The Effects of Borojo on Regulating The Anti-tumor Activity of Human Vγ9Vδ2-T Cells and Its Underlying Mechanisms

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Abstract

γδ T cells are a unique and conserved population of lymphocytes that have been the subject of a recent explosion of interest owing to their essential contributions to many types of immune response and immunopathology. At present, the existing in vitro amplification system has been unable to meet the needs of the development of immunotherapy. How to improve the quantity and quality of anti-tumor immune cells in vitro by optimizing conventional amplification methods has become a hot issue in the current immunotherapy research. In this experiment, the effect of Borojo on Vγ9Vδ2-T cells expanded in vitro was studied by cell proliferation assay, killer assay and cytokine assay. The results showed that under the effect of 100μg/mL Borojo, the viability of Vγ9Vδ2-T cells could be enhanced, and they could secrete higher levels of INF-γ, TNF-α, GranzymeA, GranzymeB, Perforin and Granulysin enhance its anti-tumor activity, which lay a good application foundation for the development of immunotherapy based on γδ-T cells.

Keywords

Borojo; Vγ9Vδ2-T cells; Antitumor.

1. Introduction

Borojo is a kind of fruit produced in tropical rain forest of South America which belongs to the species of Rubiaceae. It includes Borojoa patinoi Cuatrec and Borojoa sorbilis Cuatrec which have long been used by local people. It's reported that Borojo has a role in natural immune function, regulating blood sugar, stabilizing blood pressure, anti-inflammatory and anti-oxidation[1]. Borojo is rich in nutrients and contains many vitamins, amino acids, polysaccharides and so on. Cernasov et al discovered that Borojo has antioxidant activity on skin[2]. γδ-T cells are the first line of defense cells in the body and play an important role in anti-infection, anti-tumor, immune surveillance, immune regulation and professional antigen presentation[3]. γδ-T cells play many important roles in human immune responses, and"bridge" between innate immunity and acquired immunity[4]. Among several subsets of γδ-T cells, Vγ9Vδ2 subset only represents in peripheral blood of human and primates[5]. Although the small population in T lymphocytes, investigations on this group of γδ-T cells during the past a few years revealed that they directly involve in both anti-tumor and anti-infections, and that they can be applied in clinical treatment of tumor and infection using adoptive infusion. Recently, more and more evidence shows that Vγ9Vδ2-T cells are one of the most attractive targets for anti-tumor immunotherapy[6]. The existing in vitro amplification system can no longer meet the needs of the growing development of immunotherapy. How to improve the quantity and quality of anti-tumor immune cells expanded in vitro by optimizing conventional amplification methods has become a hot topic in current immunotherapy. At present, a lot of researches are focusing on whether it is possible to establish an amplification system that can simultaneously ensure that the amplified Vγ9Vδ2-T cells have high anti-tumor cytotoxic activity. The traditional amplification system is mainly based on RPMI-1640 medium by adding various kinds of interleukin, mainly IL-2, so as to achieve the purpose of amplifying immune cells in vitro. However, RPMI-1640 medium can only supply the basic nutrients needed for cell expansion in vitro. For example, in the RPMI-1640 medium

which purchased on the market at present contain 20 essential amino acids, vitamin B12,glucose, folic acid,Nicotinamide inositol and so on[7].According to our previous experience, this kind of nutritional composition can only meet the purpose of culturing immune cells in vitro for a period of time. In the process of culturing effector cells for immunotherapy, in order to obtain enough number of cells for clinical treatment, we need to extend the amplification time in vitro. Therefore, the requirements for nutrient in the medium are more stringent.

2. Experimental Detail

2.1 Materials and Reagents

Borojo is provided by the Ecuadorian Oriental Food Group and is identified as the mature fruit of the B.sorbilis.Cuatrec; K562 cell line was preserved by the Laboratory of Antibody Engineering of Jinan University; Lymphocyte Separation Medium is purchased from axis-shield Company; Pamidronate (PAM) is purchased from Pamisol Company; Human recombinant interleukin 2 (IL-2) was purchased from PeproTech Company; RPMI-1640 and FBS were purchased from Gibco;CCK-8 kit purchased from Dojindo; TCRγ/δ^{+}T cell selection kit purchased from Miltenyi Company; CountBright™ Absolute Counting Beads purchased from Invitrogen company; Fixation / Permeabilization Solution Kit is purchased from BD Company; CFSE cell division tracker kit and LEGENDplex™ CD8/NK Panel kits are purchased from Biolegend Company; Pacific Blue™ anti-human CD3, PerCP anti-human TCR Vδ2, FITC anti-human Perforin, PE anti-human GranzymeB, FITC anti-human IFN-γ,PE anti-human TNF-α,FITC Mouse IgG1, κ Isotype Ctrl, PE Mouse IgG1, κ Isotype Ctrl are all purchased from Biolegend Company.

2.2 Methods

2.2.1 Preparation of Borojo Solution

Freshly picked ripe Borojo, preferably, washed, peeled seeds, pulp and water, homogenized, pulp, pectinase enzymatic hydrolysis, filtration; Adjust pH, heat Enzyme, Concentrate in vacuum at low temperature, add β-Cyclodextrin, spray-dried into powder. Take appropriate amount of Borojo powder, mix it with water to 1mg/m L, adjust PH to 7.0, sterilize by filtration, and store at -20°C after packing. Dilute with RPMI-1640 complete medium before use.

2.2.2 Isolation of Human Peripheral Blood Mononuclear Cells

Normal human venous blood was collected, lymphocyte separation solution was added, and the mononuclear cell layer was separated by density gradient centrifugation. The collected cells were washed with PBS. Resuspend the cells in RPMI-1640 medium containing 10%FBS and adjust the cell concentration to 2×10^{6} cells/mL

2.2.3 Effects of Borojo on the Cytotoxicity and In Vitro Growth of Human PBMCs

Isolated PBMC cells were resuspended at 2×10^{6} cells/mL in RPMI-1640 medium containing 10%FBS and 100μL/well of cell suspension was plated in 96-well plates. Add different concentrations of Borojo (final mass concentrations of 10μg/mL, 50μg/mL, and 100μg/mL, respectively), 3 replicates in each group, and blank wells with no drugs or cells. After 72h incubation at 37°C and 5%CO2, colony formation was observed under an inverted microscope. Afterwards, 10μL of CCK-8 was added to each well and the culture was continued for 1-4 h. The microplate reader measures the absorbance at a wavelength of 450 nm.

2.2.4 Effects of Borojo on In Vitro Expansion of Vγ9Vδ2-T Cells

Isolated PBMC cells were resuspended at 2×10^{6} cells/mL in RPMI-1640 medium containing 10%FBS. The PAM was added at day0 to a final concentration of 9μg/mL, While stimulating, add different concentrations of Borojo (final mass concentrations of 10μg/mL, 50μg/mL, and 100μg/mL, respectively). The percentage of Vγ9Vδ2-T cells was detected on day0. On day3, the medium was changed and PAM concentration 9μg/mL and IL-2 (100IU/mL) were added. Change medium and supplement IL-2 or IL-2 and Borojo every 3 days, Percentage of Vγ9Vδ2-T cells detected by flow cytometry every 5 days.

2.2.5 Effects of Borojo on The Proliferation of Vγ9Vδ2-T Cells

While measuring the percentage of Vγ9Vδ2-T cells by flow cytometry, 100μL of cells from each treatment groups were collected every 5 days. collected Vγ9Vδ2-T cells were labeled with Pacific Blue™ anti-human CD3, PerCP anti-human TCR Vδ2 and resuspended in 300μL PBS, then add 10μL of pre-warmed CountBright™ Beads, vortexed, and analyzed by flow cytometry. Calculation formula: $\frac{A}{B} \times \frac{C}{D}$= Sample concentration (cells/μL) A=Number of cells collected; B=Beads number; C=Absolute number of added Beads; D=Volume of sample (μL)

2.2.6 The effects of Borojo on Immunophenotypes of Vγ9Vδ2-T Cells

Vγ9Vδ2-T cells were collected after treated with and without Borojo for 30 days. The expressions of TRAIL, FasL, Fas and NKG2D on the cell surface, intracellular Perforin and granzyme B were detected by flow cytometry.

2.2.7The Determination of the Killing Ability of Borojo on Vγ9Vδ2-T cells

The Vγ9Vδ2-T cells treated with and without Borojo were collected and the number of cells was determined. According to the instructions of the Miltenyi Company's TCRγ/δ^+T cell selection kit, magnetic beads were used to sort out the Vγ9Vδ2-T cells that treated with and without Borojo. K562 cells were collected and labeled with CFSE. Vγ9Vδ2-T cells and K562 were co-incubated for 6h at a 10:1 effect-to-target ratio in a 37°C, 5% CO_2 incubator, then stained with PI, and detected the percentage of PI^+ in CFSE labeled K562 cells by flow cytometry, that is, the efficiency of killing K562 by Vγ9Vδ2-T cells.

2.2.8 Effect of Borojo on Cell secretory Factors Secreted by Vγ9Vδ2-T Cells

Vγ9Vδ2-T cells were collected after treated with and without Borojo for 30 days. According to the instructions of the Miltenyi Company's TCRγ/δ^+T cell selection kit, magnetic beads were used to sort out the Vγ9Vδ2-T cells that treated with and without Borojo. K562 and purified Vγ9Vδ2-T cells were co-cultured at 37°C in a 5%CO_2 incubator for 12h.collect the cell supernatant. Follow the instructions of LEGENDplex™ CD8/NK Panel kits and test the concentration of TNF-α, INF-γ, Granzyme A, Granzyme B, Perforin, and Granulysin in the cell culture supernatant.

3. Results and Discussion

3.1 Toxicity Test of Borojo

CCK-8 is a method of determining the number of live cells in a cell proliferation or toxicity assay using a colorimetric assay. WST-8 is reduced to a highly water-soluble yellow formazan product by dehydrogenases in the cell's mitochondria. The amount of formazan is positively related to the number of live cells. After intervention with Borojo for 72h, it showed that there was no significant difference between the group treated with 50μg/mL and 100μg/mL Borojo and the control group, while that with 10μg/mL Borojo was significantly different from the control group. It showed that the high concentration of Borojo had no toxic effect on human PBMC, and it may have a pro-proliferative effect on PBMC at a certain concentration range.

Freshly isolated PBMCs were treated with 10μg/mL, 50μg/mL and100μg/mL of Borojo. The OD value at 450nm was detected by ELISA after 72h. The absorbance is positively correlated with the number of live cells. The date were shown as mean±SEM.*$p<0.05$, **$p<0.01$.

3.2 Effect of Different Doncentrations of Borojo on The Viability of Human PBMC Duing in Vitro Culture

PBMCs were cultured in vitro with 10μg/mL, 50μg/mL, 100μg/mL Borojo for 72h We found that the colony formation in the Borojo treatment group was significantly enhanced compared with the control group. It was demonstrated that Borojo can promote the proliferation of PBMC during in vitro culture.

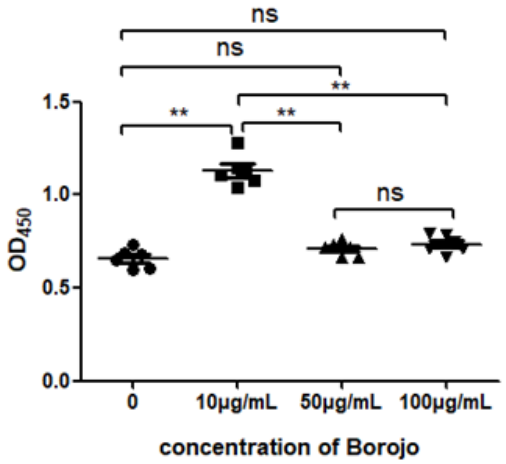

Fig 1. Toxicity test of Borojo

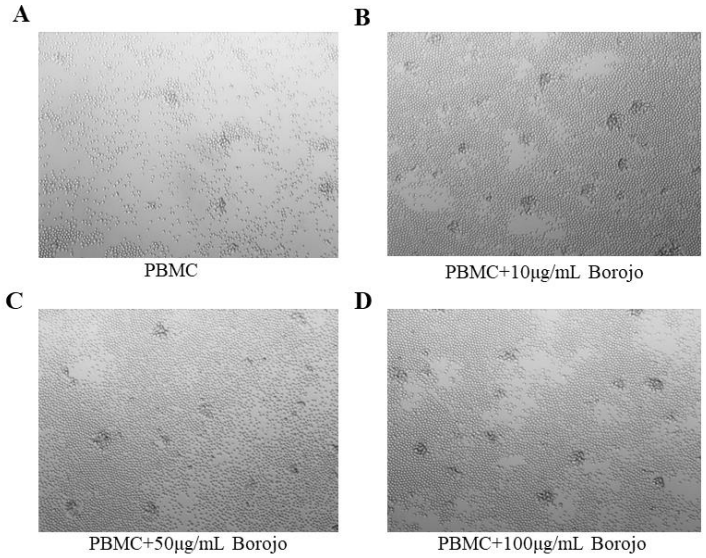

Fig 2. Effect of different concentrations of Borojo on the viability of human PBMC duing in vitro culture

FigA-D show the influence of different concentrations of Borojo on the viability of human PBMC after 72h intervention in vitro. A: human PBMCs were un-treated by Borojo. B: human PBMCs were treated by 10μg/mL of Borojo. C: human PBMCs were treated by 50μg/mL of Borojo. C: human PBMCs were treated by 100μg/mL of Borojo.

3.3 Effect of Borojo on Stimulation And Amplification of Vγ9Vδ2-T Cells In Vitro

As a natural product, Borojo is rich in nutrition, and a large number of experimental studies have demonstrated the regulatory effects of related nutrients on the immune system and immune cells，so whether or not Borojo can be used as a kind of "Nutrient" plays an important role in the culture of Vγ9Vδ2-T cells? Is it possible to prolong the culture time of Vγ9Vδ2-T cells during prolonged proliferation in vitro? Based on the above considerations, in the process of PAM and IL-2 stimulating the expansion of Vγ9Vδ2-T cells, 10μg/mL, 50μg/mL, 100μg/mL Borojo were added.The result showed that there was no significant difference in the percentage of amplification and the absolute number of cells in each group of Vγ9Vδ2-T cells in the first 20 days of culture after the addition of the Borojo (Fig3.3A, B). However, when the cells were cultured to the 25th day, although the number of Vγ9Vδ2-T cells in different treatment groups showed a decreasing trend, the trend of decline was slower in the 100μg/mL Borojo treated group (Fig3.3C,D). It was demonstrated that 100μg/mL

Borojo could significantly enhance the viability of Vγ9Vδ2-T cells in the culture system, thus providing certain advantages for the continued acquisition of more Vγ9Vδ2-T cells for clinical treatment. Therefore, 100μg/mL was selected as the appropriate concentration for the following experiment.

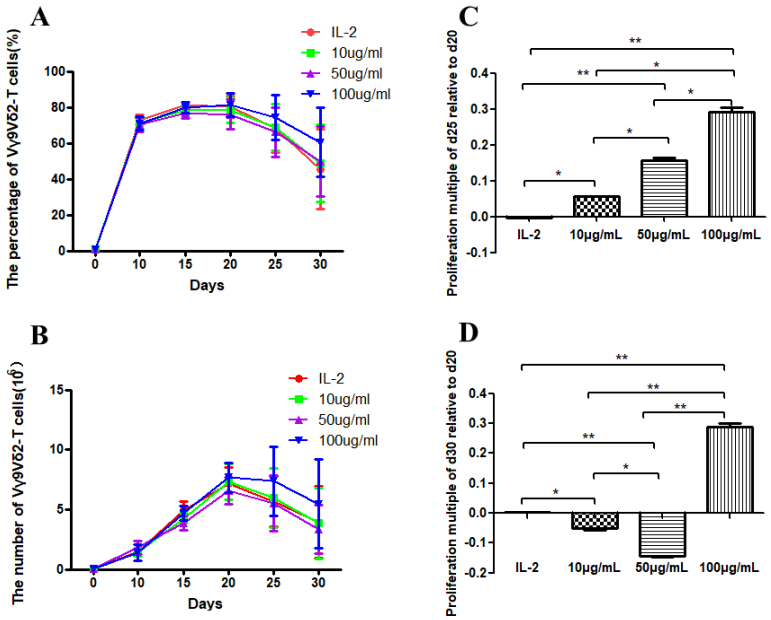

Fig 3. Effect of Borojo on stimulation and amplification of Vγ9Vδ2-T cells in vitro

Figure (A, B) show that when PAM combined with IL-2 is used to stimulate and amplify Vγ9Vδ2-T cells, 10μg/mL, 50μg/mL and100μg/mL of Borojo are added for treatment. During amplification, the percentage of Vγ9Vδ2-T cells was detected by flow cytometry every 5 days, and the absolute number of cells was calculated. C and D represent the increment multiples of day 25 and day 30 relative to day 20, respectively. The date were shown as mean±SEM. *p<0.05, **p<0.01.

3.4 The Effects of Borojo on Immunophenotypes of Vγ9Vδ2-T Cells

In order to investigate the effect of Borojo on the immunophenotype of Vγ9Vδ2-T cells, the surface and intracellular molecular markers of Vγ9Vδ2-T cells after 30 days of treatment with Borojo were detected. We found that Borojo had no significant effect on the expression of TRAIL, FasL, NKG2D and Perforin (Fig3.4); However, the expression of Granzyme B was maintained at a high level in Vγ9Vδ2-T cells that treated with Borojo. At the same time, Borojo has a significant effect on the expression of Fas on Vγ9Vδ2-T cells, and its expression has a significantly decreasing trend (Figure3.4), It indicates that after contacting with tumor cells expressing FasL, the Vγ9Vδ2-T cells treated with Borojo can significantly reduce the level of apoptosis regulated by Fas/FasL pathway due to the low expression of Fas.

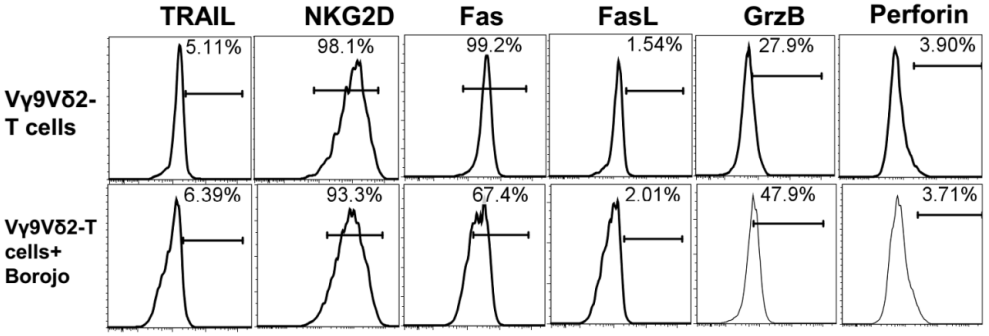

Fig 4. The effects of Borojo on immunophenotypes of Vγ9Vδ2-T cells

Vγ9Vδ2-T cells were treated by 100μg/mL Borojo or PBS during the in vitro expansion. Phenotypic characteristics and cytokine profile of Vγ9Vδ2-T cells were analyzed at day 30 after expansion. The

number shown in the figure represent expression levels of TRAIL, NKG2D, Fas,FasL ,Granzyme B and Perforin in Vγ9Vδ2-T cells after comparing with the isotype control. The data are representative of 4 independent experiments.

3.5 The Borojo Manintain Cytotoxic Ability of Vγ9Vδ2-T Cells

In the previous in vitro amplification experiments, we found that Borojo can significantly enhance the viability of Vγ9Vδ2-T cells in the culture system, thus providing certain advantages for continuously obtaining more Vγ9Vδ2-T cells for clinical treatment. However, in addition to cell activity, whether the expanded Vγ9Vδ2-T cells have a high cytotoxic activity is one of the important things that must be investigated. Therefore, we further examined the effect of Borojo on the killing capacity of Vγ9Vδ2-T cells. Vγ9Vδ2-T cells that have been expanded in vitro for 30 days were purified by MACS, and then incubated with CFSE labeled K562 cells at a 10:1 target ratio (E:T) for 6 h. The ratio of $CFSE^+PI^+$ cells in the total target cells was analyzed by flow cytometry to obtain K562 mortality. The results showed that the percentage of killing K562 by Vγ9Vδ2-T cells was 13.43%±1.8% in the group that treated without Borojo, while the percentage of killing K562 by Vγ9Vδ2-T cells treated with Borojo was 31.18%±3.6% (Figure 3.5) .It was demonstrated that the killing capacity of Vγ9Vδ2-T cells cultured with Borojo remained high after 30 days of amplification, and the mortality rate of K562 cells increased by nearly 18% when the target effect ratio was 10:1. This shows that the vitamin c, vitamin e, polysaccharide and other substances contained in the Borojo can not only maintain the survival activity of Vγ9Vδ2-T cells, but also maintain the tumor killing ability of Vγ9Vδ2-T cells cultured in vitro for a long time.

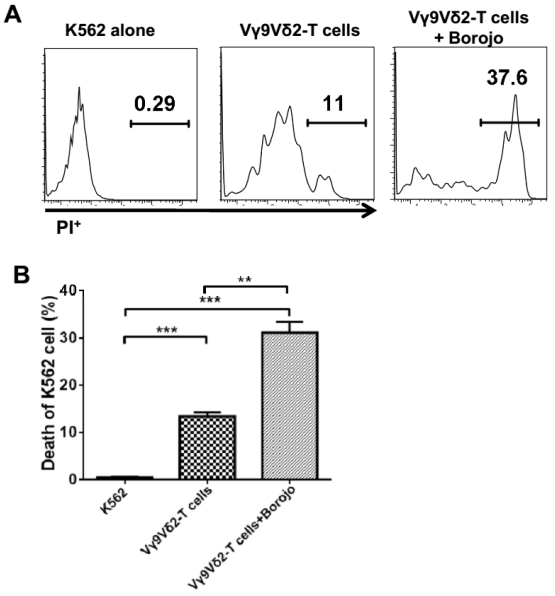

Fig 5. The Borojo manintains cytotoxic ability of Vγ9Vδ2-T cells

During the in vitro expansion, Vγ9Vδ2-T cells were treated by 100μg/mL Borojo solution, and using PBS as control. After 30 days of expansion, Vγ9Vδ2-T cells purified by negative selection then co-cultured with K562 cells at a E:T ratio of 10:1 for 6h.The percentage of dead K562 cells in the CFSE stained-target cells were identified as $CFSE^+PI^+$. The data are representative of 4 independent experiments (A). The date were shown as mean ± SEM (B), **p<0.01， ***p<0.001.

3.6 Mechanism of Borojo Regulating The Killing Ability of Vγ9Vδ2-T Cells

In previous studies, we found that after treatment with Borojo, the ability of Vγ9Vδ2-T cells to kill tumor cells was still well maintained after long-term expansion in vitro. However, the specific molecular mechanism by which Borojo maintains the killing activity of Vγ9Vδ2-T cell tumors is not clear. In order to clarify the way in which Borojo can protect Vγ9Vδ2-T cells from killing tumor cells, we purified Vγ9Vδ2-T cells after 30 days of treatment with Borojo and co-cultured them with K562 cells at a ratio of 1: 1 for 12h, we detected the expression of various cytokines secreted into the

supernatant of cell culture by Cytometric Bead Array.The results showed that compared with PBS control group, the level of cytokines secreted into the cell culture supernatant by the Borojo treated Vγ9Vδ2-T cells was significantly increased, such as TNF-α,INF-γ, Granzyme A, Granzyme B,perforin and Granulysin. It was demonstrated that Vγ9Vδ2-T cells treated with Borojo contacted with tumor cells by maintaining high levels of secreted factors such as TNF-α, INF-γ, GranzymeA, GranzymeB, Perforin and Granulysin that ensure the kill tumor cell activity of Vγ9Vδ2-T cells.

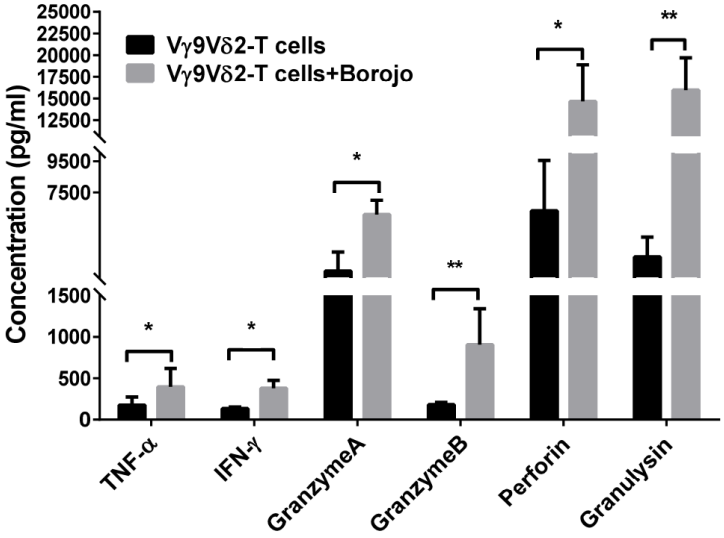

Fig 6. The mechanisms using by Borojo in sustaing cytotoxicity of Vγ9Vδ2-T cells

In vitro expanded Vγ9Vδ2-T cells were treated by Borojo solution or PBS. Vγ9Vδ2-T cells were purified by negative selection then co-culturedwith K562 cells at 1:1 ratio for 12 h. And then the supernatant of cell culture were collected. The CBA was used for detecting levels of multiple effector molecules in the samples. The gray columns represent the secretion levels of TNF-α, INF-γ, Granzyme A, Granzyme B, Perforin and Granulysin in the Borojo solution-treated Vγ9Vδ2-T cells. The black columns represent secretion levels of different effector molecules in the control group. The data were shown as mean ± SEM, *$p<0.05$, **$p<0.01$.

4. Conclusion

In this study，Borojo has no cytotoxicity to human PBMC, and can promote the proliferation of PBMC in vitro at a certain concentration range. With the concentration of 100μg/mL Borojo, Vγ9Vδ2-T cells can be cultured in vitro for a longer time, and their antitumor activity can be enhanced by secreting higher levels of INF-γ, TNF-α, GranzymeA, GranzymeB, Perforin and Granulysin. It indicates that Borojo can lay a solid foundation for promoting the wide application of Vγ9Vδ2-T cells in immunotherapy in the future.

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