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Original Article

Antitumor effect of intravenous immunization with malaria genetically attenuated sporozoites through induction of innate and adaptive immunity

Xu-Feng Deng[1*], Hong Zheng[2*], Dong Zhou[1], Quan-Xing Liu[1], Yan Ding[3], Wen-Yue Xu[3], Qian Chen[4], Bin Hou[1], Jia-Xin Min[1], Ji-Gang Dai[1]

[1]Department of Thoracic Surgery, Xinqiao Hospital, The Third Military Medical University, Chongqing, China; [2]Institute of Immunology of PLA, The Third Military Medical University, Chongqing 400038, China; [3]Department of Pathogenic Biology, The Third Military Medical University, Chongqing, China; [4]Department of Pathology, Southwest Hospital, The Third Military Medical University, Chongqing, China. [*]Co-first authors.

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Abstract: Lung cancer is remained the common malignancy in world and its high morbidity and mortality means that no effective therapeutic methods are available. Therefore, developing new therapeutic strategies for lung cancer is urgently needed. In this experiment, we aimed to provide an original immunotherapy that whether intravenous immunization with malaria genetically attenuated sporozoites (GAS) has antitumor effect. Our result revealed that GAS inhibited Lewis lung cancer (LLC) growth and prolonged the survival of tumor-bearing mice. Immunohistochemistry analysis of tumors from mice injected with GAS revealed that angiogenesis was inhibited, meanwhile increased terminal deoxynucleotidyl transferase-mediated (TUNEL) staining and Ki-67 expression in tumors. Through cytokine assays and flow cytometry, we found that antitumor effects of GAS by inducing both a potent antitumor innate immune response, including the secretion of interferon γ (IFN-γ), interleukin-6/12 (IL-6/12) and tumor necrosis factor α (TNF-α) and adaptive antitumor immunity with increasing cytolytic activity of $CD8^+$ T cells. Notably, GAS significantly suppresses LLC growth via induction of innate and adaptive antitumor responses in a mouse model. These data suggest that the malaria parasite may provide a novel strategy or therapeutic vaccine vector for anti-lung cancer immune-based therapy.

Keywords: Malaria genetically attenuated sporozoites, Lewis lung cancer, immunotherapy, vector

Introduction

Lung cancer remains the most common cancer in the world, both in term of new cases (1.8 million cases, 12.9% of total) and deaths (1.6 million deaths, 19.4%) because of the high case fatality [1]. Non-small-cell lung cancer (NSCLC) accounts for about 85% of all lung cancer cases [2]. Despite recent advances in surgery, chemotherapy and radiotherapy, the prognosis of patients with lung cancer is still poor [3]. Therefore, it is necessary to develop new approaches to replace or complement the current therapies. In the current decade, some new insights in the interaction between tumors and the immune system have led to the development of immunotherapy as a fundamentally new concept for the treatment of NSCLC [4]. Immunotherapy consists of mainly therapeutic vaccination designed to induce or amplify the immune responses directed against tumor-associated antigens. But, it is now clear that lung cancer often present a tolerogenic microenvironment that hampers effective antitumor immunity. Therefore, the historical results of current cancer vaccination for non-small-cell lung cancer (NSCLC) were disappointing [5]. In animal models it has been shown that certain parasites were able to overcome immunosuppressive environment and inhibit the cancer growth [6-9]. Thus, parasites may serve as a therapeutic strategy of cancer in future clinic trial.

Malaria via deletion of preerythrocytic-stage-expressed genes (UIS3, UIS4 and FabB/F) that

play essential roles for it's growth [10], it may result in significant growth defects in a hepatocytes and avoided malaria infection [11]. This defective malaria of preerythrocytic-stage was named genetically attenuated sporozoites. Furthermore, GAS has been reported to stimulate host immune responses such as promoting IFN-γ, TNF-α production, activating natural killer (NK) cells, γδT cells and NKT cells, inducing the maturation of dendritic cells (DCs), and stimulating T-cell proliferation to protection against malaria challenge [12]. We hypothesized that whether GAS induced immune response could treat for lung cancer.

To clarify this issue, and because of the need for more effective treatment strategies in patients with NSCLC, here, we show that experimental immunization of mice with Plasmodium yoelii fabb/f(-) (Pyfabb/f(-)), a genetically attenuated rodent malaria parasite that arrests late in the liver stage, induced functional host innate and adaptive immunity that significantly inhibited the growth of Lewis lung cancer (LLC).

Materials and methods

Mice, cells, and parasites

Female 6- to 8-week-old C57BL/6 mice were purchased from the Vital River Experiment Animal Limited Company (Beijing, China). Mice were kept under specific pathogen-free (SPF) conditions. The murine LLC cell line obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China). The attenuated P. yoelii BY265 strain was provided by department of Pathogenic Biology, Third Military Medical University, China. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Xinqiao Hospital, the Third Military Medical University.

Mice immunization and tumor volume measure

12 C57/BL6 Mice were randomly divided into two groups (n = 6/each group). In intravenous immunization group (n = 6), each mouse was administered with 200 μl of GAS vaccines in 1.5×10^5/ml concentration. In non-immunization group (n = 6), each mouse was given 200 μl phosphate buffer saline (PBS) as background blank control. After 2 weeks administration, subcutaneous inoculated 5×10^5 LLC to each mouse of all groups. To judge the inhibition of tumor growth, animals were examined daily until the tumors became palpable, tumor volume was determined by measuring the diameter of the tumors using calipers. The volume was calculated following formula: tumor volume (mm^3) = 1/2 × a (mm) × b^2 (mm^2), where 'a' means the long axis of the tumor and 'b' denotes the short axis [13]. All experiments were performed three times with similar results.

Cytokine analysis by BD™ cytometric bead array (CBA)

CBA was utilized in this study. This newly developed technique uses uniform-size microparticle-based flow cytometry to measure a panel of five murine cytokines (IL-12, IFN-γ, TNF-α, and IL-6) simultaneously in a single peripheral blood sample. Blood was collected from GAS group (n = 10) and PBS group (n = 10) of mice by eyeball bleeding on day 1, 3, 5 7, 9 after immunization. Blood samples were transported immediately to the laboratory for processing. Serum was separated by centrifugation (5800×g for 10 min) at 4°C and stored at -80°C until analysis was carried out. The assay kits provide a mixture of five microbead populations with distinct fluorescent intensities (FL-3) and were precoated with capture antibodies specific for each cytokine. Analysis was carried out in two sets, one set was maintained for standards and the second set was for the samples (serum). 50 μl of standard cytokines (set one) and 50 μl of serum were added to the premixed microbeads in 12 mm × 75 mm Falcon tubes (BD). After the addition of 50 μl of a mixture of PE conjugated antibodies against the cytokines, the mixture was incubated for 3 h in the dark at room temperature. The mixture was washed and centrifuged at 500×g for 5 min and the pellet was resuspended in 300 μl of wash buffer. The FACS-LSR flow cytometer (BD Pharmingen) was calibrated with setup beads and 3000 events were acquired for each sample. Data were acquired and analyzed using Becton Dickinson (BD) cytometric bead array (CBA) software. Forward vs side scatter gating was employed and data were displayed as two-colour dot plots (FL-2 vs FL-3) such that the five discrete FL-3

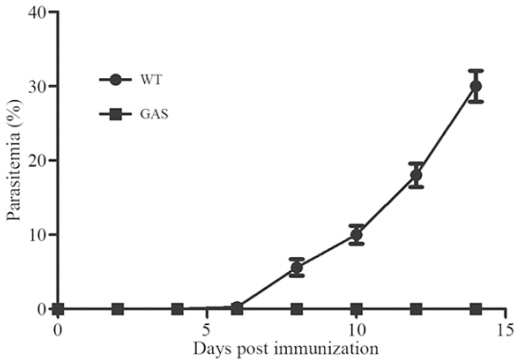

Figure 1. Malaria genetically attenuated sporozoites is a safely, potential vaccine. Mice were divided into two groups. Each group of mouse administration of 30×10^3 numbers of genetically attenuated sporozoites (GAS) and wild type (WT) sporozoites respectively. In WT group mice, at day 6 we could find parasitemia, and at day 14 the level of parasitemia up to 30%. But in GAS group, the level of parasitemia is always zero.

microparticle dye intensities were distributed along the y-axis. All experiments were performed three times with similar results.

Flow cytometry

Blood was collected from GAS group (n = 6) and PBS group (n = 6) of mice by eyeball bleeding on day 1, 7, 14, after immunization and leukocytes were purified for flow cytometry using red blood cell lysis solution (eBioscience, San Diego, CA) following the manufacturer's protocol. Cells were labeled with antibodies for anti-mouse CD8-APC (eBioscience), CD11c-FITC (eBioscience), following the manufacturer's protocol. Data was collected on the BD FACS Calibur platform (BD Biosciences, San Jose, CA) using CellQuestPro (v5.1, BD Biosciences) and exported for analysis via FlowJo (v.7.6.5, Tree Star, Inc, Ashland, OR). All experiments were performed three times with similar results.

Immunohistochemistry analysis

Mice tumor were dissected and washed once in PBS, submerged in neutral buffered 10% formalin, and transferred to 70% ethanol for paraffin embedding. Semiserial sections were cut at 4 μm, placed on positively charged slides, and fixed in cold acetone. Serial paraffin sections were used for immunohistochemistry and hematoxylin and eosin (H&E) staining as previously described [14]. Briefly, all slides were stained in Richard Allan Scientific Hematoxylin (Thermo Scientific, Waltham, MA) and Eosin-Y (Thermo Scientific) with the Leica Autostainer (Leica Biosystems, Buffalo Grove, IL). Immunohistochemistry was performed with antibodies for CD31 (Cell Signaling Technology USA), Ki-67 (Cell Signaling Technology USA) and TUNEL (Promega Corporation USA) following the manufacturer's protocol. All experiments were performed three times with similar results.

Statistics

All numerical data were expressed as means ± SEM. Statistical differences were determined by unpaired two-tailed Student t tests; for non-parametric data, differences between groups were analyzed with the Mann-Whitney test. Survival curves were analyzed by a log-rank test. Results were considered to be statistically significant when $P < 0.05$. Statistical analysis was performed with SPSS19 for windows (SPSS Inc.) and Excel 2010 (Microsoft Inc.). Figures are made by Graph Pad Prism software.

Results

Malaria genetically attenuated sporozoites is a safely, potential vaccine

Immunization with genetically attenuated sporozoites (GAS) would be an attractive alternative approach. In this study, we present data on safety and protective efficacy using attenuated sporozoites with deletions of P. yoelii fabb/f genes. In immunization group, each mouse after administration of 30×10^3 number of attenuated sporozoites without resulting in blood stage infections by tail vein blood smear. But in control group, after administration equal concentrations of wild type (WT) sporozoites, at day 6 we could find parasitemia (**Figure 1**).

Malaria genetically attenuated sporozoites inhibited LLC growth

To determine the effect of malaria attenuated sporozoites immunization on the growth of LLC cells, we tail vein immunization mice with P. yoelii fabb/f By265 or with an equivalent number of PBS. After 2 weeks, both groups of mice implanted with a subcutaneous injection of LLC cells. The result revealed that the growth of tumor cells was significant suppressed in the GAS group compared to the PBS group (**Figure**

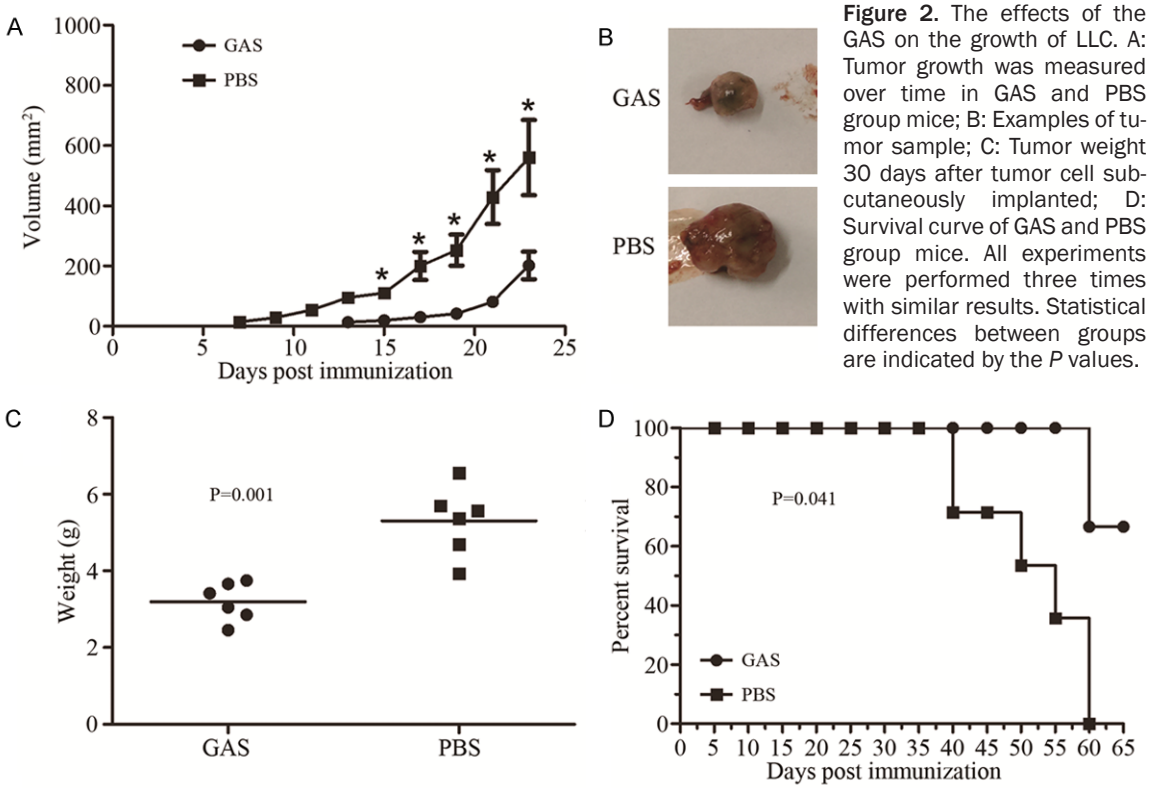

Figure 2. The effects of the GAS on the growth of LLC. A: Tumor growth was measured over time in GAS and PBS group mice; B: Examples of tumor sample; C: Tumor weight 30 days after tumor cell subcutaneously implanted; D: Survival curve of GAS and PBS group mice. All experiments were performed three times with similar results. Statistical differences between groups are indicated by the *P* values.

2A-D). The tumor volumes ($P < 0.05$, **Figure 2A**) and tumor sample and tumor weights ($P = 0.001$, **Figure 2B**, **2C**) were clearly decreased in the GAS group mice as compared with the PBS group. In addition, mice of GAS group survived much longer than PBS group ($P = 0.041$, **Figure 2D**).

Effect of malaria genetically attenuated sporozoites on tumor cell proliferation, apoptosis, and angiogenesis

We used the proliferation marker Ki-67 staining for mice tumors shows significant suppression of proliferation in the GAS group mice (**Figure 3A**). Meanwhile, an evidenced by TUNEL staining of tumors shows apoptosis cells were clearly increased in tumors of the GAS group mice (**Figure 3B**). Moreover, Staining of endothelial cells in blood vessels with CD31 showed slightly tumor vasculature in tumor nests in the GAS group mice (**Figure 3C**).

Malaria attenuated sporozoites induced innate and adaptive antitumor immunity

The potent anti-tumor activity of attenuated sporozoites greatly encouraged us to further explore its profound mechanism. The first line of defense against metastatic invasion is the innate immune system that provides immediate defense by produce protective cytokines [15, 16]. We found that malaria attenuated sporozoites at early stage led to rapid increases in IFN-γ, TNF-α and IL-6/12 levels, peaking 3 days after immunization (**Figure 4A-D**). Meanwhile, the peripheral blood mononuclear cells (PBMCs) were examined by flow cytometry. Both $CD4^+$ and $CD8^+$ T cells populations were increased with $CD8^+$ T cells representing the dominant cell type in our study. We found that malaria attenuated sporozoites immunization markedly increased the percent of $CD8^+$ T cells in GAS group mice. Moreover, the percent of $CD8^+$ T and CD11a T cells were significantly increased in GAS group mice versus PBS group mice ($P < 0.05$, **Figure 5**).

Discussion

During immunosurveillance, the immune system is able to recognize malignant cells as foreign, as these cells may express specific tumor antigens and destroy them; however, tumors can escape elimination by the immune system through activation of inhibitory feedback loops

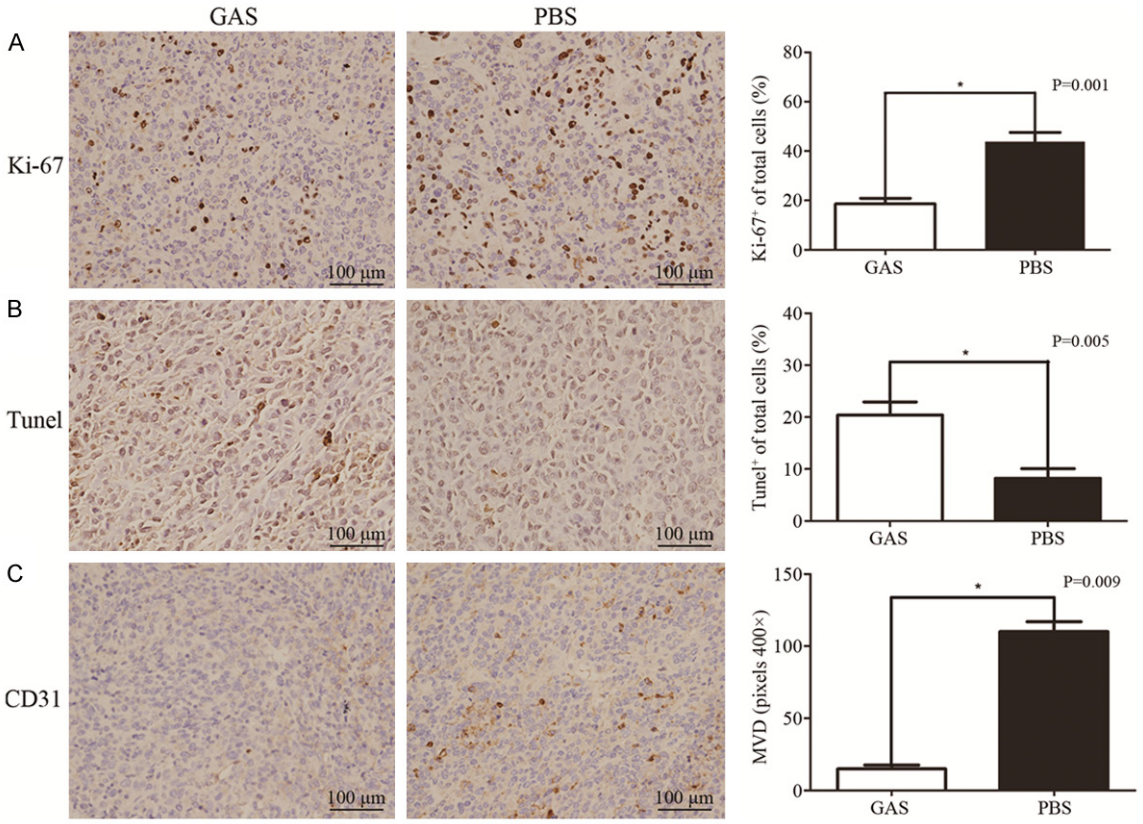

Figure 3. Effect of GAS on tumor cell proliferation, apoptosis, and angiogenesis. Immunohistochemical staining for Ki-67 (A), TUNEL (B), and CD31 (C) analyses of the tumor samples. Each symbol corresponds to an individual animal.

or (so-called immunological brakes) that are essential to avoid autoimmune events, and so can create barriers to T-cell activation and tumor rejection [17, 18]. Therefore, it is important to augment host immune response and overcome the tumor immunosuppressive and tolerogenic status for cancer therapies.

Cancers and parasites have some common properties. When parasites infect an organism, they infiltrate its tissues and organs, trying to avoid the host's defense systems. Eventually, they will find their niche, proliferate, and develop a full infection if the immune response is insufficient. This behavior resembles the colonization of metastatic sites by cancer cells and their adaptation to the metastatic niche, suggesting that both processes may share similar mechanisms [19]. Means both parasites and tumor cells need to interact with the innate immune system and evade leukocyte surveillance in order to achieve successful colonization. In addition, anti-parasitic drug could also possibly be used as anti-cancer therapies [20, 21]. Therefore, it is reasonable that parasites could be antitumor. The ability of parasites to suppress tumor growth has been well documented [6, 9, 22, 23].

Lung cancer is the most common cause of cancer death across the world [24]. Non-small-cell lung cancer (NSCLC) accounts for about 85% of all lung cancer cases, histological subtypes, and half of patients present with incurable metastatic disease at the time of diagnosis [25, 26]. A treatment with radical intent can be offered to patients with non-metastatic stages, but even then, a large number of patients will relapse and die of their cancer. Its high fatality means that no effective treatment is available. Although, cancer immunotherapy may represent one new effectively approach that has low toxicity and high specificity for cancer. It is to augment the weakened host immune response against tumors using specific and/or nonspecific immune stimulants. Therefore, lung cancer

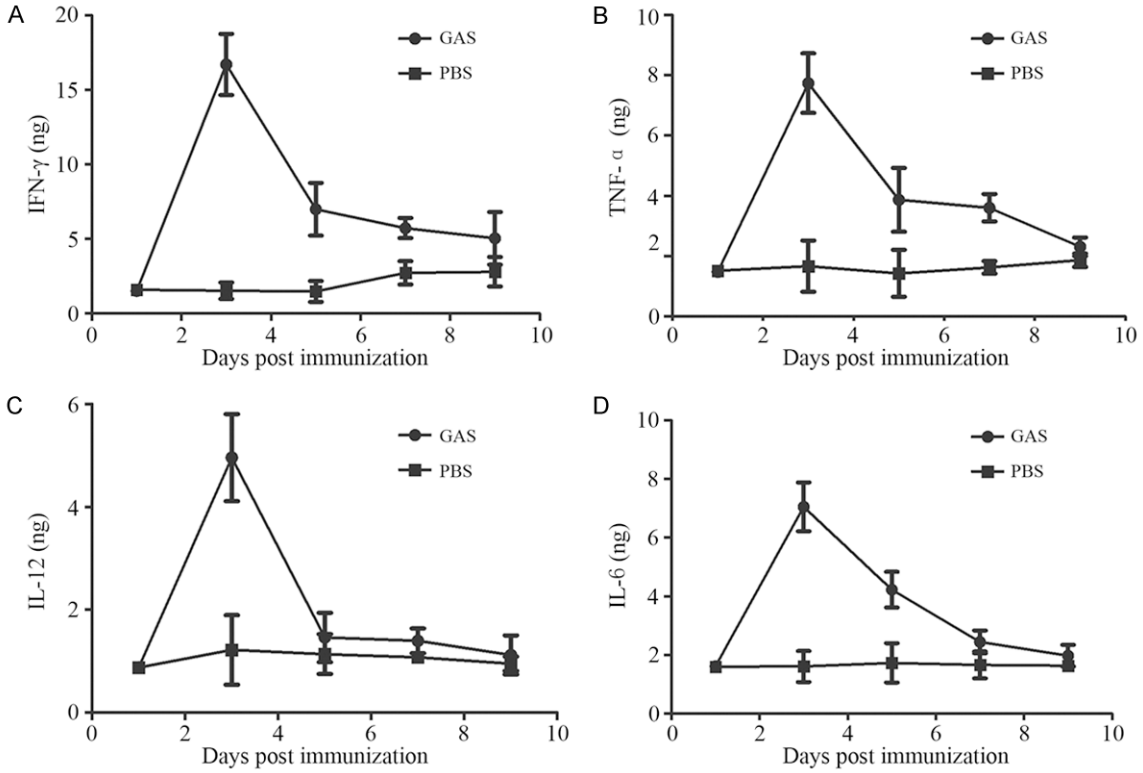

Figure 4. Malaria GAS induces the production of Th1-type cytokines. (A-D) Levels of IFN-γ (A), TNF-α (B), IL-12 (C) and IL-6 (D) in peripheral blood measured by Cytokine analysis by BD™ Cytometric Bead Array (CBA).

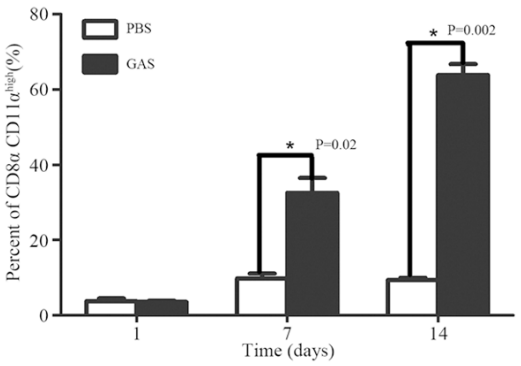

Figure 5. Malaria GAS induces anti-tumor-specific immune responses. The percent of $CD8^+$ T and CD11a T cells were significantly increased in GAS group mice.

immunotherapy may represent one new approach that has low toxicity and high specificity, but implementation has been a challenge due to poor antigenic characterization and the ability of lung cancers to escape immune responses [27, 28]. Traditional biological immunoadjuvants leads to affirmative efficacy in the treatment of some cancers [29]. However, the induction of the desired immune responses is weak, responses are short lived, and memory formation is defective. New approaches to the treatment of lung cancer are urgently needed.

In the present study, we examined antitumor activities of malaria attenuated sporozoites immunization in subcutaneously implanted murine LLC model. Malaria attenuated sporozoites inhibited LLC growth and prolonged the survival of tumor-bearing mice. Further histological analysis of tumors from mice intravenously immunization with malaria attenuated sporozoites revealed that angiogenesis was inhibited and the proportion of proliferative cells was decreased, whereas apoptotic cells were increased. Subsequently, we suggested the mechanisms of antitumor effects in this study.

The immune response has been artificially divided into innate immunity (resistance) and adaptive immunity (specific). It is closely linked between innate and adaptive immunity. Innate immune signals are essential for the initiation of adaptive immune responses. Activation of

innate immunity stimulates the adaptive immunity system [30]. Attenuated sporozoite induces mice innate immune system, significantly increased the secretion of IFN-γ, TNF-α, IL-6 and IL-12 in GAS group on the day 3 after administration. Those cytokines are crucial cytokines to induce lymphocytes to produce another set of cytokines, which in turn activate effector responses meanwhile it also induce potent antitumor responses in a variety of animal cancer models [28, 31]. Intravenously immunization with malaria attenuated sporozoites result in a strong cell-mediated immune response in human model, especially the T-cell-mediated response [12]. Attenuated sporozoites induce tumor-specific T-cell proliferation, and cytolytic activity of $CD8^+$ T cells that exhibit high cytotoxic activity in the tumor microenvironment. In the present study, the percentages of $CD8^+$ T-cells were significantly increased in mice of GAS group than non-immune mice of PBS group. Moreover, the percentage of both of CD8α and CD11a maintain high level after attenuated sporozoites intravenously immunization. As documented has been well reported that the expression of CD11a and CD8α can be used to distinguish naive from Ag-experienced (effector and memory) CD8 T cells after infection or vaccination [32]. Indicating attenuated sporozoites could induce long-lasting systemic immunity.

These results indicate that malaria attenuated sporozoites induce innate and adaptive immune responses were depressed in Lewis lung cancer. But, the Activation of innate immunity, the production of cytokines such as IFN-γ, TNF-α, IL-12, and IL-6, play an important role initiation of adaptive immune responses. Moreover, lymphocytes activate by those cytokines could induce to produce another set of cytokines that can arrest proliferation of malignant cells and prevent the angiogenesis necessary for tumor growth [27, 28, 33-36]. Notably, malaria attenuated sporozoites induce not only nonspecific antitumor immunity but also tumor-specific immunity. In addition, malaria attenuated sporozoites induce effective and long-lasting local and systemic antitumor immunity.

Malaria parasite infections profoundly provoke the host immune system, inducing polyclonal activation, massive proliferation and differentiation of lymphocytes with parasite-unrelated specificities as well as immune responses to host antigens released during malaria parasite infection [36-41], which may serve as enhanced immune surveillance mechanisms against lung cancer. Possibly, in our study the pre-existing cytokines and pre-activated non-tumor specific responses together with the tumor-Ag-specific humoral and cellular responses initiated after the parasite infection might inhibit early stage tumorigenesis. In addition, here there is candidate molecules in malaria attenuated sporozoites that could act as adjuvant components. The circumsporozoite protein (CSP), a key component of the sporozoite stage of the malaria parasite, is believed to be an important factor in antitumor activation by block NF-κB then suppresses the growth of SW480 [42].

Conclusions

Our present results suggest that attenuated sporozoites in LLC-bearing mice induce antitumor activity against lung cancer through the induction of host innate and adaptive immune response. Moreover, this antitumor immune response could inhabit angiogenesis and increase the percentage of apoptotic cells and decrease the proportion of proliferative cells. Our study provides a novel immunotherapy strategy for lung cancer. We suggest that the malaria attenuated sporozoites may be an effective lung cancer vaccine vector and to identify the active cellular components of malaria parasite for immunotherapy for lung cancer. In our future study we will insert a specific gene of lung cancer in attenuated P. yoelii BY265 (fabb/f(-)) and build a recombinant non-pathogenic attenuated P. yoelii BY265 clone of as a vaccine vector to induce vigorous and specific and long-term T cell-mediated immunity. More broadly, this strategy could be used to elicit a long-term T cell-mediated immunity and used for prophylaxis or therapy of chronic infectious diseases.

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Disclosure of conflict of interest

None.

Abbreviations

GAS, genetically attenuated sporozoites; PBS, phosphate buffer saline; LLC, Lewis lung cancer; TUNEL, terminal deoxynucleotidyl transferase-mediated; WT, wild type.

Address correspondence to: Dr. Ji-Gang Dai, Department of Thoracic Surgery, Xinqiao Hospital, The Third Military Medical University, Chongqing 400037, China. Tel: +86 23 68774724; Fax: +86 23 68774724; E-mail: cnxfdoc@163.com

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