrhizinic acid the main active component in *Glycyrrhiza glabra rhizome* also show significant reduced levels of reactive oxygen species in macrophages and improved the inflammatory response in mice with variant asthma[25]. Scolopendra is the main active component of *Centipede*. Experiments using bombesin to induce the inflammatory response of pancreatic vesicle cells have been used to establish a cell model of acute pancreatitis. In these experiments Scolopendra effectively inhibited inflammation and reduced cell necrosis[26]. *Centipede* also has a strong reparative effect on joint inflammation[27]. All of the above findings are in agreement with our results of Sanliangsan treatment of skin rashes in rats.

In our study we used both photographs and itch score analysis to assess changes in skin inflammation reactions and their response to Sanliangsan treatment. It is not surprising that Sanliangsan, a traditional Chinese medicine formula containing *Astragali radix*, *Lonicera japonica* and *Glycyrrhiza glabra rhizome* have a therapeutic effect on the inflammatory response to rash caused by an EGFR inhibitor such as gefitinib as each remedy have been shown to have both anti-inflammatory and antiviral effects[20-25].

Sanliangsan administration led to significantly down-regulated expression of inflammation-related factors associated with macrophage downstream secretion: CINC-2 (MIP-1), CINC-3 (MIP-2) and TREM-1. Recent studies imply that ferulic acid can inhibit the expression of MIP-2 secreted by macrophages and improve the inflammatory response of macrophages[28-29]. In a study that established a cell model of allergic dermatitis by infecting keratinocytes with hairy bacteria, it was suggested that glycyrrhizic acid could significantly reduce the expression of MIP-2 and improve the inflammatory reactions of keratinocytes[30]. Another study suggested that glycyrrhizinic acid is not only a good anti-inflammatory agent, but also protects hepatocytes mainly by reducing the expression of MIP-1α[31]. The IL-17A signaling pathway is one of the key signaling pathways in the inflammatory response of skin T cells, B cells and macrophages. Skin inflammation mediated by IL-17A is thought to involve the transformation of macrophages from the M2 type to the M1 type[15]. In a study investigating the mechanism of EGFR inhibitor-related rash, it was found that IL-17A expression in the skin of waved 2 (*Egfr*^{wa2}) mice treated with EGFR inhibitors was significantly increased, indicating that IL-17A plays a key role in the induction of skin rash[32].

Our present findings also demonstrated that IL-17A was the most likely signaling pathway involved in the anti-inflammatory mechanism of Sanliangsan in gefitinib-induced skin rash. Immunohistochemistry showed that the strongest IL-17A staining was in the skin erosion area and epidermis. Treatment with Sanliangsan significantly reduced the number of IL-17A positive cells in the skin. Our findings further supports the suggestion that the anti-inflammatory mechanism of this traditional Chinese medicine formula is closely related to the IL-17 signaling pathway. At the same time other studies also found that traditional Chinese medicine not only plays an important role in the treatment of skin diseases, they also have good therapeutic prospects in treatment of hepatitis B, leukemia, and disorders of the central nervous system[33-35].

In conclusion, our results demonstrate that the anti-inflammatory effects of Sanliangsan on gefitinib-induced skin rash occurred *via* the modulation of macrophages in close association with the IL-17A signaling pathway (Figure 6).

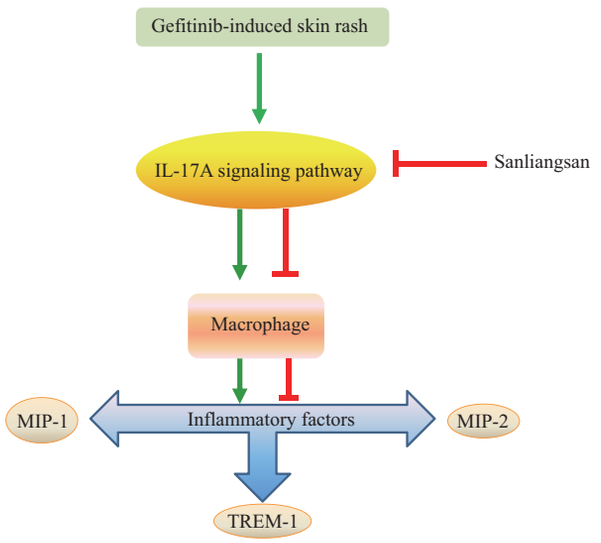

Fig. 6　Proposed mechanism of action of the anti-inflammatory effect of Sanliangsan on gefinitib-induced skin rash in rats

Up-regulation (green arrow) and down-regulation (red line).

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“三两三”通过巨噬细胞发挥对吉非替尼所致皮疹的抗炎作用*

万亮琴[1,2)**]　宋晨晨[1)**]　谭　琰[1)]　何　芳[1)]　张亚丽[1)]　王雅蕾[1)]　陈紫薇[1)]
张　策[1)]　谷若曦[1)]　张丁阳[1)]　王　旭[1)]　华　茜[1)***]
（[1)] 北京中医药大学，北京 100029；[2)] 北京市鼓楼中医医院，北京 100009）

摘要　吉非替尼所致的皮疹是治疗癌症中的难题，而中药验方三两三对于该皮疹具有较好的临床疗效. 由于三两三治疗皮疹的机制尚不清楚，本文探究了该中药验方对吉非替尼所致皮疹的抗炎作用. 将Brown Norway（BN）大鼠随机分为5组：野生型对照组、吉非替尼皮疹模型对照组、皮疹模型三两三低剂量组、中剂量组、高剂量组. 采用吉非替尼（上午）和三两三（下午）同天给药4周. 三两三低、中、高剂量组分别按照2 mg/kg/day、4 mg/kg/day、8 mg/kg/day 的剂量对BN模型大鼠进行灌胃，对照组给予纯净水. 使用流式细胞仪对巨噬细胞进行分类；免疫组化检测蛋白质的表达；蛋白芯片检测与炎症相关的信号通路和炎症因子. 结果表明，与野生型对照组相比，吉非替尼皮疹模型对照组中巨噬细胞炎症蛋白（MIP）-1、MIP-2、髓细胞触发受体-1（TREM-1）和IL-17A的表达显著增加. 三两三干预组与吉非替尼皮疹模型对照组相比，MIP-1、MIP-2、TREM-1和IL-17A的表达明显降低，且三两三对吉非替尼所致皮疹的抗炎作用与巨噬细胞的IL-17A信号通路密切相关.

关键词　三两三，吉非替尼，皮疹，巨噬细胞，IL-17A，炎症
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** 并列第一作者.
*** 通讯联系人.
Tel：010-64286192，E-mail：hqianz@aliyun.com，huaq@bucm.edu.cn
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