Anti-tumour and immune enhancing activities of MLAA-22$_{379–387}$ on acute myeloid leukemia

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We have earlier demonstrated that MLAA-22$_{379–387}$ is a novel, acute, monocytic, leukemia-associated antigen epitope *in vitro*. In this study, the effect and mechanism of MLAA-22$_{379–387}$ on animals have been further examined. We found that tumour weight and volume had significantly decreased in SCID-injected THP-1 mice with MLAA-22$_{379–387}$ treatment for two weeks. MLAA-22$_{379–387}$ induced cytotoxic T lymphocytes (CTL) activity in A549, MCF-7, THP-1, U937 and T2 cells, especially significant CTL activity at effector/target ratio of 50 : 1 in THP-1 cells. The percentage of CD3 + CD8 + T cells had significantly increased, while the percentage of CD4 + CD25 + T cells had significantly decreased in MLAA-22$_{379–387}$ treatment group compared to other groups. Levels of IL-2, IFN-γ and IgG had significantly increased, but levels of TGF-β and IL-10 had significantly decreased after MLAA-22$_{379–387}$ vaccination for two weeks. Thus, we may conclude that MLAA-22$_{379–387}$ treatment effectively improves the immune system, thus indicating tumouricidal capacity in leukaemic mice. These findings highlight the potential application of MLAA-22$_{379–387}$ as an efficient target for immunotherapy in acute myeloid leukemia.

Keywords: Acute myeloid leukemia, anti-tumour activity, immunotherapy, mice.

ACUTE myeloid leukemia (AML) is a clinically heterogeneous disorder[1]. It is characterized by clonal proliferation and differentiation arrest of neoplastic myeloid hematopoietic precursor cells in the bone marrow and peripheral blood[2]. AML treatment over the past few decades includes high-dose chemotherapy[1,3]. Novel therapeutic strategies are needed for AML treatment, such as immune-based therapies or immunotherapies.

Immunotherapy, which may generate an efficacious anti-tumour immune response (IR), is the best therapeutic option for AML[4,5]. Immunotherapeutic treatments include cytokine therapy, monoclonal antibodies with or without conjugation, AML vaccines and T cell-based immune therapies. T cells play an important role in targeting residual leukemia. Recent studies demonstrate that T cell-based immune therapies represent an exciting new area of study in AML treatment[6,7]. AML arises from leukemia stem cells (LSCs) which are resistant to various forms of therapies like irradiation and cytotoxic drugs[6,8]. Cytotoxic T cells (CTLs) as a 'killer' or cytotoxin that produces a specific antigenic response, can specifically target LSCs and play a crucial role in the control of leukemia[9]. CTLs express CD8 on their surface and can bind directly to tumour-associated antigen (TAA)-derived killer peptides to result in cancer cell death[10]. For leukemia, this TAA is leukemia-associated antigen (LAA).

Several LAAs have been identified in AML patients, e.g. PR1, Proteinase-3 (ref. 11), BRAP, HSJ2 and MPP11 (ref. 12), WT1, survivin and TERT[13]. Our previous studies have shown that *MLAA-22* is a novel, acute, monocytic LAA gene[14]. It is predominantly expressed in AML, lesser in chronic myeloid leukemia (CML), but not in gastric and renal carcinoma, and normal adult tissues[14,15]. Based on the ProPred1 and SYFPEITHI bioinformatics tools, we have identified that MLAA-22$_{379–387}$ (abbreviation: MLAA1) is a potential LAA target for AML immunotherapy *in vitro*[16]. However, the effect and mechanism *in vivo* remain largely unknown.

In this study, SCID injected THP-1 mice were used to evaluate the effect and mechanism of MLAA-22$_{379–387}$. The effects on tumour growth and CTL activity were evaluated for anti-tumour activity. The changes of peripheral blood T cell subsets, and levels of IgG and cytokines were also studied. The findings suggest that MLAA-22$_{379–387}$ treatment effectively improves the immune system of leukemic mice, thus indicating the tumouricidal capacity. This, MLAA-22$_{379–387}$ highlights the potential for future anti-cancer drug development against AML.

Materials and methods

Animals

C.B17 severe combined immunodeficiency (SCID) mice (6–8 weeks, 20 ± 2 g) were provided by the Animal Experiment Center of Xi'an Jiaotong University, China. Feed and drinking water as well as padding for mice were administered by high-pressure sterilization. Experimental

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procedures on mice were carried out in a super clean bench. IgG levels of all the animals were measured by ELISA method. Mice were considered as 'not immune leakage' under IgG < 1 μg/ml. The experiment was conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals (USA, Washington, DC). The protocols were approved by the Ethics Committee on Animal Research of Xi'an Jiaotong University, Shaanxi, China.

Cells and culture

A549, MCF-7, THP-1, U937 and T2 cells were purchased from the Chinese Academy of Science, Shanghai Institute of Cell Biology, Shanghai, People's Republic of China. The cells were cultured in RPMI-1640 containing 10% foetal bovine serum and incubated at 37°C with 5% CO_2. Cultures were continuously maintained by routine harvesting of cells at 70–80% confluence using 0.05% trypsin-EDTA.

Animal model construction

The immune system was reconstructed in mononuclear cell leukemia SCID mice model using the natural killer cells (NK cells) inhibitor of Anti Asialo GM1 (Rabbit). The inhibitor (1 ml inhibitor was dissolved in 1 ml saline) was intraperitoneally injected in mice (20 μl/each mouse) to block NK cells' activity. Peripheral blood lymphocytes (8×10^7/ml) were intraperitoneally injected to reconstruct that immune system. After 24 h, THP-1 cells (1×10^7/ml) were subcutaneously injected to establish the animal model. The mice were vaccinated when the tumour volume was about 100 mm^3.

Next, 25 mice were randomly divided into five groups with five animals in each group. The first group was treated only with incomplete Freund's adjuvant (IFA, Con group). The second group was treated with a universal T helper cell epitope (tetanus endotoxin) and IFA (Th1+IFA, Th1 group). The third group was treated with inactivated THP-1 cell vaccine, universal T helper cell epitope and IFA (Inactive THP-1+Th1+IFA, INA THP-1 group). The fourth group was treated with WT1 polypeptide, universal T helper epitope and IFA (WT1+Th1+IFA, WT1 group). The fifth group was treated with the MLAA-$22_{379–387}$ polypeptide, universal T helper epitope and IFA (MLAA-$22_{379–387}$ + Th1+IFA, MLAA-$22_{379–387}$ group, abbreviated as MLAA1 group). The amino acid sequence of MLAA-$22_{379–387}$ polypeptide is 'LLPNAIYKV' (ref. 15). SCID mice were sacrificed after two weeks of vaccination.

Transplanted tumour in SCID mice assay

Before the SCID mice were sacrificed, retro-orbital bleeding (ROB) was used to collect blood from them mice for the following experiment[17]. Then the SCID mice were sacrificed, tumour tissues and spleens were immediately excised. Tumour weights were measured, and tumour volumes were calculated using the following formula: $V = 0.5 \times D \times d^2$, where D is the long diameter of the tumour, while d is its short diameter. The tumour tissues were fixed with 10% neutral formalin for 24 h, embedded, sectioned and hematoxylin-eosin stained. The pathological features of transplanted tumours were observed under a microscope (Olympus, CX21, Japan).

Determination of CTL activity

CTL activity was determined by the following steps: (1) Preparation of effector cells – A single cell suspension of spleen was prepared from different groups under aseptic condition. The cells were adjusted to a concentration of 5×10^6/3 ml/well with RPMI 1640 medium (containing 10% FCS) in a six-well plate for culture. Simultaneously, THP-1 cells were inoculated to the previous six-well plate at the concentration of 1×10^6/well. Recombinant human IL-2 of 20 U/ml was added to the culture medium (added once every three days). After five days, the cell culture medium was centrifuged at 1000 rpm for 5 min; then the cells were collected and used as effector cells. (2) Preparation of target cells – The previous cultured A549, MCF-7, THP-1, U937, T2 cells were used as target cells and collected. The concentration of target cells was adjusted to 1×10^5/ml with 5% FCS-containing RPMI 1640 medium. (3) Co-culture effector/target cells – 50 μl of effector cells and 50 μl target cells were added to 96-well culture plates according to the different effector/target ratios (50 : 1, 25 : 1, 12.5 : 1 and 6.25 : 1). After 4 h of incubation, CTL activity was detected by LDH method as previously reported[15].

Detection of CD3 + CD4+, CD3 + CD8 + T cells, CD4 + CD25 + Treg cells

A triple antibody of 10 μl PerCp-CD3/FITC-CD4/PE-CD8 was added to 50 μl blood containing heparin sodium, which was incubated at 4°C for 30 min in the dark. Then the solution was hemolysed and washed, and detected using a flow cytometer (FACSCanto II). The changes of CD3 + CD4+, CD3 + CD8 + T cells were analysed using BD FACSDiva software. To detect Treg cells, CD4, CD25 monoclonal antibody was added to label the cells; the same FACS method was used in the experiment.

Detection of IFN-γ, TGF-β, IL-10, IL-2 and IgG

No anticoagulant blood samples through ROB were collected. The blood samples were centrifuged at 1500 rpm for 20 min, and stored at –70°C. Serum levels of IL-2,

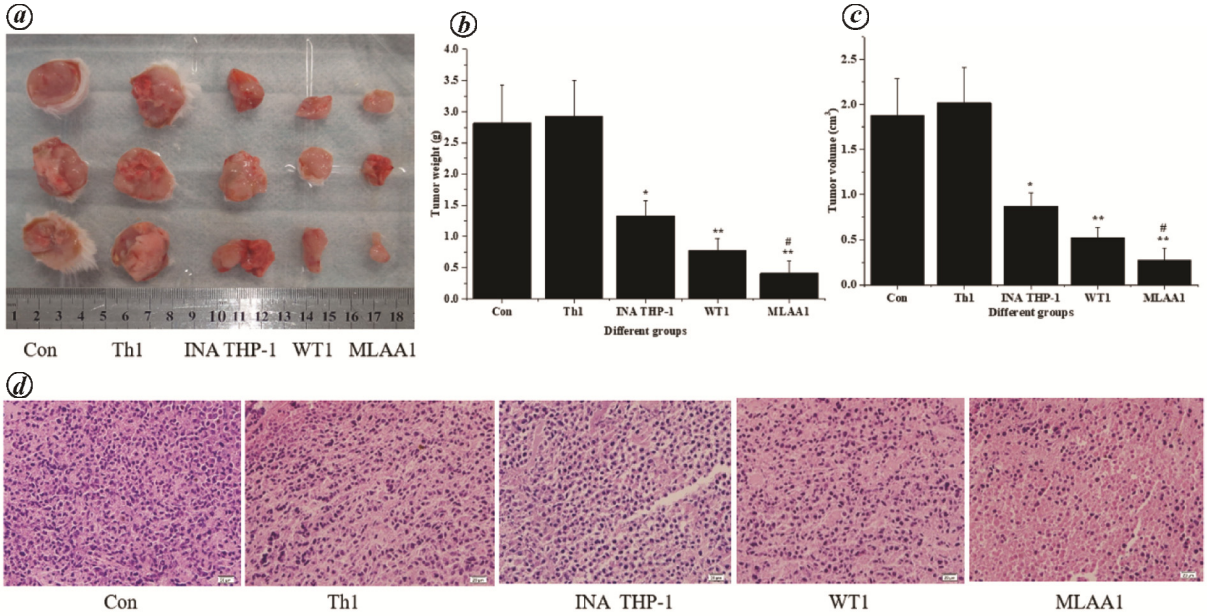

Figure 1. Effect of MLAA-$22_{379–387}$ and other vaccines on THP-1-derived tumour growth. THP-1 cells were subcutaneously injected and tumour xenografts were allowed to grow to an average size of 100 mm^3; then different vaccines were inoculated for two weeks. ***a***, Representative tumours from nude mice. ***b***, Tumour weight. ***c***, Tumour volume. ***d***, Representative photographs of HE staining. Data are presented as mean ± SD from at least three independent repeats. $*P < 0.05$ versus Con group, $**P < 0.01$ versus Con group respectively. $^{\#}P < 0.05$ represents MLAA1 group compared with other groups. Note: MLAA1 represents MLAA-$22_{379–387}$.

IFN-γ, TGF-β, IL-10 and IgG were measured by ELISA kit, according to the manufacturer's instructions.

Statistical analysis

Data were analysed using the statistical package SPSS version 16 and presented as mean ± standard deviation. The statistical significance of differences between groups was compared with one-way analysis of variance. A P value <0.05 was considered significant.

Results

Inhibition of MLAA-$22_{379–387}$ on tumour growth

Humanized monocytic leukemia SCID mice was established by injection THP-1 cells. Figure 1 *a* shows a photograph of tumours after two weeks of vaccination. The tumours are small in INA-THP-1, WT1 and MLAA1 groups. The tumour weights and volumes decreased significantly ($P < 0.05$, $P < 0.01$) in INA-THP-1, WT1, MLAA1 groups compared with the control group. Additionally, significant reductions ($P < 0.05$) of tumour volume and weight in MLAA1 group were observed compared to the other groups (Figure 1 *b* and *c*). Pathological changes, such as tumour cell atypia and larger nucleus/cytoplasm ratio, were observed in Con and Th1 groups. Some tumour cells were observed to be necrotic and apoptotic in INA-THP-1 and WT1 treatment groups. Inflammatory cell infiltration and degeneration, necrotic or apoptotic cells in a large number of tumour cells were observed in MLAA-$22_{379–387}$ treatment group. These results demonstrate the inhibition of MLAA-$22_{379–387}$ on THP-1 cell-derived tumour growth.

MLAA-$22_{379–387}$ induced CTL activity in different cancer cells

To determine whether the inhibition of tumour growth in SCID mice can enhance CTL activity, we measured CTL activity at different ratios of effector/target cells. The spleen is an important lymphoid organ which has hematopoietic and hemofiltration functions. It also plays an important role in lymphocyte migration and immune response after antigen stimulation. Thus, effectors cells derived from spleen cells stimulated by THP-1 were used in the experiment. As shown in Figure 2 *a–e*, specific killer rate (%) increased with MLAA-$22_{379–387}$ and other vaccine treatments in THP-1, A549, MCF-7, T2 and U937 cancer cell lines. The results showed the effector/target ratio dependent manner. The mean specific killer rate (%) was the following ascending order, Con < Th1 < INA THP-1 < WT1 < MLAA1. More importantly, MLAA-$22_{379–387}$ group indicated significant increase ($P < 0.05$, $P < 0.01$, $P < 0.001$) in specific killer rate compared with other antigen peptide treatment groups. The results demonstrate that MLAA-$22_{379–387}$ induces significant CTL activity in the examined cancer cell lines. When we compared the CTL activity of MLAA-$22_{379–387}$

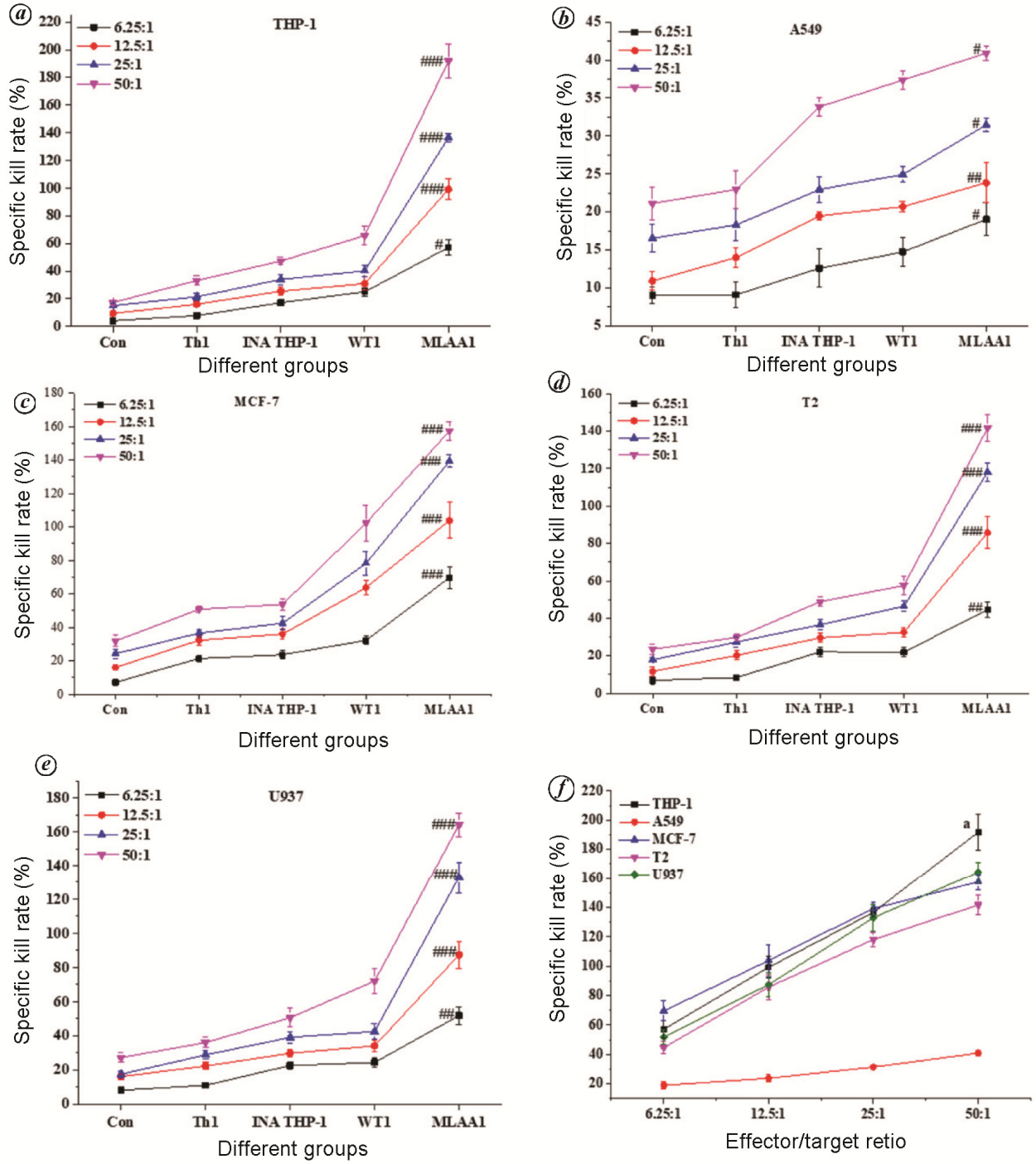

Figure 2. Effect of MLAA-$22_{379–387}$ and other vaccines on CTL activity of THP-1, A549, MCF-7, T2 and U937 cancer cell lines. Co-culture effector/target cells were cultured in six-well plates in the ratio 6.25 : 1, 12.5 : 1, 25 : 1 and 50 : 1 for 4 h respectively. CTL activities were measure by LDH method. ***a***, THP-1; ***b***, A549; ***c***, MCF-7; ***d***, T2; ***e***, U937; ***f***, CTL activity of MLAA-$22_{379–387}$ at 50 : 1 E/T ratio treatment in different cancer cells. Data are presented as mean ± SD. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ indicate statistical significance when MLAA-$22_{379–387}$ is compared with various other vaccine treatment groups. $^{a}P < 0.05$ represents THP-1 versus other cells of MLAA-$22_{379–387}$ at 50 : 1 E/T ratio treatment. Note: MLAA1 represents MLAA-$22_{379–387}$.

treatment in the examined cells, the A549 cells showed the lowest CTL activity. With the effector/target ratio at 50 : 1, CTL activity in THP-1 cells showed a significant increase than the other examined cells (Figure 2 *f*).

MLAA-$22_{379–387}$ modulated peripheral blood T cell subsets in SCID mice

When blood was taken from SCID mice after two weeks of vaccination, we found that the percentage of

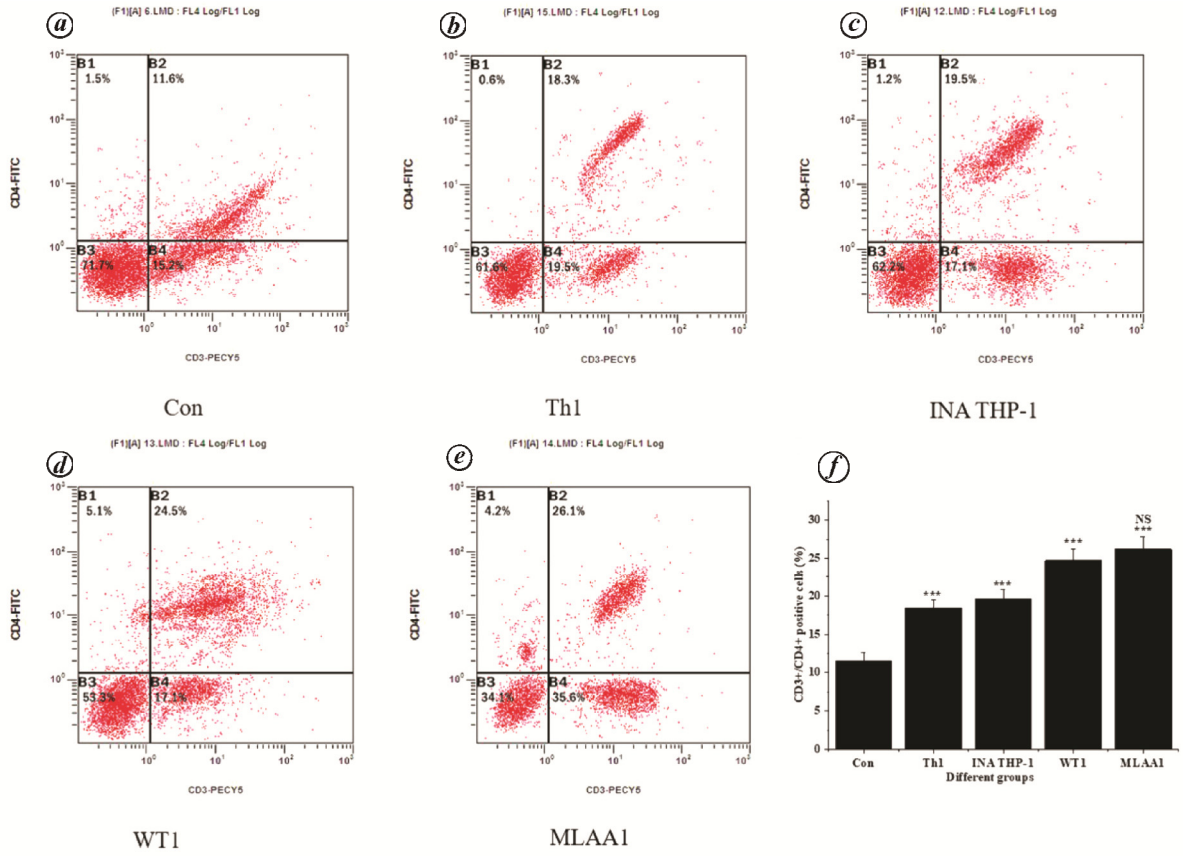

Figure 3. Flow cytometry analysis of CD3 + CD4 + T cells in blood derived from Con and different treatment groups. Triple antibody of PerCp-CD3/FITC-CD4/PE-CD8 was used to label the cells which were analysed by flow cytometry. ***a*–*e***, Representative examples of staining for CD3 + CD4 + T cells. Gates are based upon isotype controls. ***f***, Statistical analysis of results. Data are expressed as mean ± SD of three independent experiments. ***$P < 0.01$ versus Con group. [NS]No significant difference in MLAA1 versus other groups. Note: MLAA1 represents MLAA-$22_{379–387}$.

CD3 + CD4 + T cells had significantly increased ($P < 0.001$) in different peptide treatment groups compared to the Con group (Figure 3). The percentage of CD3 + CD8 + T cells had also significantly increased in MLAA-$22_{379–387}$ group compared to the Con group ($P < 0.001$) and other peptides treatment groups ($P < 0.001$) (Figure 4). However, significant decrease was observed in the CD4 + CD25 + T cells when compared with the Con group ($P < 0.001$) and other peptide treatment groups ($P < 0.001$) (Figure 5).

MLAA-$22_{379–387}$ modulated secretion of IgG and cytokines

To determine the further varied T lymphocytes mechanism, we measured the contents of IgG, IL-2, IFN-γ, TGF-β and IL-10 cytokines in the blood. As shown in Figure 6, the levels of IL-2, IFN-γ, IgG had significantly increased ($P < 0.05$, $P < 0.001$) in INA-THP-1, WT1 and MLAA-$22_{379–387}$ groups. The levels of TGF-β and IL-10 had significantly decreased ($P < 0.001$) in INA-THP-1, WT1 and MLAA-$22_{379–387}$ groups, while those of TGF-β in Th1 group had significantly decreased ($P < 0.05$) compared to the Con group. Importantly, the levels of IgG, IL-2 and IFN-γ had significantly increased ($P < 0.001$) while those of TGF-β and IL-10 had significantly decreased ($P < 0.001$) in MLAA-$22_{379–387}$ treatment group compared with other vaccine treatment groups.

Discussion

Therapeutically targeting immune pathways has radically changed the treatment paradigm for solid and lymphoid tumours, but is yet to be approved for myeloid malignancies[18]. In this study, we found that MLAA-$22_{379–387}$, a potential LAA target, shows anti-tumour and immune enhancing effects through CTL activity, CD3+CD8+ T cells, levels of IgG, IL-2 and IFN-γ. The results demonstrated that MLAA-$22_{379–387}$ could activate specific immune cells to re-establish the immune surveillance of the immune system against these cancer cells[5].

In this study, anti-peripheral blood mononuclear cell was used to reconstitute SCID mice *in vivo*, and THP-1 tumour cells were inoculated later. The results show that

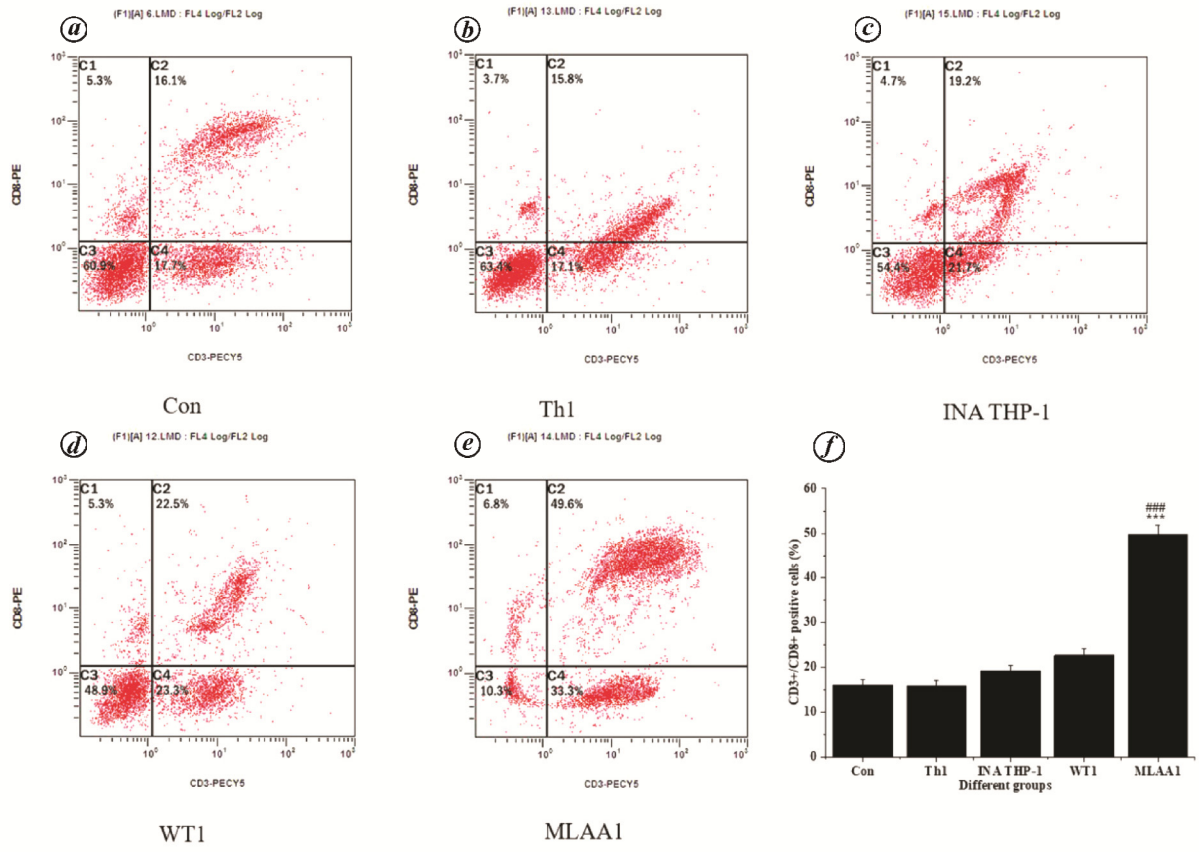

Figure 4. Flow cytometry analysis of CD3 + CD8 + T cells in blood derived from Con and different peptide treatment groups. Triple antibody of PerCp-CD3/FITC-CD4/PE-CD8 was used to label the cells which were analysed by flow cytometry. ***a–e***, Representative examples of staining for CD3 + CD8 + T cells. Gates are based upon isotype controls. ***f,*** Statistical analysis of results. Data are expressed as mean ± SD of three independent experiments. ***$P < 0.001$ versus Con group. $^{###}P < 0.001$ represents MLAA1 versus other groups. Note: MLAA1 represents MLAA-$22_{379–387}$.

human CD3+, CD4+, CD8+ T cells were detected in peripheral blood of SCID mice. Thus PBMC can ensure immune reconstitution in SCID mice[19–21]. We observed inhibition in the growth of tumour in monocytic leukemia SCID mice by significant decrease in tumour volume and weight of the MLAA-$22_{379–387}$ treated group. Simultaneously, histopathological changes, including degeneration and necrosis of large tumour cells and partial apoptotic cells, were observed in MLAA-$22_{379–387}$ treated mice. These findings further confirm the inhibited effects of MLAA-$22_{379–387}$ *in vivo* for monocytic leukemia.

We have previously reported that MLAA-$22_{379–387}$ is the tumour antigen of MLAA-22 HLA-A*0201 restricted CTL epitope[15]. The present study further proves that MLAA-$22_{379–387}$ induces significant CTL activity in THP-1, A549, MCF-7, T2 and U937 cancer cell lines. We here confirmed with human THP-1 cells that MLAA-$22_{379–387}$ induced the specific cytolytic activity at an effector/target ratio of 50 : 1. This may be due to the fact that specific epitope of MLAA-$22_{379–387}$ induced specific CTL cells, which are widely distributed in peripheral blood, partially into cellular immune organs (such as spleen, lymph nodes, etc.) and tissues, which continue to proliferate and degenerate[22,23]. Moreover, the epitope of MLAA$22_{379–387}$ was recognized by the specific CTL. These CTL cells additionally recognize the THP-1 cells and rapidly mediate the production of CTL cellular immune effect after THP-1 cells presenting the specific epitope exposure. It has been reported that WT1 polypeptide and inactivated THP1 can be effectively applied for AML treatment[24]. The present study also confirms that WT1 polypeptide and inactivated THP1 treatment show certain anti-AML therapeutic effects. This is in accordance with significant reductions of tumour weight and volume in mice under INA-THP-1 and WT1 treatments.

The CTL activities are closely related to variations of different T lymphocytes. Thus, we further determined the effects on T cell subsets. Helper T lymphocytes (CD3+CD4+) play an intermediate role in immune response by activating proliferation of other immune cells for direct immune response, and participating in T cell regulation or 'assisting' other lymphocytes[25]. Cytotoxic T lymphocytes (CD3+CD8+) can kill target cells that produce a specific antigen response. The major surface

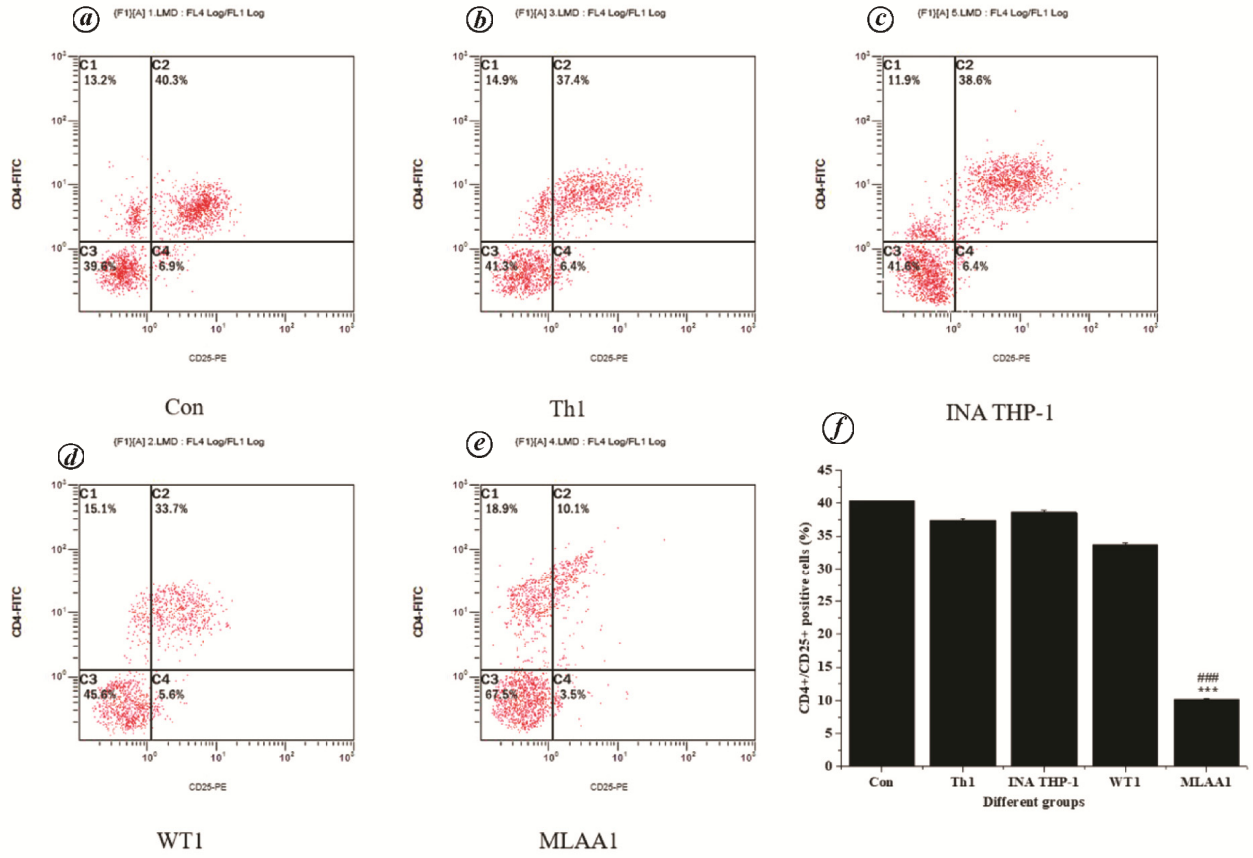

Figure 5. Flow cytometry analysis of CD4 + CD25 + T cells in blood derived from Con and different peptide treatment groups. CD4, CD25 mAb was used to label the cells which were analysed by flow cytometry. ***a*–*e***, Representative examples of staining for CD4 + CD25 + T cells. Gates are based upon isotype controls. ***f***, Statistical analysis of results. Data are expressed as mean ± SD of three independent experiments. $^{***}P < 0.001$ versus Con group, $^{###}P < 0.001$ represents MLAA1 versus other groups. Note: MLAA1 represents MLAA-$22_{379–387}$.

marker for cytotoxic T cells is CD8, also known as killer T cells[26]. In this study, the percentage of both CD3 + CD4 + T cells and CD3 + CD8 + T cells significantly increased in mice administered with MLAA-$22_{379–387}$, especially significantly higher than those in other groups for CD3 + CD8 + T cells. These findings suggest that MLAA-$22_{379–387}$ can significantly activate the CD8 + T cell immune effect *in vivo* and stimulate a more effective anti-tumour immune effect. Additionally, naturally occurring regulatory T cells of CD4+CD25+ are pivotal to the maintenance of self-tolerance and immunosuppression[27]. The CD4+/CD25 + T cells can simultaneously inhibit the anti-tumour activity of CD8 + T cells and CD4 + T cells. Removal or reduction of CD4+/CD25 + T cells significantly enhances antitumour activity[28,29]. On the contrary, increased numbers of Tregs are reported in patients with solid tumours and haematological malignancies, and are considered to facilitate disease progression[30,31]. Our *in vivo* test results show that the level of CD4+/CD25+ Treg cells is significantly lower in MLAA-$22_{379–387}$ group than those in other treatment groups. Thus, our results suggest that MLAA-$22_{379–387}$ may enhance the anti-tumour CTL effect in AML.

Alterations in the functional cytokines secreted by T lymphocytes play an important role in CTL activity. IL-2 is a cytokine that induces activation of naïve helper T lymphocyte proliferation and differentiation to effector T helper cells (Th1) to produce non-specific anti-tumour immune response[32,33]. IFN-γ secreted by Th1 cells plays a pivotal role in promoting innate and adaptive immune response in the host defence against tumours[34]. IgG plays an important role in protective immunity in body[35]. In this study, significant high levels of IgG, IFN-γ and IL-2 indicate greater tumouricidal capacity of MLAA-$22_{379–387}$ than other vaccine treatments. IL-10 has been shown to mediate immunosuppression, and to inhibit the proliferation of T cells and in the production of IFN-γ and IL-2 (ref. 36). TGF-β inhibits the proliferation of T and B cells that depend on IL-2, and inhibits the production of IFN-γ and TNF-α in peripheral blood mononuclear cells[37,38]. Inhibition of IL-10 and TGF-β can escape immune tolerance, stimulate the synthesis and secretion of cytokines, promote Th1 cell differentiation and CTL proliferation, and enhance cytotoxicity[39]. The variations of IgG and cytokines are in accordance with the significant inhibition in tumour growth for the MLAA-$22_{379–387}$ treatment group.

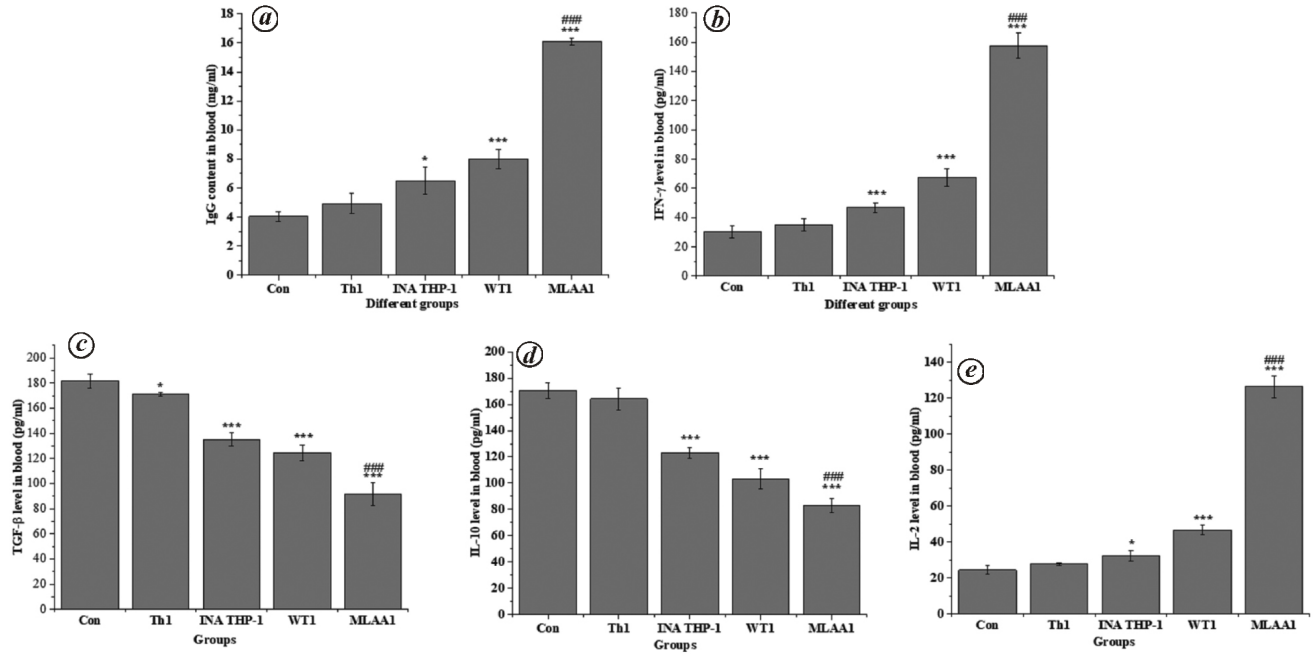

Figure 6. Effect of MLAA-$22_{379–387}$ and other vaccine treatments on IgG and cytokines. After SCID mice construction, MLAA-$22_{379–387}$ and other vaccines were treated for two weeks. Blood samples were collected and serum levels of IL-2, IFN-γ, TGF-β, IL-10 and IgG were measured by ELISA method. ***a***, IgG; ***b***, IFN-γ; ***c***, TGF-β; ***d***, IL-10; ***e***, IL-2. Results are expressed as mean ± SD of three independent experiments. $^{***}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus Con group respectively. $^{\#\#\#}P < 0.001$ indicates statistical significance when MLAA-$22_{379–387}$ is compared with various other vaccine treatment groups. Note: MLAA1 represents MLAA-$22_{379–387}$.

In conclusion, MLAA-$22_{379–387}$ can effectively induce CTL activity to promote degeneration and necrosis in THP-1 cells, increase CD3 + CD8 + T cells, and IFN-γ, IL-2, IgG levels, decrease IL-10 and TGF-β levels, and down-regulate immunosuppressive factors of CD4+/CD25 + T cells. Our results confirm the enhanced immune effect and tumouricidal capacity of MLAA-$22_{379–387}$ in SCID mice derived from THP-1 cells. These findings suggest that this epitope can be used as a highly efficient target molecule for immunotherapy in AML.

Conflict of interest: The authors declare no conflict of interest.

Research involving animals: Animal studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Animals (USA, Washington, DC). In this study, the protocols on mice were followed and approved by the Ethics Committee on Animal Research at Xi'an Jiaotong University, Shaanxi, China.

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