A model for anticancer surveillance was pharmacologically developed to evaluate vitality principle in breast cancer rats

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METHODS: The breast cancer in rats was replicated with 7,12-Dimethylbenz[a]anthracene (DMBA, i.g., 100 mg/kg) at d0. The anticancer surveillance was defined as the intervals between the primary sensitization and the first challenge stirred with complete Freund's adjuvant (CFA), the various intervals (k = 0.80) were dominated from d10 (600.00 h) to d10 (2288.82 h). The optimal surveillance status was confirmed with the median effective interval (EI) from tumor volume regressive curve, for developing the pharmacodynamic model. The tumor and tumor infiltrating lymphocyte histopathology was used to confirm the immune surveillance being affected with CFA in breast cancer tumorigenesis. The availability of this model was confirmed with Shugan Liangxue prescription (SLP), from the vitality principle, and assured further from interleukin-12 levels.

RESULTS: The regressive curve was set up between the intervals and tumor volumes, the EI in SLP-treated rats (1475.00 h, \( Y_{up} = 0.1026 + 0.8780/(1 + 10^{0.1475 \cdot 0.3050/3}) \)) was postponed, which was 1.87 multiple of the EI in CFA rats (791.40 h, \( y = -0.0525 + 0.9452/(1 + 10^{0.4870 \cdot 10.5271/3}) \)), so did prepone the curve between the intervals and the immunological biomarker, serum interleukin-12 levels, the EI in SLP-treated rats (744.90 h, \( Y_{up} = -0.0145 + 0.7455/(1 + 10^{0.7396 \cdot 10.1341/3}) \)) be 0.78 multiple of the EI in CFA rats (960.10 h, \( Y_{up} = 0.2460 + 0.7270/(1 + 10^{0.7159 \cdot 10.521/3}) \)), this immunological action being mediated the anticancer prognosis. Tumor histology was confirmed the more tumor infiltrating lymphocytes activated in SLP rats with CFA stirred immunity than rats only received CFA.

Abstract

OBJECTIVE: To evaluate vitality principle in breast cancer rats by pharmacologically developing a model for anticancer surveillance.
CONCLUSION: The model for anticancer surveillance was pharmacologically established as the optimal interval (791.40 h) between the primary sensitization and the first challenge stirred with complete Freund’s adjuvant. This available model was confirmed with SLP, from the vitality principle, for evaluating immunological effects against breast cancer.

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Keywords: Breast cancer; Pharmacology; Drug screening assays; antitumor; Freund’s adjuvant; Shugan Liangxue prespriction

INTRODUCTION

Breast cancer (BC) is the first cause of malignant diseases in women. BC immune escape partly originates from the oncocytes inhibiting immune surveillance, consequently, immune system promotes BC pathogenesis rather than prevents BC development in general. Inhibiting immune escape has been successfully used for BC therapies. This effectiveness has been evaluated with the quantification of tumor infiltrating lymphocyte (TIL). The vitality principle (VP) has been defined as a therapy strategy for enhancing individual ability to defend any harmful attacking in Traditional Chinese Medicine (TCM), especially, inhibiting immune escape in BC. From TCM VP strategy, Shugan Liangxue prespriction (SLP), composed of eleven herbal medicines, was used for inhibiting BC immune escape. Complete Freund’s adjuvant (CFA) was used to amplify cell-mediated immunity for benefitting BC prognosis in experimental BC rats, especially TILs, such as T helper and effector lymphocytes. Anticancer surveillance in BC rats was designed as CFA-stirred immunity in this study. This anticancer degree was depended on the interval between the primary sensitization and the first challenge of CFA. The optimal interval (EL0) was regressed from the BC tumor volumes at various endpoints, establishing an available pharmacodynamic model for evaluating VP immunological effects. The specificity of immunological surveillance in this model was assured with the administration of SLP.

MATERIALS AND METHODS

Reagents

Preparation of Shugan Liangxue prescription: Shugan Liangxue Prescription is composed of Chaihu (Radix Bupleuri Chinensis), Huangqi (Radix Astragali Mongolici), Gancao (Radix Glycyrrhizae), Chenpi (Pericarpium Citri Reticulatae), Yujin (Radix Curcumae Wenyuqin), Mudanpi (Cortex Moutan Radicis), Huaithua (Flos Sophorae), Zicao (Radix lithospermii), Ezhu (Rhizoma Curcumae Phaeocaulis), Xiakucao (Spica Prunellae Vulgaris) and Danshen (Radix Salviae Miltiorrhizae). All herbal materia medica were purchased from Beijing Tongrentang Drugstore (Beijing, China). According to Pharmacopoeia of the People’s Republic of China (2015), the decoction (3.0 g/mL) of SLP was extracted by the pharmaceutical department, Beijing University of Chinese Medicine. The main and relatively constant components of SLP: saikosaponins a, saikosaponins d, rutin and hesperidin were used for quality control by high-performance liquid chromatography. Complete Freund’s adjuvant: CFA is composed of paraffin oil, mannide monooleate and heat-killed, dried Bacillus Calmette-Guerin (BCG). CFA was purchased from Sigma-Aldrich (USA), which had been used to stir immunity in several researches.

Animals

Two hundred and ten female SD rats [(185 ± 9) g, body weight, N = 210] were obtained from Animal Centre of the Chinese Academy of Medical Sciences. Animals were kept under a 12h-light/dark cycle, temperature [(25.0 ± 0.2) °C] and humidity (45% ± 2%) controlled specific pathogen free environment. The animals were acclimatized for 3 d, fed standard rodent pellets and allowed free access to filtered water. All experimental procedures were approved by the Ethics Committee of the Institute of Medicinal Plant Development, CAMS & PUMC (Ethical approval No. SLXD-2017011329).

Experimental design

The intervals designed between the primary sensitization and the first challenge was considered as immune surveillance against BC stirred with CFA. The optimal interval of CFA group was used to establish as a pharmacodynamic model for evaluating VP. The interval of SLP group was designed to confirm the model available to evaluate VP immunological effects against BC. Being equalized with body weight, 210 rats were randomly divided into 3 groups (n = 70). At d0, the rats in two oncogenic groups were once administrated intragastrically with a single dose of 7,12-Dimethylbenz[a]anthracene (DMBA, 100.0 mg/kg; Sigma, St. Louis, MO, USA), so did control rats with olive oil (OO, 10.0 mL/kg). At d0, d10, d20, for primary sensitization at the medial side of proximal limbs, the rats in two oncogenic groups were daily administrated subcutaneously with CFA (0.05 mL/rat, Sigma, St. Louis, MO, USA), so did the rats in control group with normal saline (NS, 0.05 mL).

In the morning at d20, the twelve nipples and their surrounding tissue of each rat were checked by palpation to assess tumorigenesis, including both precancerous lesions and cancerous tumors in lateral or bilateral mammary glands. The precancerous lesions were accounted as the number of palpated hardness around each nip.
ple, and the cancerous tumors were quantified as both tumor numbers and tumor volumes, long (a) and short diameters (b) of each tumor were gauged using calipers to calculate tumor volume using the formula: \( V = \frac{\pi a b^2}{6} \) (cm³).

A total carcinogenic score had been derived from all parameters with a coefficient to modify itself contribution in the pathological process (total sum of all coefficients was dominated as 1.00). Each rat was sequenced according to itself total carcinogenic score for it being further located in subgroups. Being equalized with total carcinogenic score, 70 rats in each group were randomly divided into 7 subgroups (\( n = 10 \)) for 7 intervals, subcontrol, subCFA and subSLP, respectively.

In the afternoon at \( d_{056} \), the rats in 7 SLP subgroups were administrated intragastrically with SLP (18.0 g/kg) daily until each endpoints, so did the rats in 7 control or CFA groups with 0.5% CMC-Na (10.0 mL/kg). According to design, the day (\( d_i \)) for initiating the first challenge of CFA was calculated from a formula 

\[ aa = 52 + 25/(0.8)^{i-1} \]  

(\( i = 1-7 \)) for each animal. At \( d_{052} \), \( d_{058} \), and \( d_{054} \), the rats in each CFA or SLP subgroups were daily administrated with CFA (0.5ml/rat) challenge subcutaneously, so did the rats in control subgroups with NS (0.5 mL). Each of 7 endpoints (\( d_{052} \)) was 50 d after the first challenge [\( bb = 52 + 25/(0.8)^{i+1} + 50 \)]. At endpoint, the subgroups in 3 groups were matched with carcinogenic score series, rats were anesthetized by 10% chloral hydrate after fasting for 12 h, blood was selected from aorta abdominalis (Figure 1).

### Detected parameters

**Tumor volume:** at each endpoint, rats were killed, whole skin was peeled off as quickly as possible in autopsy, and the numbers and position of each tumor were recorded. Two long (a) and short (b) diameters were gauged using calipers to calculate tumor volume using the formula: \( V = \frac{\pi a b^2}{6} \) (cm³). The time-effective curve of DMBA induced BC in 3 groups was respectively set up from the regression between the 7 means of total tumor volumes in 7 subgroups and the 7 design.

**Figure 1 Design of pharmacodynamic model for evaluating vitality principle against breast cancer**

At \( d_{052} \), 210 rats were randomly divided into 3 groups (\( n = 70 \)) based on body weight, and administrated intragastrically with DMBA (Oncogenic group) or olive oil (Control group) once. At \( d_{052} \), \( d_{058} \), and \( d_{054} \), the rats were administrated subcutaneously with CFA (Oncogenic group) or normal saline (Control group) for primary sensitization. At \( d_{052} \), each rat was sequenced according to itself total carcinogenic score, 70 rats in each group were randomly divided into 7 subgroups (\( n = 10 \)) for 7 intervals, rats were administrated intragastrically with SLP (SLP group) or 0.5% CMC-Na (control and CFA group) until each endpoints. \( d_i \) for initiating the first challenge of CFA was calculated from a formula 

\[ aa = 52 + 25/(0.8)^{i-1} \]  

(\( i = 1-7 \)). At \( d_i \) (\( d_{052} \), \( d_{058} \), and \( d_{054} \)) the rats were daily administrated with CFA (0.5 mL) challenge (CFA and SLP groups) or NS (0.5 mL) (control group). At each of 7 endpoints (\( d_i \)), [\( bb = 52 + 25/(0.8)^{i+1} + 50 \)], 3 groups were matched with carcinogenic score series, anesthetized and blood was selected from aorta abdominals. OO: olive oil; DMBA: 7,12-Dimethylbenz[a]anthracene; ig: intragastric administration; NS: normal saline; sc: subcutaneous administration; CFA: complete Freund’s adjuvant; CMC: carboxymethylcellulose; SLP: Shugan Liangxue Prescription. Score: marked as tumor volume; pathology: marked as tumor and tumor infiltrating lymphocytes pathological changes; Immunology: marked as IL-12 level.
signed intervals. The length of the antitumor main peak was located as the EIi in each group, the shift of SLP curve from CFA curve was calculated as the EIi change, indicating SLP prolonging survival duration.

Tumor histology: all mammary tumors were fixed in 4% formalin buffer for 24 h and embedded in paraffin. Sections were cut at 2.0 µm and stained with hematoxylin and eosin (HE staining) for histopathologic examinations using standard protocols, all of the images were acquired with a Digital Pathology system Aperio CS2 (Leica, Germany) for assessment the immune surveillance being affected with CFA or SLP in BC tumorigenesis.13

Cytokine measurement: the blood samples were centrifuged at 3000 rpm for 15 min at 4.0°C, the supernatant was aliquoted into microtubes (100 µL) as the serum stored at −80.0°C until the assays. The level of interleukin-12 (IL-12) in serum was measured using enzyme-linked immunosorbent assay kits (Boster, Wuhan, China) according to the manufacturer’s guidelines as the previous study.14

Statistical analysis
The primary data were analyzed using SPSS 16.0 (SPSS Inc. Released 2007. SPSS for Windows, Version 16.0. Chicago, IL, USA), and expressed as mean ± standard deviation (X ± s) in each subgroups, student t test was carried out between every both groups, P < 0.05 as statistically significant. The potency data were defined from the formula [X = (Xmax - Xmin)/Xmin] in each group, X was a data from a rat, Xmax was the maximum or maximum datum in a detected parameter. Each of the potency data (X) from rats was ranged from 0.00 to 1.00. The potency data were expressed as mean ± standard deviation (X ± s) in each subgroup. 7 means at 7 endpoints were analyzed with 7 intervals, as logarithmic value of the duration (ln T - ln T0 × 24) between the primary sensitization and the first challenge of CFA, using GraphPad Prism 5 in the manner as non-linear regression with both of variable slope and 95% confidence intervals, the equation, EIi and its 95% confidence intervals were expressed in CFA group and SLP group, respectively at the same orthogonal coordinate system, the shift of SLP curve from CFA curve was calculated as the EIi change, indicating SLP proponing or postponing. A Pearson’s correlation coefficient test was used to determine the correlation of BC tumor volume and the intervals between the primary sensitization and the first challenge of CFA. A P < 0.05 was the significant level.

RESULTS
Optimal interval for CFA-stirred immunity against breast cancer
The morbidity of BC tumor was 0% in control rats, more than 85% in oncogenic rats. Being indicated as the primary data (Table 1), the increase means of tumor volume were depended on the lengths of the intervals or the endpoint duration in both CFA subgroups (y = 0.001x + 0.5181, n = 7, R2 = 0.626, P < 0.05) and SLP subgroups (y = 0.0005x-0.0032, n = 7, R2 = 0.8864, P < 0.01). At each interval, the average volume in SLP rats was smaller than that in CFA rats, especially at the three interval (P < 0.05), at 937.50 h (0.6 ± 0.6 vs 21.1 ± 1.7), 1171.88 h (0.4 ± 0.5 vs 2.0 ± 2.3), 1464.84 h (0.8 ± 0.9 vs 2.4 ± 2.1), respectively (Table 1).

### Table 1 Interval leading BC tumor volume (TV) in CFA or SLP rats (X ± s)

<table>
<thead>
<tr>
<th>Interval (h)</th>
<th>CFA n, TV</th>
<th>SLP n, TV</th>
</tr>
</thead>
<tbody>
<tr>
<td>600.00</td>
<td>9, 5.0±0.5</td>
<td>10, 0.4±0.4</td>
</tr>
<tr>
<td>750.00</td>
<td>9, 1.0±1.5</td>
<td>8, 0.3±0.4</td>
</tr>
<tr>
<td>937.50</td>
<td>2.1±1.7</td>
<td>7, 0.6±0.6</td>
</tr>
<tr>
<td>1171.88</td>
<td>2.0±2.3</td>
<td>8, 0.4±0.5</td>
</tr>
<tr>
<td>1464.84</td>
<td>2.4±2.1</td>
<td>9, 0.8±0.9</td>
</tr>
<tr>
<td>1831.06</td>
<td>2.1±3.4</td>
<td>10, 1.0±1.0</td>
</tr>
<tr>
<td>2288.82</td>
<td>2.5±5.8</td>
<td>9, 1.1±2.6</td>
</tr>
</tbody>
</table>

Notes: the tumor volume in BC rats (DMBA, 100 mg/kg) with CFA (0.05 mL/rat for sensitization, 0.5 mL/rat for challenge) and BC rats with CFA and SLP (18 g/kg), t-test, vs CFA, P < 0.05, at the same interval. DMBA: 7,12-Dimethylbenz[a]anthracene; BC: breast cancer; CFA: complete Freund’s adjuvant; SLP: Shugan Lìngxīue presprcriptions.

Being indicated as the potency data (Table 2) in CFA or SLP group, the increase means of tumor volume potencies were depended on the logarithmic values of the intervals, either CFA subgroups (y = 1.5626x − 4.1396, n = 7, R2 = 0.7668, P < 0.01) or SLP subgroups (y = 1.6842x − 4.7557, n = 7, R2 = 0.8241, P < 0.01). Being normalized with the range from the minimum to the maximum in CFA or SLP group, the average volume potency in SLP rats was similar to CFA rats at each interval (P > 0.05), respectively.

### Table 2 Interval leading BC tumor volume potency (TVP) in CFA or SLP rats (X ± s)

<table>
<thead>
<tr>
<th>Log[Interval (h)]</th>
<th>CFA n, TVP</th>
<th>SLP n, TVP</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.778151</td>
<td>9, 0.0±0.3</td>
<td>10, 0.1±0.5</td>
</tr>
<tr>
<td>2.875061</td>
<td>9, 0.3±0.8</td>
<td>8, 0.0±0.5</td>
</tr>
<tr>
<td>2.971971</td>
<td>10, 0.8±0.9</td>
<td>7, 0.3±0.7</td>
</tr>
<tr>
<td>3.068881</td>
<td>9, 0.7±1.2</td>
<td>8, 0.1±0.6</td>
</tr>
<tr>
<td>3.165791</td>
<td>9, 1.0±1.0</td>
<td>9, 0.6±1.1</td>
</tr>
<tr>
<td>3.262701</td>
<td>9, 0.8±1.7</td>
<td>10, 0.8±1.2</td>
</tr>
<tr>
<td>3.359611</td>
<td>8, 1.0±2.9</td>
<td>9, 1.0±3.4</td>
</tr>
</tbody>
</table>

Notes: the tumor volume potency in BC rats (DMBA, 100 mg/kg) with CFA (0.05 mL/rat for sensitization, 0.5 mL/rat for challenge) and BC rats with CFA and SLP (18 g/kg), TVP: tumor volume potency; DMBA: 7,12-Dimethylbenz[a]anthracene; BC: breast cancer; CFA: complete Freund’s adjuvant; SLP: Shugan Lìngxīue prespiration.

From the potency data, the non-linear equation was regressed with GraphPad Prism 5 in the same orthogonal coordination, the potency data were expressed as mean ± standard deviation (X ± s) in CFA group (n = 7, r = 0.9727), or y = 0.1026 ± 0.8780/[1 + 10^(-0.2750±0.5656)] in SLP group (n = 7, r = 0.9595).
The Ei50 with its 95% confidence interval was 791.4 h (630.5-993.4 h) in CFA group, or 1475.0 h (1064.0-2045.0 h) in SLP group. The right shift of SLP Ei50 from CFA Ei50 indicated the sensitive duration was nearly double ($10^{0.271} = 1.87$).

The time-effective regression was tested with Pearson's correlation coefficient (95% confidence interval) as 0.8759 (0.3606-0.9815) in CFA group ($P < 0.01$), and 0.9073 (0.4867-0.9864) in SLP group ($P < 0.01$), respectively (Figure 2).

![Figure 2](image)

Figure 2 SLP postponing nearly double the interval from CFA antitumor

The non-linear equation was regressed in the same orthogonal coordination, $y = -0.0525 + 0.9452/[1 + 10^{10.0403}e^{0.0027}]$ with the Ei50 (its 95% confidence interval) 791.4h (630.5-993.4 h) in CFA group ($n = 7$, $r = 0.9727$) (red curve), or $y = 0.1026 + 0.8780/[1 + 10^{27.1454}e^{0.0059}]$ with the Ei50 (its 95% confidence interval) 1475.0 h (1064.0-2045.0 h) in SLP group ($n = 7$, $r = 0.9959$) (blue curve). The right shift of SLP Ei50 from CFA Ei50 indicated SLP postponing double the interval from CFA antitumor ($10^{0.271} = 1.87$).

**DMBA-induced BC histopathology in rats with CFA-stirred anticancer immunity**

Normal mammary: in control rats, the mammary gland is mainly composed of ducts with few acini. The normal epithelia of mammary glands included in three types located at the interlobular ducts, terminal lobular ducts and acini. The epithelia at interlobular ducts were the pseudostratified columnar epithelia, one or two layers of nuclei, the plasma of each cell contacted to the basement membrane, if they had been considered as three dimensions. The nucleus-to-cytoplasm ratio may approach to 3/5 with the nucleus near the basement membrane. There were collagen fibers, myoepithelia, blood vessels, fibroblasts and infiltrated resident inflammatory cells outside the basement membrane, as the mesenchyma structure of the interlobular ducts (Figure 3B). The epithelia at terminal lobular ducts were the cubic or columnar epithelia, as monolayer of nucleus. The nucleus-to-cytoplasm ratio may approach next to 2/5 with the nucleus near the basement membrane. There were vessels, collagen, myoepithelia and fibroblasts, as the mesenchyma (Figure 3C). The epithelia at acini were the cone epithelia bordering the lumens, one layer of nucleus. The nucleus-to-cytoplasm ratio is next to 1/5 with multiple vesicles in cytoplasm.

There were vessels, white adipocytes, fibroblasts and collagen as the mesenchyma (Figure 3D).26

Carcinogenic mammary: in the rats with DMBA-induced BC and CFA-stirred immunity, the differentiated oncocytes in the malignant nests originated from interlobular ducts, terminal lobular ducts and acini with various pleomorphic atypia (reversing to differentiation form tubule structure), invading into the stroma through the basement membrane and surrounding desmoplastic response. Some pre-malignant breast lesions nearby the tumor suggested the earliest events from the originated epithelia. The oncocytes from interlobular ducts were the columnar, multilayer, tubule-forming nests with massive collagen surrounding. Their nuclei were larger, thick-staining, and in dysforming shape, with less mitosis, about 4/5 as the nucleus-to-cytoplasm ratio. Their cytoplasm were richier, basophil staining, and more protrusion, without multiple vesicles or clear bordering. Various pleomorph of oncocytes were found as different volume and in multiple shape. There were collagen, myoepithelia, blood vessels, fibroblasts and infiltrated lymphocytes, as the mesenchyma structure (Figure 3E). The oncocytes from terminal lobular ducts were the cubic, thick multilayers, tubule-forming nests with richer preserved myoepithelia and new growing capillaries surrounding. Being spread throughout terminal ducts, the monomorphic oncocytes could extend as invasive lesions distorted the primary nests. The nucleus with ranged grades were in dysforming shape and larger thick-staining, with less mitosis, above 5/9 as the nucleus-to-cytoplasm ratio. The cytoplasm was basophil protrusion, without multiple vesicles or clear bordering. The oncocyte pleomorph was found as rapid proliferation with smaller volume and pressed shape. Some intraepithelia spaces were evenly distributed and regular in shape (cookie cutter-like), as papillary or micropapillary growing manners with rarely central necrosis or small calcifications. There were richer capillaries, myoepithelia, infiltrated lymphocytes, fibroblasts, and some collagen in the mesenchyma (Figure 3F). The oncocytes from acini were the nests with cone epithelia, thick multilayer, tubule-forming with smaller lumens, and some white adipocytes surrounding. The nucleus as a monolayer was larger, thick-staining, and in dysforming shape, with less mitosis, about 4/9 as their nucleus-to-cytoplasm ratio. The cytoplasm was monomorphic, acidophilous staining, and less protrusion, with multiple vesicles or clear bordering.4 There were white adipocytes, richest capillaries, infiltrated lymphocytes and fibroblasts in the mesenchyma (Figure 3G).

Therapy caicinogenic mammary: in SLP rats with DMBA-induced BC and CFA-stirred immunity, the differentiated oncocytes in the malignant nests originated from three locations with moderated atypia, limited invasion and less tubule-forming. It was obvious that monocytic response. Some pre-malignant breast lesions in SLP rats with DMBA-induced BC and CFA-stirred immunity, the differentiated oncocytes could extend as invasive lesions distorted the primary nests. The nucleus with ranged grades were in dysforming shape and larger thick-staining, with less mitosis, above 5/9 as the nucleus-to-cytoplasm ratio. The cytoplasm was basophil protrusion, without multiple vesicles or clear bordering. The oncocyte pleomorph was found as rapid proliferation with smaller volume and pressed shape. Some intraepithelia spaces were evenly distributed and regular in shape (cookie cutter-like), as papillary or micropapillary growing manners with rarely central necrosis or small calcifications. There were richer capillaries, myoepithelia, infiltrated lymphocytes, fibroblasts, and some collagen in the mesenchyma (Figure 3F). The oncocytes from acini were the nests with cone epithelia, thick multilayer, tubule-forming with smaller lumens, and some white adipocytes surrounding. The nucleus as a monolayer was larger, thick-staining, and in dysforming shape, with less mitosis, about 4/9 as their nucleus-to-cytoplasm ratio. The cytoplasm was monomorphic, acidophilous staining, and less protrusion, with multiple vesicles or clear bordering.4 There were white adipocytes, richest capillaries, infiltrated lymphocytes and fibroblasts in the mesenchyma (Figure 3G).

Therapy caicinogenic mammary: in SLP rats with DMBA-induced BC and CFA-stirred immunity, the differentiated oncocytes in the malignant nests originated from three locations with moderated atypia, limited invasion and less tubule-forming. It was obvious that more TILs accompanied with less smaller nests, and
less oncocytes density in nests. The oncocytes from interlobular ducts were the columnar, monolayer, tubule-forming nests with micropapillary growth and less collagen. The nucleus was larger, thick-staining, and in fixed shape, without mitosis and nucleolus, about 4/5 as the nucleus-to-cytoplasm ratio. The cytoplasm was richer, basophil staining, with some multiple vesicles or clear bordering. Oncocyte pleomorphism did as multiple shape and formed smaller volume. There were myoepithelia, fibroblasts, collagen, blood vessels and TILs in mesenchyma (Figure 3H). The oncocytes from terminal ducts were the cubic, thicker multilayer, tubule-forming nests with myoepithelia and rich capillaries. The monomorphic oncocytes could extend as invasive lesions throughout terminal ducts. The nuclei with ranged grades were thick-staining and in various shape, with less mitosis and nucleolus, more than 1/2 as the nucleus-to-cytoplasm ratio. The cytoplasm were basophil, with some multiple vesicles or clear bordering. The oncocyte pleomorphism was found as limited proliferation with smaller nests. Some intraepithelia spaces were regular in shape, as micropapillary growing manners with rarely central necrosis or small calcifications. There were richer capillaries, myoepithelia, TILs and some collagen in mesenchyma (Figure 3I). The oncocytes from acini formed the nests with cone epithelia, multilayers, tubule-forming with smaller lumens, and some white adipocytes surrounding. The nucleus as a monolayer were larger and thick-staining, without mitosis and nucleolus, less than 1/2 as the nucleus-to-cytoplasm ratio. The cytoplasm was monomorphic, and acidophilous staining, with more multiple vesicles and central necrosis. There were white adipocytes, richest capillaries and infiltrated lymphocytes in the mesenchyma (Figure 3J). 

**TIL phenotype of BC in the rats with CFA-stirred anticancer immunity**

For evaluating SLP potency, the TILs in stroma were more significant than all lymphocytes in tumor in this BC antitumor immunological experiment.

Normal mammary: in control rats, the resident mature...
lymphocyte was scatterly observed in lobule histological structure of mammary gland. At the interlobular ducts, the lymphocytes were floating distribution and at silent status, demonstrated as round large dark-staining nucleus (condensed chromatin) with vague nucleoli, less basophilic cytoplasm with clear bordering, and 7/9 nucleus-to-cytoplasm ratio (Figure 4B). At the terminal ducts, those were scatterly distributed, as round large dark nucleus, some basophilic cytoplasm with clear perinuclear zone (or halo), and 5/9 nucleus-to-cytoplasm ratio (Figure 4C). At the acini, the lymphocytes were active in some degree, demonstrated as round large nucleus with vague nucleoli, more basophilic cytoplasm with thin staining, and 4/9 nucleus-to-cytoplasm ratio, some advanced into the epithelia of the acini through the basement membrane (Figure 4D).  

Carcinogenic mammary: in the rats with DMBA-induced BC and CFA-stirred immunity, the TILs were observed as large thick-staining nucleus, 4/5 nucleus-to-cytoplasm ratio at the nests from interlobular ducts (Figure 4E). Those were observed as rounded larger thick nucleus, larger basophil cytoplasm, and decreased nucleus-to-cytoplasm ratio at the nests from terminal ducts (Figure 4F). Those were observed as rounded large thick nucleus, large basophil cytoplasm, and 3/5 nucleus-to-cytoplasm ratio at the nests from acini. They were activated enough to attack the malignant oncocytes obviously, accompanied with their karyopycnosis with dissolved cytoplasm consequently (Figure 4G). The TILs around nests from interlobular ducts to acini, had been activated gradually, being indicated as the decreased nucleus-to-cytoplasm ratio, and the malignant cellular necrosis exacerbated.  

Therapy carcinogenic mammary: in the rats with DMBA-induced BC, CFA-stirred immunity and SLP administration, the TILs were observed as large thick nucleus with more karyopycnosis, 2/5 nucleus-to-cytoplasm ratio in the nests from interlobular ducts, located around the vessels more (Figure 4H). The TILs in the nests of terminal ducts were observed as larger thick nucleus, 4/9 nucleus-to-cytoplasm ratio, swollen basophilic cytoplasm with dissolved vesicles. Those TILs were activated enough to attack into the nests through the basement membrane, accompanied with malignant cellular karyopycnosis (Figure 4I). The TILs in nests from acini were also defined as the larger thick nucleus with more karyopycnosis, swollen eosinophilic cytoplasm with dissolved vacuoles. The nests from acini were severely destroyed, lytic oncocytic necrosis and obviously interstitial edema (Figure 4J). From the pathological changes, the TILs in SLP rats had fully been activated.  

**Clinic biomarker for immunological mechanism against breast cancer**

IL-12 exhibits antitumor function itself and regulates T cells activity especially CD8+ T cells (Table 3). IL-12 level in control rats was independent (y =
0.0041x + 44.186, n = 7, R² = 0.2334, P > 0.05), while the decrease means of IL-12 in CFA group (y = −0.0087x + 58.895, n = 7, R² = 0.5013, P < 0.05) or the increase IL-12 means in SLP group (y = 0.0045x + 49.938, n = 7, R² = 0.5915, P < 0.05) was depended on the lengths of the intervals or the endpoint duration. The average of IL-12 in CFA rats was larger than control rats at 600.00 h (56.1 ± 15.3 vs 40.9 ± 4.6, P < 0.05), while since 1171.88 h, CFA rats showed a lower IL-12 average level than that of control rats (P < 0.01), especially at 1171.88 h (42.7 ± 6.1 vs 50.5 ± 2.5), 1464.84h (37.5 ± 4.4 vs 44.4 ± 0.4) and 2288.82 h (P < 0.05) (42.0 ± 10.0 vs 54.5 ± 13.6), respectively. At each interval, SLP rats showed a higher IL-12 average level than that of control rats, especially at the four intervals, 750.00 h (53.6 ± 3.9 vs 49.4 ± 0.4, P < 0.05), 1171.88 h (57.4 ± 6.5 vs 50.5 ± 2.5, P < 0.01), 1464.84 h (56.1 ± 8.2 vs 37.5 ± 4.4, P < 0.01), 1831.06 h (56.2 ± 5.8 vs 51.7 ± 0.4, P < 0.05), respectively. Compared with CFA group, IL-12 means in SLP group were higher since 937.50 h (P < 0.01), especially at the three intervals at 1171.88 h (57.4 ± 6.5 vs 42.7 ± 6.2), 1464.84 h (56.1 ± 8.2 vs 37.5 ± 4.4), 2288.82 h (60.7 ± 3.3 vs 42.0 ± 10.0) (Table 3).

Being indicated as the potency data (Table 4) in the three groups, the IL-12 potencies showed the same tendency with the IL-12 level data in each group (control, y = 0.8992x − 2.1539, n = 7, R² = 0.2622, P > 0.05), (CFA, y = −1.4115x + 4.8516, n = 7, R² = 0.6116, P < 0.05), (SLP, y = 1.2309x − 3.3032, n = 7, R² = 0.6749, P < 0.05). After being normalized with the range from the minimum to the maximum in each group, the average IL-12 level potencies in CFA rats were larger at log (interval) 2.778151 (0.9 ± 0.8 vs 0.0 ± 0.3, P < 0.05), while smaller than that in control rats at 3.068881 (0.3 ± 0.3 vs 0.7 ± 0.2, P < 0.01), 3.165791 (0.0 ± 0.2 vs 0.2 ± 0.0, P < 0.05), respectively. Potencies in SLP rats were smaller at log (interval) 2.778151 (0.0 ± 0.9 vs 0.9 ± 0.8, P < 0.05) and 2.875061 (0.4 ± 0.3 vs 1.0 ± 0.6, P < 0.05), while much larger than that in CFA rats at 3.359611 (1.0 ± 0.3 vs 0.2 ± 0.5, P < 0.01) (Table 4).

From the potency data (Table 4), the non-linear equation was regressed with GraphPad Prism 5 in the same orthogonal coordination, y = 0.2460 + 0.72701[1 + 10^{−(0.546−2.32)x}](n = 7, r = 0.9242) in CFA group, or y =

<p>| Table 3 Interval leading IL-12 in CFA or SLP rats (x ± s) |
|---------------------------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Interval (h)</th>
<th>Control</th>
<th>CFA</th>
<th>SLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>600.00</td>
<td>5</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>750.00</td>
<td>6</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>937.50</td>
<td>5</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>1171.88</td>
<td>6</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>1464.84</td>
<td>6</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>1831.06</td>
<td>6</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>2288.82</td>
<td>6</td>
<td>5</td>
<td>6</td>
</tr>
</tbody>
</table>

Notes: the IL-12 level in control rats, BC rats (DMBA, 100 mg/kg) with CFA (0.05 ml/rat for sensitization, 0.5 ml/rat for challenge) and BC rats with CFA and SLP (18 g/kg), t-test, vs control, P < 0.05, P < 0.01, vs CFA, P < 0.01, at the same interval. DMBA: 7,12-Dimethylbenz[a]anthracene; BC: breast cancer; CFA: complete Freund’s adjuvant; SLP: Shugan Lianxue prescryption; IL-12: interleukin-12.

<p>| Table 4 Interval leading BC tumor volume potency in CFA or SLP rats (x ± s) |
|--------------------------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Interval (h)</th>
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<th>CFA</th>
<th>SLP</th>
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<tr>
<td>2.778151</td>
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<tr>
<td>3.359611</td>
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</tbody>
</table>

Notes: The IL-12 level potency in control rats, BC rats (DMBA, 100 mg/kg) with CFA (0.05 ml/rat for sensitization, 0.5 ml/rat for challenge) and BC rats with CFA and SLP (18 g/kg), t-test, vs control, P < 0.05, P < 0.01, vs CFA, P < 0.01, at the same interval. DMBA: 7,12-Dimethylbenz[a]anthracene; BC: breast cancer; CFA: complete Freund’s adjuvant; SLP: Shugan Lianxue prescryption; IL-12: interleukin-12.
The time-effective regression was tested with Pearson’s correlation coefficient (95% confidence interval) as $-0.7844 (-0.9665-0.0721)$ in CFA group ($P < 0.05$), 0.8209 (0.1776-0.9727) and in SLP group ($P < 0.05$), respectively (Figure 5).

Underlying mechanism

For evaluating BC candidates from anticancer surveillance, the pharmacodynamic model was developed as the optimal interval of CFA in BC rats. Its availability was confirmed with SLP administration, outcome with both of the postponing survival duration (limited tumor growth) and the preponing TILs activation (Figure 4). This potential action might be more effective in adult human beings, for BCG vaccination had been received for tuberculosis prevention during the ages early to BC occurrence. Besides, DMBA led to the mammary epithelia injuries as BC carcinogen and the immunotoxicity as deficient anticancer surveillance.3

In primary sensitization, BCG was recognized and presented to immune system.34 Meanwhile, the circulating tumor cell from DMBA-induced BC might be also arrived to immune system, so that the CFA-stirred immunity in BC rats was magnified more than that in control rats without BC.35,36 It was memory T lymphocytes 4 weeks after the primary sensitization that the effective T lymphocytes be activated more as proliferating, maintaining and effecting, at the first CFA challenge.37-38 In conclusion, the model for anticancer surveillance was pharmacologically developed to evaluate candidates in BC rats, as an optimal interval between the primary CFA sensitization and the first CFA challenge. The availability of the model was confirmed with SLP administration, as the outcome postponing survival duration.

DISCUSSION

Research strategy

CFA or BCG was effectively administrated for BC patients from the principle of immune surveillance,37 and for bladder cancer patients38,39 with a wide dose range.20-23 This was similar to clinic VP practice in traditional Chinese medicine, especially SLP in BC therapy. The underlying mechanisms of SLP-modified BCG immunity should be further explored in animal experiments. A pharmacodynamic model in the rats with BC was developed as the stirred immunity with CFA (as a common dose in rats)4 for evaluating the candidates in BC immunity therapy. The potencies of CFA-stirred immunity were depended on the intervals between the primary sensitization and the first challenge from clinical or experimental data.27,41,42 An optimal interval of BCG immunity in BC rats had been regressed from the time-effective curve with designed intervals ($k = 0.80$), its availability for assessing was ensured with SLP administration for enhancing T lymphocyte infiltration in tumors.4

Core significance

The CFA-stirred immunity in the BC rats was demonstrated as the EI$_{50}$ of time-effective curve from tumor volume. The EI$_{50}$ with its 95% confidence interval in CFA rats was 791.40 h (630.5-993.4 h). So did that in SLP rats 1475.0 h (1064.0-2045.0 h). It was 1.87 multiple of CFA rats, accompanied with decreased tumor volumes at three intervals (937.50, 1171.88, 1464.84 h), indicating a prolong survival duration.

This outcome was confirmed with the pathological change of BC and the TILs in BC tissues. The BC oncocytes were originated from the epithelia at interlobular ducts, terminal lobular ducts or acini in rats with DMBA-induced BC and CFA-stirred immunity. The scatter resident lymphocytes at normal mammary as background in the control rats, the more activated TILs around nests indicated as thick larger nucleus and basophil cytoplasm in the BC rats with CFA-stirred immunity, so did these most and activated in the BC rats with both of CFA and SLP. Being indicting the prognosis, the CFA stirred immunity in BC rats was demonstrated as the EI$_{50}$ of time-effective curve from IL-12 level. The IL-12 EI$_{50}$ with its 95% confidence interval in CFA rats was 960.1 h (605.1-1523 h). So did that in SLP rats 744.9 h (550.7-1007 h). 0.78 multiple compared with CFA rats, accompanied with increased IL-12 at three intervals (937.50, 1171.88, 1464.84, 2288.82 h), indicating the preponing surveilling occurrence.
ration (as tumor volume) and prepping anticancer surveillance (as IL-12). More studies should be carried out for confirming the findings further.

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