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Sunitinib impairs the proliferation and function of human peripheral T cell and prevents T-cell-mediated immune response in mice ☆

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Abstract Sunitinib (sunitinib malate; SU11248; SUTENT) is a novel multi-targeted receptor tyrosine kinase inhibitor currently approved for the treatment of metastatic renal cell carcinoma. To analyze the possible use of this compound in combination with immunotherapeutic approaches, we investigated the effects of sunitinib on the human peripheral T cells and the induction of primary immune responses in mice. Sunitinib inhibited the proliferation of primary human T cells from normal healthy volunteers as well as from renal cell carcinoma (RCC) and other cancer patients. The inhibition was recoverable after drug withdrawal because sunitinib did not induce T-cell apoptosis even at 0.8 μM. In addition, sunitinib led to accumulation in G_0/G_1 phase of the cell cycle, inhibition of cytokine production, downregulation of activation markers expression and blockade of Zap-70 signaling in the T cells. Sunitinib significantly reduced the ear swelling induced by picryl chloride in mice. In light of these findings, the effects of sunitinib on the immune system should be emphasized for the therapy of metastatic renal cell carcinoma patients to avoid the impairment of T lymphocytes.

Introduction

Renal cell carcinoma (RCC) accounts for 2% to 3% of all malignant tumors in adults [1]. Patients with untreated metastatic RCC (mRCC) have an overall median survival of no more than 12 months and a 5-year survival of less than 10% [2,3]. Metastatic RCC is usually considered to be resistant to conventional chemotherapy and radiation, and cytokine-based immunotherapy using interferon-α (IFN-α) and interleukin-2 (IL-2) was the only effective treatment in the past 15 years [4,5]. Until recently, two novel tyrosine kinase inhibitors, sorafenib and sunitinib, were introduced in the treatment of RCC patients.

Sunitinib (marketed as Sutent, and previously known as SU11248) is an oral, small-molecule, multi-targeted receptor tyrosine kinase (RTK) inhibitor that was approved by the FDA

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for the treatment of RCC and imatinib-resistant gastrointestinal stromal tumor (GIST) on January 26, 2006 [3,6,7]. Sunitinib has become the standard of care for both of these cancers and is currently being studied for the treatment of many others [8–10]. These effects were associated with the blockade of receptor tyrosine kinase signaling by vascular endothelial growth factor receptors (VEGFR-1,VEGFR-2, VEGFR-3), platelet-derived growth factor receptor (PDGFRα, PDGFRβ), c-KIT, Fms-like tyrosine kinase-3 receptor (FLT3) and the receptor encoded by the ret proto-oncogene (RET) [7,11].

It was found that sorafenib, another outstanding agent for the treatment of mRCC, but not sunitinib, has a detrimental effect on DC phenotype and inhibits cytokine secretion, migration ability and T-cell stimulatory capacity [12]. Finke JH and his colleagues reported that sunitinib impaired regulatory T-cell function [13]. However, until now the effect of sunitinib on function of human peripheral T cells and immune responses have not been evaluated in detail. The aim of this study was to examine the effects of sunitinib on the function of T cells both *in vitro* and *in vivo*. We show here that sunitinib inhibits human peripheral blood T-cell proliferation, activation and cytokines production in vitro and reduces the T-cell-mediated immune response in mice.

Materials and methods

Animals

Female BALB/c and C57BL/6 mice (6–8 weeks old, 18–22 g) were supplied by the Model Animal Genetics Research Center (Nanjing, China). They were maintained in specific pathogen-free condition at 25 ± 2 °C and kept on a 12-h light–dark cycle. Animal welfare and experimental procedures were carried out strictly in accordance with the "Guide for the Care and Use of Laboratory Animals" (The Ministry of Science and Technology of China, 2006) and the related ethical regulations of our university. All efforts were made to minimize the animals' suffering and to reduce the number of animals used.

Cells and reagents

Peripheral blood mononuclear cells (PBMC), collected from normal healthy volunteer donors, RCC and other cancer patients, were purified by Ficoll density gradient centrifugation. Normal human T cells were enriched from PBMC by R&D Systems' Human T Cell Enrichment Columns (R&D, MN). The purification rate of $CD3^+$ T cells was 90 ± 5%. The cells were incubated in RPMI 1640 medium (Gibco, MD) supplemented with 10% New Born Calf Serum (NBS) in a humidified atmosphere of 5.0% CO_2 at 37 °C.

Sunitinib (Pfizer, NY) was dissolved in dimethyl sulfoxide (DMSO) to a concentration of 20 mM as stock solution, which was stored at −20 °C until use. Phytohemagglutinin (PHA), myelin oligodendrocyte protein (MOG) and staphylococcal enterotoxin B (SEB) were obtained from Sigma (MO).

Cell proliferation assay

Cell proliferation was determined by two methods. For H^3-TdR incorporation assay, the cells were incubated with graded concentrations of sunitinib for 60 h, pulsed with 1 μCi tritiated thymidine per well, and incubated for another 12 h before they were harvested, and incorporated radioactivity was then quantified. For carboxyfluorescein diacetate succinimidyl ester (CFSE) proliferation assay, purified human PBMCs were suspended in PBS containing 0.1% BSA and labeled with the vital dye CFSE at a final concentration of 2.5 μM for 10 min at 37 °C. After labeling, the cells were washed three times in NBS-rich medium and resuspended in PRMI 1640 medium (2.5×10^6 cells/ml). The CFSE-labeled cells were pre-incubated with sunitinib for 1 h as indicated and then cultured for 96 h in the presence of 5 μg/ml PHA or were stimulated with 10 μg/ml anti-CD3 and 1 μg/ml anti-CD28 for 72 h . In the case for examining the effect of sunitinib on the T lymphocytes activated by specific antigens, an experimental autoimmune encephalomyelitis (EAE) was induced in C57BL/6 mice as previously described [14], and the mice were monitored for clinical symptoms and graded from 1 to 4 as follows: 0, no clinical expression of disease; 1, floppy tail without hind limb weakness; 2, hind limb weakness with or without flaccid tail; 3, hind leg paralysis and floppy tail; and 4, hind leg paralysis accompanied by floppy tail and urinary or fecal incontinence. Animals that progressed to a clinical score of 4 were euthanized on day 14 and lymph nodes were collected. The cells from EAE mice were then stimulated by 2 μg/ml MOG with various concentrations of sunitinib for 96 h. The proliferation was evaluated using H^3-TdR incorporation assay.

Cell cycle analysis

Approximately 1×10^5 cells were collected at specified time points after culture, washed twice with PBS and fixed in ice-cold 70% ethanol for 30 min and then incubated with 10 μg/ml propidium iodide (PI) and 10 μg/ml RNase for 30 min. Thereafter, the cells were analyzed by a flow cytometer (BD, CA).

Detection of apoptosis

An annexin V-fluorescein isothiocyanate (FITC)/PI double-stain assay was performed in accordance with the manufacturer's protocol (Jingmei Biotech, Shenzhen, China). Briefly, the T cells (2×10^5) were collected and resuspended in 100 μl of binding buffer containing 1 μl of annexin V-FITC and 1 μl of PI then incubated for 15 min in the dark at room temperature. Analysis was immediately performed using a flow cytometer.

IL-2 assay

Human blood T cells (1×10^5 cells/well) were cultured in 96-well plates with or without sunitinib for 24 h, in the presence or absence of anti-CD3 and anti-CD28 antibodies. Interleukin 2 (IL-2) production was measured with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Biosource, SA) according to the manufacturer's instructions.

Cytokine analysis

PBMCs were stimulated with PHA in the presence or absence of sunitinib, as indicated in the text. Super-

natants were analyzed for cytokines using the Proteome Profiler Array (R&D Systems) according to manufacturer's instructions.

Protein analysis by Western blotting

Western blotting analysis was performed as described previously. Monoclonal antibodies specific for GAPDH (as control of protein loading, Santa Cruz, CA), CDK6, Cyclin D3, Cyclin D1, P38 and P38-PO4 (Cell Signaling Technology, MA), immunopurified polyclonal rabbit antibodies specific for LCK Y505-PO4, ZAP-70, ZAP-70 Y319-PO4 and Rb S807/811-PO4 (Cell Signaling Technology, MA), and polyclonal goat antibodies to rabbit IgG and mouse IgG, both coupled to HRP (KPL Biotechnology, ML), were used for detection of proteins and phosphoproteins.

Picryl chloride-induced contact hypersensitivity

Mice were sensitized by painting 100 μL of 1% picryl chloride (WAKO, Japan) in ethanol onto the shaved skin of their abdomens. Five days after sensitization, they were challenged on the right ear with the painting of 30 μL of 1% picryl chloride in olive oil. Ear swelling was evaluated by the difference in thickness between the right and left ears, as measured with an engineer's micrometer 24 h after the challenge. The swelling-negative group was normally sensitized and painted with the vehicle olive oil when challenged. The control animals with contact hypersensitivity were given saline instead of drugs.

Histological analysis

Formalin-fixed, paraffin-embedded mouse ear tissues were sectioned at 5 mm in thickness, and the sections were stained with hematoxylin and eosin. Histopathologic scoring was done by using a range from 0 (no change) to 4 (most severe) to evaluate congestion, edema and inflammatory cell infiltration, etc.

Statistics

The *P* values between two experimental groups were tested by two-tailed Student's *t* test. The level of significance was set at a *P* value of 0.05. Where applicable, data were reported as the mean ± SD.

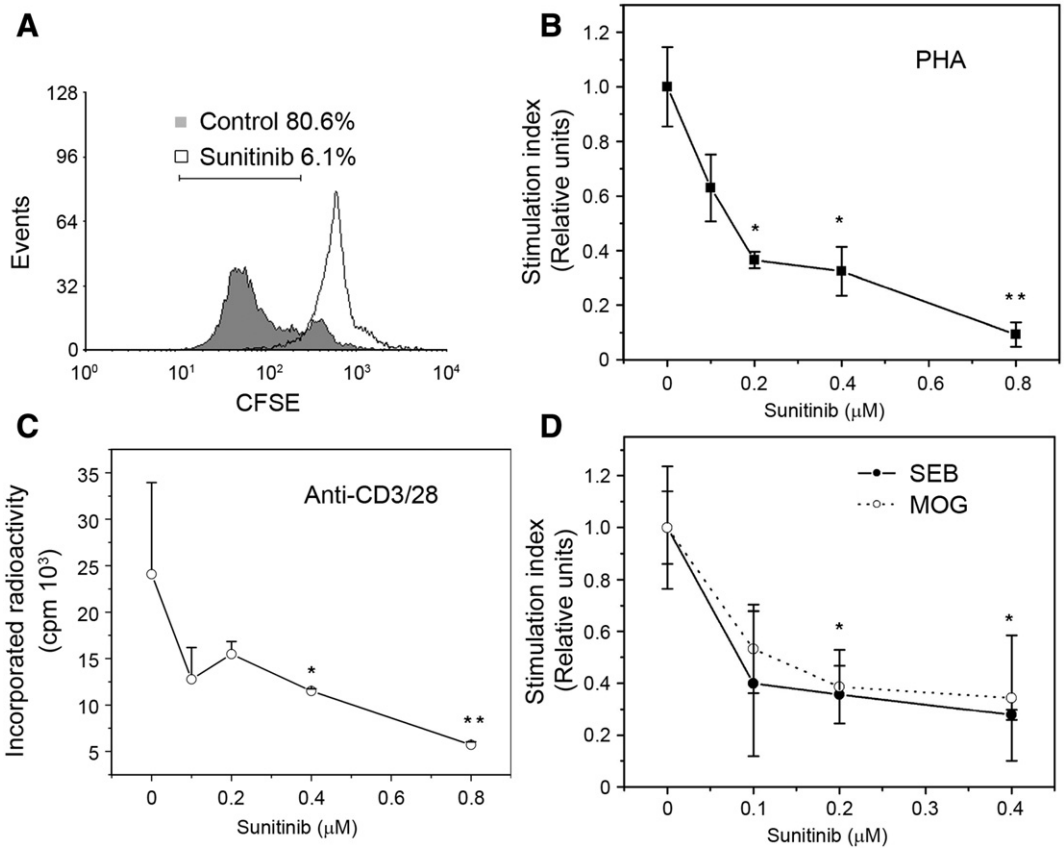

Figure 1 Sunitinib inhibits T-cell proliferation. (A) PBMC was labeled with 2.5 μM CFSE and pre-incubated with or without 0.4 μM sunitinib for 1 h. Then, they were stimulated with PHA (5 μg/ml) for 96 h. The numbers gated indicated the dividing $CD3^+$ cell population; T cells from PBMC were stimulated by 5 μg/ml PHA (B), 5 μg/ml anti-CD3 and 0.5 μg/ml anti-CD28 antibodies (C) or 10 μg/ml SEB (D), respectively, with various concentrations of sunitinib for 96 h. (D) Lymph node cells from EAE mice were stimulated by 2 μg/ml MOG with various concentrations of sunitinib for 96 h. The proliferation was evaluated using H^3-TdR incorporation assay. The results were expressed as the mean stimulation indices ± SD of three experiments. *$P<0.05$ and **$P<0.01$ versus positive control.

Table 1 Percentage of $CFSE^{low}$ $CD4^+$ T cells from cancer patients after culture.

Patients	Type	Age	Sex	Control (%)	Sunitinib treated (%)
1	Renal cell carcinoma	51	Male	66.4	42.3
2	Renal cell carcinoma	46	Female	73.8	34.7
3	Renal cell carcinoma	49	Male	84.2	42.6
4	Renal cell carcinoma	58	Male	75.6	21.4
5	Renal cell carcinoma	53	Female	64.3	35.1
6	Lung cancer	71	Male	46.9	35.6
7	Lung cancer	68	Male	41.8	10.7
8	Lung cancer	56	Male	46.2	16.8
9	Liver cancer	66	Male	60.4	47.9
10	Liver cancer	53	Male	69.1	50.5
11	Cervical cancer	77	Female	82.1	33.8
12	Stomach cancer	65	Male	75.5	27.6
13	Stomach cancer	69	Male	81.9	32.8

PBMCs from the patients were labeled with 2.5 μM CFSE and pre-incubated with or without 0.2 μM sunitinib for 1 h. Then, they were stimulated with 10 μg/ml anti-CD3 and 1 μg/ml anti-CD28 for 72 h, respectively. The percentage indicated the dividing $CD4^+$ T-cell population.

Results

Sunitinib inhibits the proliferation of human peripheral blood T cells

To investigate the effect of sunitinib on T cells, we stimulated human peripheral blood T cells from healthy donors with PHA and CD3/28 antibody. As shown in Figs. 1AB and C, sunitinib inhibited T cells proliferation in a dose-dependent manner. The inhibitory effect was evident at sunitinib as low as 0.4 μM and complete inhibition of $CD3^+$ T cells proliferation with 0.8 μM sunitinib. To investigate whether sunitinib can inhibit specific antigen or bacterial antigens induced T-cell proliferation, MOG and SEB were used as stimulus. Similar results were obtained as those in the proliferation assay with PHA (Fig. 1D). We further examined the inhibition of sunitinib on T cells from cancer patients and the proliferation was also inhibited by sunitinib at the dose of 0.2 μM (Table 1).

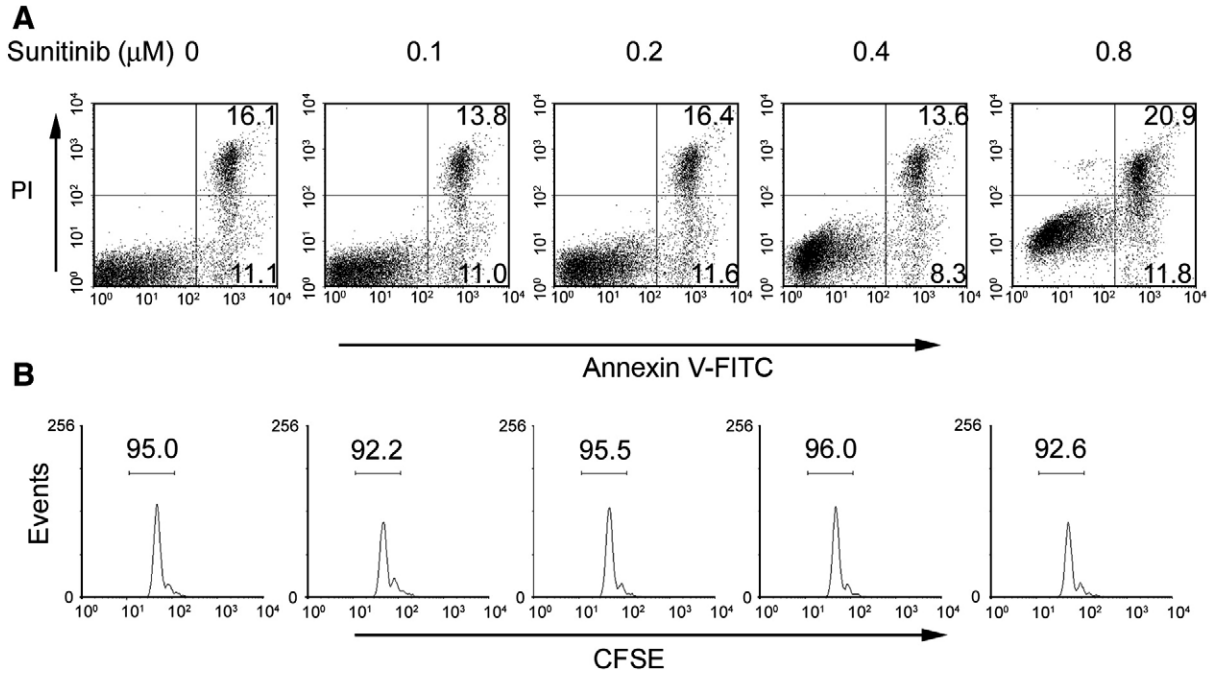

Figure 2 Sunitinib does not induce apoptosis in human blood T cells. (A) Human blood T cells were cultured for 48 h with various concentrations of sunitinib in the presence of 5 μg/ml PHA. Then the cells were assayed for the percentages of Annexin V-positive populations. (B) The CFSE-labeled PBMC cells were treated with or without sunitinib for 12 h. The cells were washed free of sunitinib and replated without it. Subsequently, all the cells were stimulated with 5 μg/ml PHA for an additional 96 h; CFSE-labeled $CD3^+$ T cells were measured as described in Fig. 1.

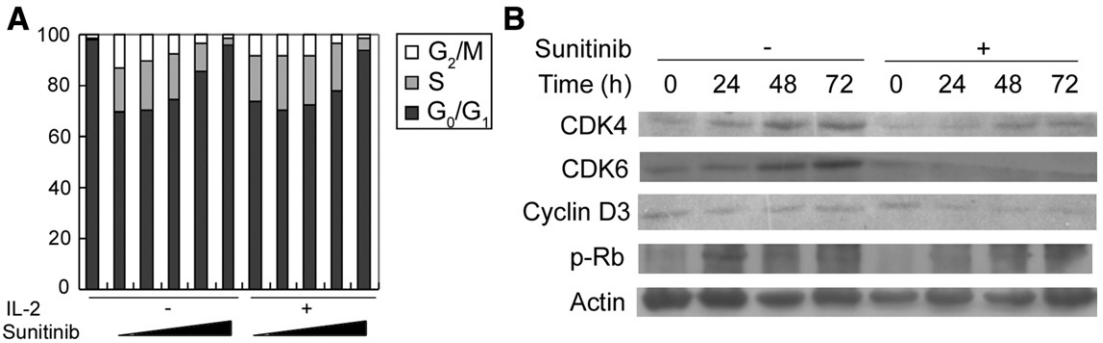

Figure 3 Sunitinib causes T-cell arrest in G_0/G_1 phase of the cell cycle. (A) Human peripheral blood T cells were treated with 0, 0.1, 0.2, 0.4 and 0.8 μM sunitinib for 96 h in the presence of 5 μg/ml PHA or 5 μg/ml PHA plus 500 U/ml IL-2. Then the cells were stained with PI and analyzed by flow cytometry; (B) T cells were stimulated by 5 μg/ml PHA with or without 0.4 μM sunitinib for up to 72 h. The expressions of cell cycle regulators were analyzed by Western blot.

Sunitinib does not induce T-cell apoptosis and cause a recoverable inhibition of proliferation after drug withdrawal

To examine whether the reduced proliferation of T cells in response to sunitinib was due to increased apoptosis, we stimulated the cells and measured apoptosis by staining with Annexin V-FITC and PI. The percentage of Annexin V-FITC-positive and PI-negative events, which corresponds to apoptotic cells, remained between 8.3% and 11.8% compared with of untreated cells. Sunitinib at a concentration up to 0.8 μM did not induce apoptosis when compared with untreated cells under these conditions (Fig. 2A).

PBMCs were incubated with and without sunitinib for 12 h. After removal of sunitinib, the cells were incubated with PHA for additional 96 h. The sunitinib pretreated T cells remained proliferated after stimulated by PHA (Fig. 2B).

Sunitinib arrests T cells accumulating in the G0/G1 phase of the cell cycle

We stimulated T cells without or with sunitinib for 96 h. Data in Fig. 3 indicate that unstimulated T cells were arrested in G_0/G_1 phase of cell cycle. PHA plus IL-2 stimulated T cells DNA synthesis and progressed cells into S phase. This effect was inhibited by sunitinib at concentrations as low as 0.4 μM, which is consistent with the data in proliferation assay.

A requirement of entry into the S phase is the phosphorylation of the retinoblastoma protein (Rb), which is regulate by the cyclin D3/CDK4/6 complex. We detected the levels of cyclin D3 protein, CDK4, CDK6 and Rb phosphorylation in the presence of sunitinib by Western blotting. At 24, 48 and 72 h after the addition of PHA, the amounts of cyclin D3 and Rb phosphoproteins were reduced. The level of phosphorylated CDK6, CDK4, was also decreased.

Sunitinib inhibits proinflammatory cytokine production in human T cells

As shown in Figs. 4A and B, the production of proinflammatory cytokines (IL-1α, IL-1β and MIF) and chemotactic factors (IL-8, MCP-1,CXCL-1, MIP-1α, MIP-1β and RANTES) was inhibited by sunitinib, while the IL-6 level slightly increased. The IL-2 level in the cell culture supernatant also decreased when pretreated with sunitinib (Fig. 4C).

Sunitinib downregulates expressions of activation markers CD25 and CD69 by blocking ZAP70 signaling

After PHA stimulation, the frequency of cells expressing CD25 and CD69 increased markedly, while the expression of these markers in resting T cells was low. As compared with the control, sunitinib downregulated expression of these T-cell activation markers in a does-dependent manner (Fig. 5A).

To investigate whether the inhibitory effects of sunitinib on the proliferation and activation of T cells could be due to the inhibition of TCR signaling transduction, the signaling events on TCR were detected by Western blotting analysis. Sunitinib inhibited the levels of phosphorylation of ZAP-70 but did not affect these of LCK and P38 (Fig. 5B).

Sunitinib prevents picryl chloride-induced delayed-type hypersensitivity (DTH) in mice

Against the swelling in control right ear, systemic treatment with sunitinib showed a significant abolishment in a dose-dependant manner (Fig. 6A). The histopathologic changes in the ear were mainly observed in the dermis as severe inflammatory infiltration, vascular congestion, and moderate edema in the control group. Against the control, the mice treated with sunitinib only showed a mild cellular infiltration and vasodilatation without edema (Fig. 6B).

Discussion

Historically, only immunotherapy showed activity in metastatic RCC [2]. Targeted therapies by using tyrosine kinase inhibitors such as sunitinib and sorafenib have been approved for use recently. Sunitinib is now regarded as the first line standard of treatment for metastatic RCC [5,6]. Current research is aimed at developing new drugs and combining available drugs to improve upon the responses and survival seen with approved single agents. Therefore, it is important to figure out the immunomodulatory activity of sunitinib. In

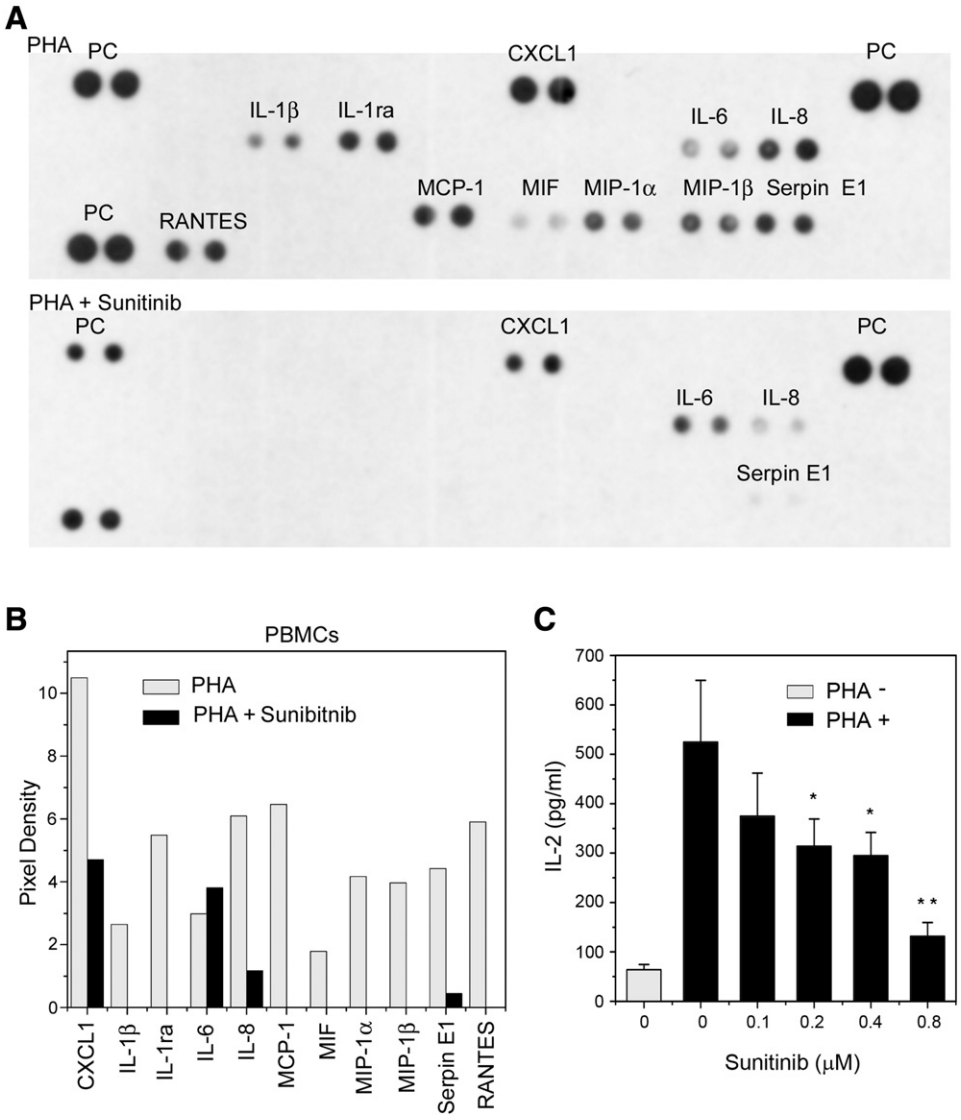

Figure 4 Sunitinib inhibits cytokines production in human T cells. (A) PBMCs were stimulated with 5 μg/ml PHA in the presence or absence of sunitinib (0.4 μM). After 24 h, supernatants were collected and cytokines in the supernatants were detected by capture antibodies spotted in duplicate on nitrocellulose membranes. The high-intensity spots in the three corners are positive controls (PC). (B)The intensity was quantified using Band Scan Software. A graph of the relative intensity is shown for selected cytokines. (C) T cells enriched from PBMC were treated with up to 0.8 μM sunitinib for 24 h in the presence of 5 μg/ml PHA. IL-2 was measured by ELISA. Each column indicates the means ± SD of three experiments and each experiment includes triplicate sets. *$P<0.05$ and **$P<0.01$ versus positive control.

the present study, we examined the effect of sunitinib on three critical parameters of T-cell response: proliferation, cytokine production and activation. As the results, all of these events were inhibited by sunitinib, suggesting that sunitinib may cause some adverse effects in its clinical practice. Importantly, such inhibition on the T-cell proliferation was also confirmed in the RCC and other cancer patients by sunitinib at the dose of 0.2 μM. On the other hand, almost all of the cytokine production was inhibited by sunitinib in our study except for IL-6. Further studies will be required to clarify the abnormal change in IL-6, which also happened in other kinds of cells. For example, in AML cells sunitnib increased the levels of c-Jun bound to the activator protein-1 site in the interleukin-6 (IL-6) gene promoter [15].

When examined the relationship between the sunitinib-induced inhibition of T-cell proliferation and the induction of apoptosis, we found the higher doses of sunitinib (0.8 μM), which almost completely inhibited the T-cell proliferation, could not induce apoptosis (Fig. 2A). The proliferation inhibition could be recovered after withdrawal of the drug (Fig. 2B). The results obtained here suggest that sunitinib did not affect the proliferation potential of T cells and that the effects of the drug are reversible. Sunitinib reduced the amount of cyclin D3 and diminished the phosphorylation of CDK6, which kept Rb combining with E2F (Fig. 3B). These effects explain the arrest of the G0/G1 phase of the cell cycle (Fig. 3A), which also implied the function of T cells could be regained after withdrawal of sunitinib.

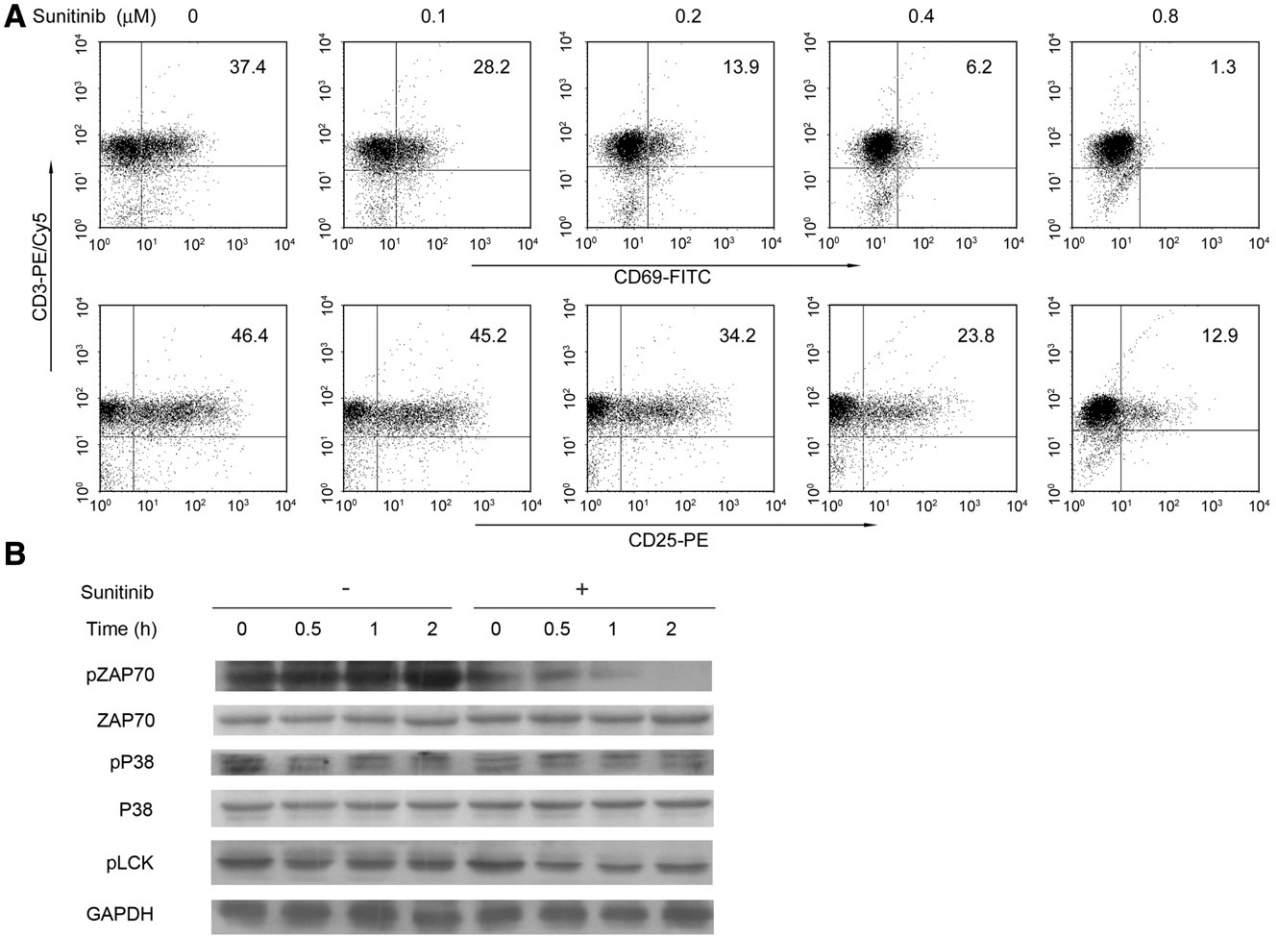

Figure 5 Sunitinib inhibits T cells activation and phosphorylation of ZAP-70. (A) PBMCs (5×10^5 /well) were stimulated with PHA 5 μg/ml for 24 h. Then they were incubated with PE/Cy5-conjugated mAb for CD3, FITC-conjugated mAb for CD69 and PE-conjugated mAb for CD25 and isotype-matched mAb. Cell surface expressions were analyzed by flow cytometry. The percentages of $CD3^+$ T cells expressing the relevant markers are shown. (B) Human peripheral blood T cells were stimulated with 5 μg/ml PHA in the presence or absence of 0.4 μM sunitinib for up to 2 h, and then whole cell lysates were analyzed by Western blot for phosphorylation of P38, LCK and ZAP70.

The effects of sunitinib on cell activation might be explained by the inhibition of early signaling events, mediated by the receptor tyrosine kinase. We examined the TCR-associated phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs). In T-cell activation, ITAMs are phosphorylated by LCK and thus promote the recruitment of ZAP70 along with its phosphorylation, which is a crucial event in TCR signaling. In this study, we have observed that sunitinib reduced the phosphorylation of ZAP70 (Fig. 5) but had no effect on the phosphorylation of LCK. From these results, we conclude that a possible mechanism explaining effects of sunitinib on T-cell activation is the inhibition of ITAMs phosphorylation and ZAP70 recruitment.

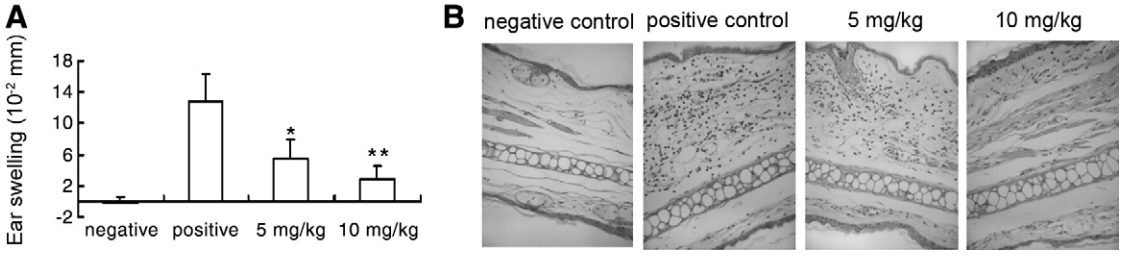

Figure 6 Sunitinib suppresses contact hypersensitivity induced by picryl chloride in mice. (A) Ear swelling was evaluated by the difference in thickness between the right and left ears 24 h after challenge. Mice were administered p.o. sunitinib each day during the experiment. Each column represents the mean ± SD of 10 animals. *$P<0.05$ and **$P<0.01$ versus positive control. (B) Ear tissues were stained with hematoxylin and eosin (original magnification 200×).

DTH is typically used for the evaluation of *in vivo* immunocompetency because it has been shown to be entirely dependent on the effects of T cells. In the experiment of contact dermatitis in mice as a DTH model, sunitinib showed a significant inhibition of the ear swelling in response to picryl chloride.(Fig. 6) The clinical implications should be noticed for the lowering of anti-infection potential, though the effects are rapidly reversed after its withdrawal.

It should be emphasized that sunitinib was much more potent at inhibiting T-cell function (IC50≈0.2 μM) than sorafenib, another molecular targeted agent for mRCC, with an IC50 of 5 μM against T-cell proliferation. It was striking that sunitinib had approximately equal potency to cyclosporine in inhibiting T-cell proliferation, with similar IC50 values. Meanwhile, there is also report that sunitinib can reverse the immune suppression by decreasing T regulatory (Treg) cells and myeloid-derived suppressor cells in a mouse model [16]. This finding seems to be inconsistent with our results, which may be due to the difference in experimental systems or in the doses of sunitinib used. In any case, the effects of sunitinib on the immune system should be emphasized for immune-based cancer therapy.

Taken together, our study demonstrated the inhibitory effects of sunitinib on T cells and provided substantial new information that improves the understanding of the effects of sunitinib on mRCC therapy. Further investigation needs to be performed for the combination of sunitinib with immunotherapeutic agents in larger clinical trials of mRCC.

Disclosure of potential conflicts of interest
No potential conflicts of interest were disclosed.

Acknowledgments

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