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Original Paper

Establish an in Vitro Immune Rejection Response for the Receptor to Realize the Transplantation of NK Cells from Different Species

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Abstract

Healthy adult grass turtle as the donor, draw 10ml of blood, prepare PBMC, isolate and extract purified grass turtle NK cell suspension.

Healthy male Big dog is a receptor. Under anesthesia, 200 milliliters of venous blood is extracted out of the body, enters the blood cell separator, extracts plasma and lymphocyte components, a total of 115 ml, and puts a blood collection bag with heparin sodium. After preserving 10ml plasma, divide the lymphocyte plasma into 3 bags of 35 ml each. Storage.

Take 1 bag of Big Dog Lymphocyte Plasma 35ml, dilute isovolume physiological saline, inject 15ml of grass turtle NK cell suspension, produce a mixed lymphocyte plasma between Turtle and Big Dog, and put it in the incubator. On the third day after mixing, the immune rejection began, and the grass turtle NK cells accounted for 76% of the dead cells. On the fifth day, the response peaked. Grass turtle NK cells accounted for 92% of the dead cells. On the 10th day, the immune rejection response was stationary, and 28% of the grass turtle NK cells survived, achieving a non-rejective peacekeeping coexistence state with the receptor Big Dog lymphocytes. When this group of grass turtle NK cells that have undergone the immune rejection test of the receptor Big dog in vitro are imported into the Big dog body with the Big dog lymphocytes, they are no longer regarded as heteroactive antigens by the receptor Big dog immune system and are not attacked by immune rejection. It became an integral part of the lymphocytes in the Big dog, and the experimental plan for the transplantation of xenogeneic NK cells from grass turtles into Big dog has been completed.

During the natural survival period of grass turtle NK cells, we can select the blood at a different time

to prepare the peripheral blood mononuclear cell PBMC of the Big dog. According to the principle of autologous NK cell retransfusion, we can isolate and cultured mixed NK cells of grass turtle and Big dog in vitro, and retransfuse them into the Beagle. Through staining comparison, grass turtle NK cells can also be specially selected from the mixed NK cells of grass turtle and Big dog for isolation and culture in vitro, and returned to the Big dog. Both methods belong to autologous NK cell retransfusion therapy.

The article expounds the clinical significance of establishing an in vitro immune rejection for the receptor, and points out that establishing an in vitro immune rejection for the receptor is a routine test that must be carried out by allogeneic immune cell transplantation and the problem of disparity between the in vivo and in vitro survival environments of immune cells.

Keywords

grass turtle NK cells, Big dog lymphocyte plasma, grass turtle and Big dog mixed lymphocyte plasma, in vitro immune rejection for the receptor, stress adjustment state of autoimmune system, surviving grass turtle cells, size of limited range, acceptable allogeneic immune cell input

1. Introduction Citation

Actually life is an organism in which the human immune cells represented by NK cells coexist with cancerous, diseased and senescent cells within a limited time frame. We live healthy every day without sick, without cancer, and not easily aging, it should be attributed that the NK cells can constantly identify and kill cancerous, diseased and senescent cells in our body,but in this normal process of immune activity, the number and activity of NK cells are gradually consumed. Although we will do everything possible to compensate for the number and activity of NK cells, in the face of the continuous invasion of cancerous, diseased and senescent cells, NK cells are ultimately a failure, once the loss of NK cell killing activity is irreparable, it may lead to a serious illness, a cancer or organ failure, in the end, those Grim Reapers who bring the soul of life to see God can be diseased cells or cancer cells or aging cells. The human immune cells are indeed very weak in maintaining and prolonging human life span. I often wonder whether we should adjust our thinking and maybe find a good way in the animal world.

As we all know, turtles live a long and healthy life. They have neither advanced medical conditions to prevent and treat diseases for them, nor expensive health care drugs for them to preserve their health and prevent aging. They rely on their superior immunity and anti-cancer ability. I often think that if the immune cells of turtles are transplanted into human beings as a new donor, then our natural immunity will become stronger than before. In the process of fighting cancer and preventing aging, our field of cellular immunity has a new magic weapon that can defeat the enemy. However, from the point of view of modern medicine, this is obviously impossible.

First and foremost, between humans and turtles, the differences in the Major Histocompatibility Complex (MHC) may trigger various types of immune rejection reactions in the human body, which

can **destroy** cell transplantation through pathological damage, dysfunction, survival risk and other aspects. At the same time, it will also cause different degrees of damage to the receptor.

How to properly deal with this problem and **realize the transplantation of** immune **cells from different species** is indeed a big challenge.

Let's seek truth from facts in practice and finally turn those seemingly impossible things into reality.

2. Experimental Part

1. Isolation, extraction and purification of grass turtle NK cells (Fuss, I. J., Kanof, M. E., Smith, P. D., & Zola, H., 2009; Zotto, G. D., Antonini, F., Pesce, S., et al., 2020; Traba, J., Waldmann, T. A., & Anton, O. M., 2020; Lal, Kerri, G., et al., 2015; Abel, A. M., Chao, Y., Thakar, M. S., et al., 2018).

1) Materials

Experimental animals: healthy adult grass turtle, weighing about 1,500 grams.

Reagents: PBS buffer, density gradient separator Ficoll, NK cell magnetic bead separation kit, ImunoSepTMBuffer, etc.

Instruments: centrifuge, flow cytometry, pipette, sterile centrifugal tube, etc.

2) Method

Sample collection:

Healthy adult grass turtle is used as a donor. After sterile disinfection of neck skin, 10ml of cervical venous blood is collected and placed in an anticoagulant tube containing sodium heparin.

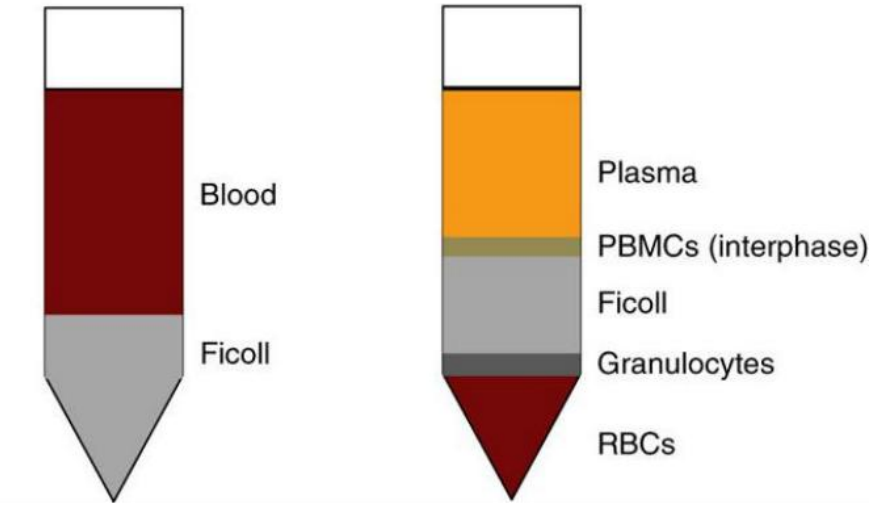
Density gradient centrifugation:

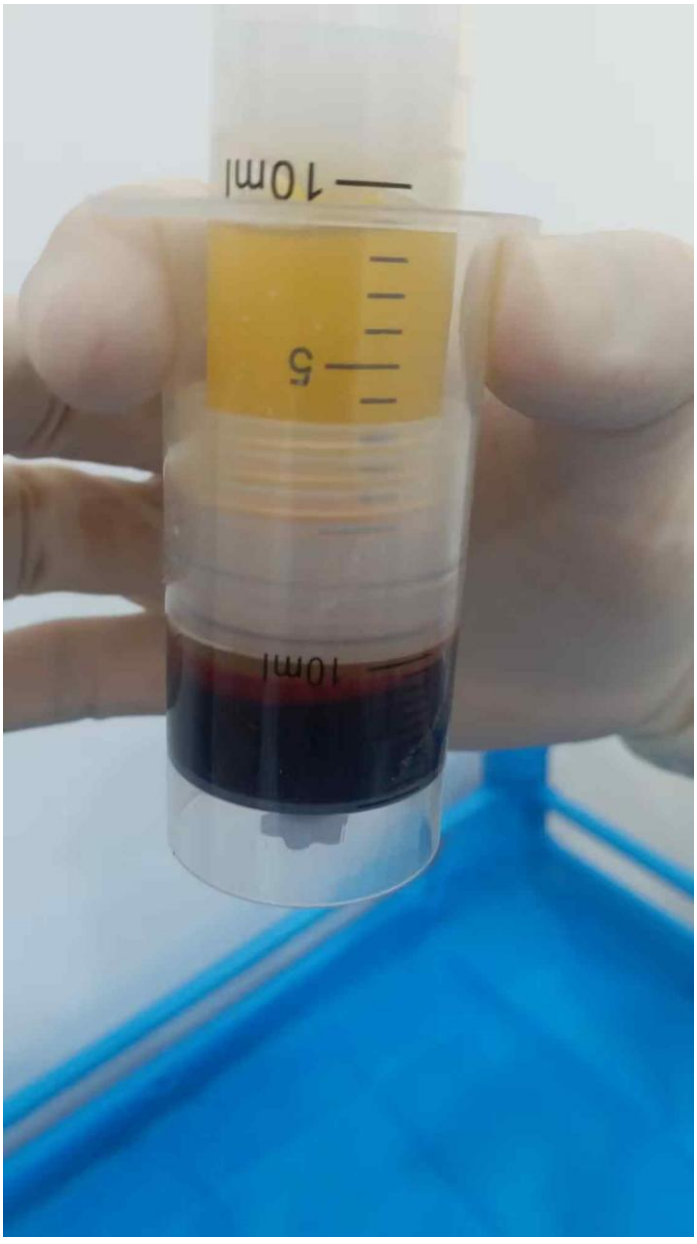
Mix the collected blood samples evenly with equal volume PBS+2% FBS buffer.

Using the Septube density gradient centrifugal tube, carefully add the density gradient separator Fillcoll below the central hole of the partition.

Slowly add diluted blood samples along the wall of the tube and put them above the level of the separator Ficoll to ensure that the blood and Ficoll separator are clearly stratified, and the end-volume ratio of peripheral blood, PBS and Ficoll isolate is 1:1:1.

At room temperature, it is 1200×g centrifugal for 10 minutes. After centrifugation, you can see that the tube is divided into three layers. The upper layer is plasma and PBS, the lower layer is mainly red blood cells and granulocytes, and the middle layer is Ficoll isolate. Between the upper and middle layers, there is a white cloud layer stenosis zone dominated by single nucle cells, that is, peripheral blood mononuclear cells PBMC, including lymphocytes and monocytes. In addition, it also contains platelets. See the figure below.


Blood
Ficoll
Plasma
PBMCs (interphase)
Ficoll
Granulocytes
RBCs


10ml
5
10ml

Collect peripheral blood single nucleus cell PBMC and PBMC cleaning:

Transfer the collected PBMC to the new sterile centrifugal tube, add PBS+2% FBS buffer, mix well and discard the topant with 300×g centrifugal for 8 minutes.

Repeat the cleaning step once to remove the remaining density gradient separator and platelets.

NK cell magnetic bead separation:

Adjust the cell concentration to 1×10^8/mL using ImunoSepTMBuffer to resuspension PBMC.

According to the instruction manual of the NK cell magnetic bead sorting kit, add an appropriate amount of sorting reagents A and B for cell incubation and washing.

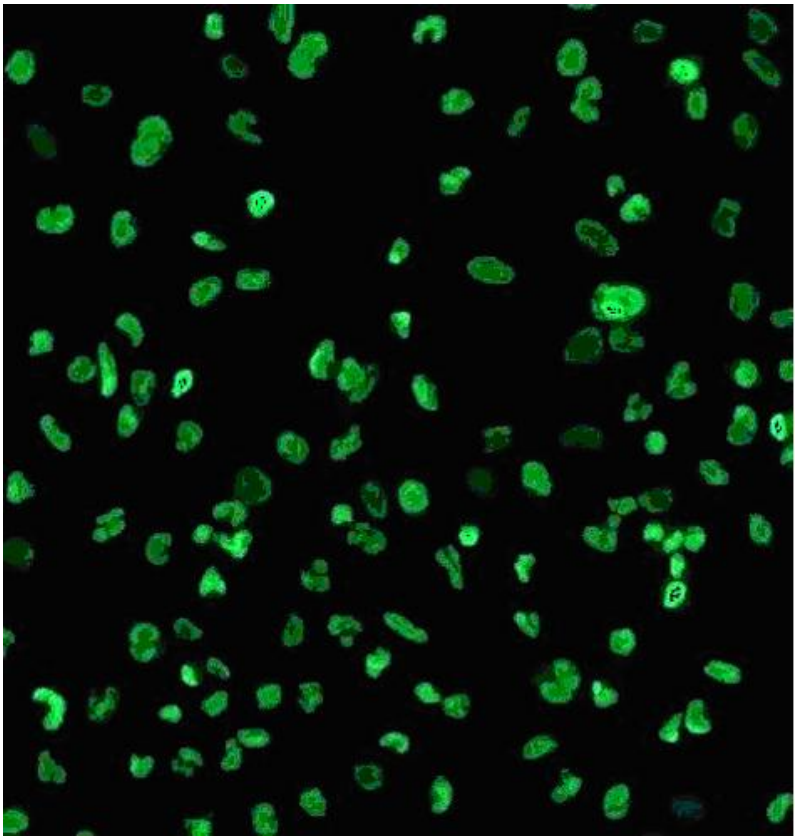
Place the cell suspension in the magnetic pole for 5 minutes to collect unmarked NK cell suspension.

In order to improve cell purity, repeat the magnetic bead separation step once.

Then, remove the collected grass turtle NK cell suspension into the 50ml centrifugal tube, add PBS 10ml, 300g centrifugal for 10 minutes, remove residual magnetic beads and impurities, then add PBS 10ml, clean centrifugal, remove residual magnetic beads and impurities. After repeatedly cleaning 3 times, PBS10ml heavy suspension cells obtain 15ml purified grass turtle NK cell suspension, spread it into the T75 culture bottle, mark it, and put it into the incubator.

Cell count and purity detection:

Fluorescent anti-CD45 antibodies were added (Kirschenbaum, D., Xie, K., Ingelfinger, F., et al., 2023), and the purified grass turtle NK cells were counted and purity tested by flow cytometry. The results show that the purified grass turtle NK cells have reached more than 90%, and the number of cells is about 1×10^6/ml. The cell morphology is smaller than that of human NK cells, semi-suspension, mostly attached walls, survival rate 95%, normal activity. Of course, the final cell activity identification still depends on the ability to attack the target cells when jointly cultured with tumor cells in the future. See the figure below.



2. Lymphoplasmapheresis in vitro (LPE) (Ke Shipeng, Li Xu, Yu Ling, et al., 2022)

25 kg male Beagle is a receptor, with regular physical examination and good health. Flat-lying operating table, fixed limbs, Shutai 0.1-0.15ml per kilogram of body weight, small saphenous intravenous injection on the outer side of the hind limbs, and the anesthesia process is smooth. Do a good job in electrocardiogram monitoring. Select the medial inferior head vein of the forelimbs as the blood collection site, local disinfection, and subcutaneous venous puncture. Connect the inlet pipeline of the extracorporeal blood cell separator and collect 200 ml of whole blood, enter the lymphocyte plasma replacement procedure on the blood cell separator, extract plasma components and lymphocyte components, a total of 115 milliliters. Leave 10 ml of plasma for storage, then divide it into 3 bags with 35 ml each, put them into sterile blood collection bags with heparin sodium anticoagulant, and store them in a blood storage refrigerator at 4±2℃. Open the return channel, return red blood cells and other blood components to the body of the Beagle dog, and input 100 ml of protein solution.

After the anesthesia is relieved, the Beagle woke up after 10 minutes and can stand.

The whole experiment took 1 hour and 40 minutes.

Below is a sample of Beagle's lymphocyte plasma before the immune rejection, it was a clear, transparent and light yellow liquid.

3. Establish an in vitro immune rejection for the receptor (Zhang Hua, Li Ming, & Wang Lihua, 2015)

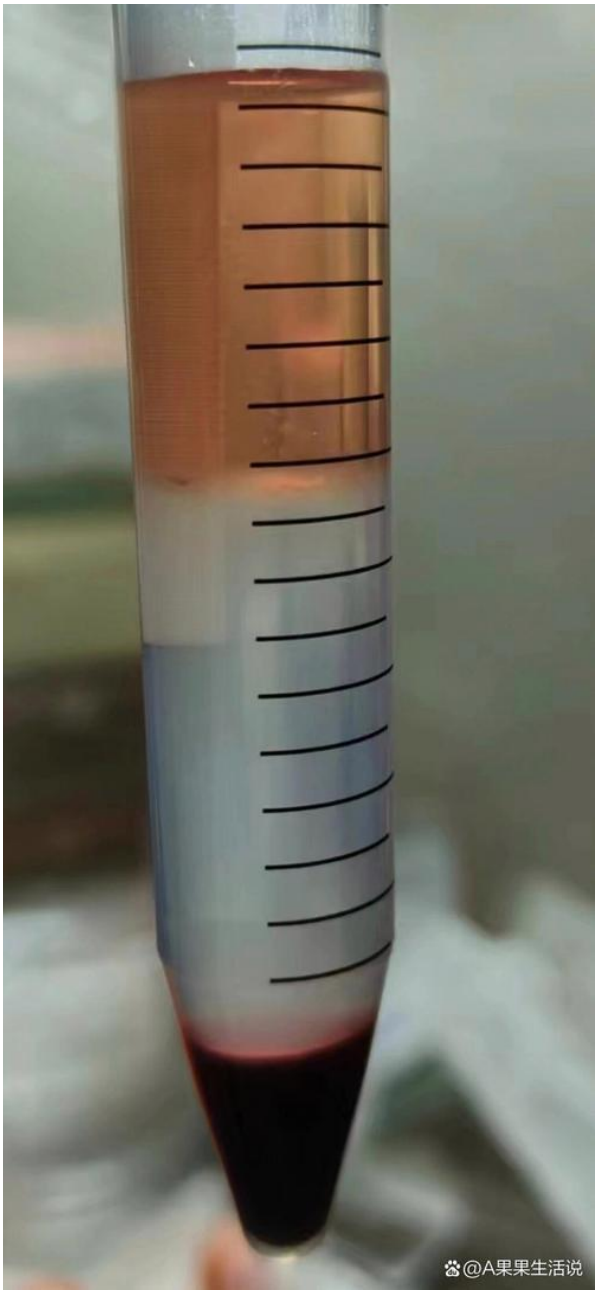
1) Take out the 35ml lymphocyte plasma blood collection bag of Beagle dog from the blood storage refrigerator, put it on the experimental operating table that has been sterile and disinfected in advance, reheat it for 1 hour, rotate the opening of the blood bag, add 0.9% of the same volume of 35ml to dilute it, slowly inject the 15ml grass turtle NK cell suspension into the blood bag, and carefully knead the mixed liquid. Put the blood collection bag flat into the 37C, 5% CO2 incubator, and observe and record the color change and liquid temperature of the mixed lymphocyte plasma every 12 hours.

2) On the 2nd day, 48h after mixing, there is no significant change in the plasma color of mixed lymphocytes, and the liquid temperature is 37C

3) On the 3rd day, 60h after mixing, the mixed lymphocyte plasma is turbid, opaque, and the liquid temperature is 37.8C. These signs indicate that the immune rejection reaction in the mixed lymphocyte plasma has already begun, as shown in the figure below.

4) Centrifugal separation of Percoll isolate to clean up dead cells

A. Take two 50 ml centrifuge tubes, inject 10 ml of Percoll separate, 30-degree inclined centrifuge tubes, and then suck 35 ml of mixed lymphocyte plasma with a 50 ml syringe thick needle and slowly inject it into the tube along the wall of the centrifuge tube. Pay attention to control the force to avoid blood rushing into the Percoll separate liquid level. Put the centrifuge tube into the low-speed centrifuge and centrifuge for 400g for 20 minutes. The liquid in the centrifuge tube is divided into four layers from top to bottom: dead cells and platelets, monocytes, lymphocytes, granulocytes and red blood cells, both the number of monocytes and red blood cells are low. as shown in the figure below.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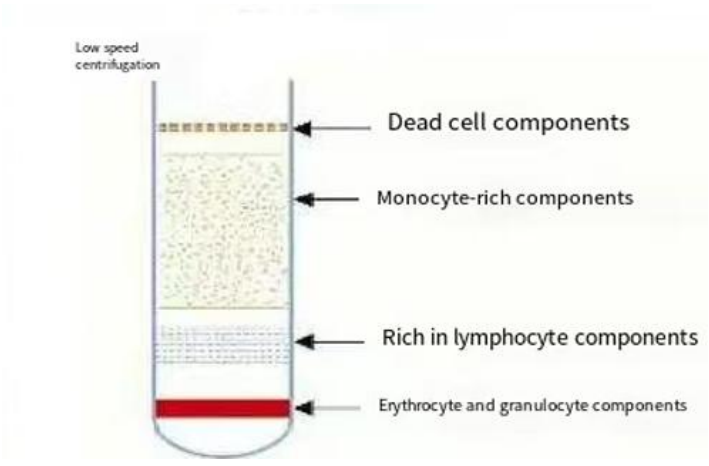

Low speed centrifugation
Dead cell components
Monocyte-rich components
Rich in lymphocyte components
Erythrocyte and granulocyte components

Use a straw to remove the dead cells suspended in the top layer of the centrifuge tube with a floc-like pale yellow bubble. During operation, it is better to suck away a little more monocyte under the dead cell layer than to clean up the dead cells without leaving any room.

B. The centrifuge tube is tilted 30 degrees, the pipette gun passes through the monocyte layer, carefully absorb the middle-layer lymphocyte mixture and injects it into a sterile 50ml centrifuge tube, add 10 ml of PBS cleaning solution, add an appropriate amount of penicillin + streptoxin + butimicarnaxin, mix the cells. Centrifuge at 300 rpm for 20 minutes, discard the supernatant, then repeat the above steps for three times, then discard the supernatant, and let it stand on the workbench.

C. Remove a new bag of 35ml of Beagle lymphocyte plasma from the blood storage refrigerator, warm it for 1 hour, dilute it with 0.9% physiological saline, etc. After thoroughly cleaning the above 3 times, carefully absorb the mixed lymphocyte fluid with a thick needle in a large syringe and move it into the new blood bag.

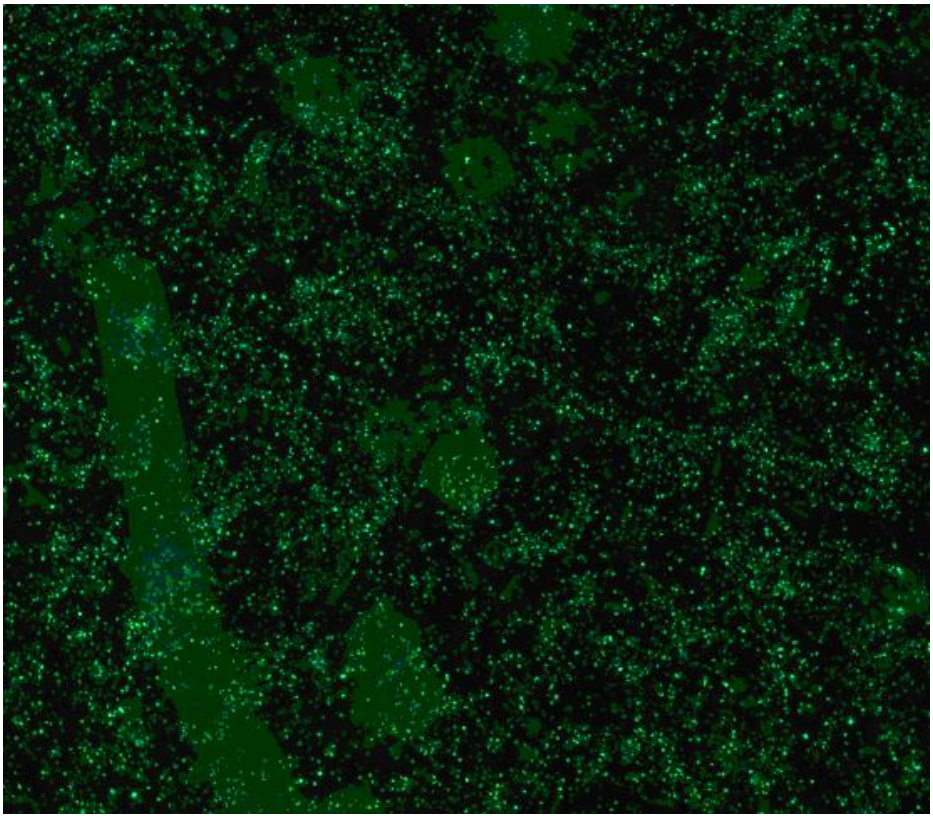
D. Repeat the above operation, clean the dead cells with another 50ml mixed lymphatic liquid, thoroughly clean them 3 times, carefully suck them with a large needle, move them into the same new blood bag, mark them, and put them flatly into the ingrators. Observe and record the color changes and liquid temperature of the mixed lymphocyte plasma regularly every 12 hours.

Dead cells Taipanlan stain count, grass turtle NK cells account for 76%

5). For the next two consecutive days, every day, the mixed lymphocyte liquid in the blood bag is divided into two 50 ml centrifugal tubes. According to the above steps, the dead cells in the upper layer are cleaned by centrifugal separation with Percoll separation liquid.

Liquid temperature, Day 4, 38c, Day 5, 38.2c; Daily dead cells Taipanlan stain count, grass turtle NK cell ratio, Day 4, 83%, Day 5, 92%.

6). On the 6th day, 144h after mixing, after cleaning up the dead cells, as mentioned above, the pipette gun passed through the monocyte layer, absorbed the mixed lymphocyte fluid, moved it into a new 50ml centrifuge tube, injected fluorescent CD45 antibodies, and detected by flow cytometry. Due to excessive cell death, the fluorescent control of grass turtle NK cells appears as scattered points in the image below.



It can be faintly seen of scattered grass turtle NK cells with complete cell membranes, full cell content, and nucleus. Repeat three times, wash with 10ml PBS plus an appropriate amount of penicillin + streptoxin + butylamine kinaxin. After centrifugation, use a large syringe thick needle to transfer this mixed lymphocyte fluid into a new bag of 35 ml of Beagle's lymphocyte plasma after warming, add 35ml of 0.9% saline to dilute, mark it, put it flat in the incubator, observe and record the color changes and liquid temperature of mixed lymphocyte plasma regularly every 12 hours.

Dead cells Taipanlan stain count, grass turtle NK cells account for 65%.

4. The immune rejection response in vitro began to weaken.

1) On the 7th day, after mixing for 168h, the plasma color of the mixed lymphocytes turns clear, small turbid, and the temperature is 37.3C. The sample was put into a 50 ml centrifuge tube for low-speed centrifugation and 300g for 20 minutes. The inner layer of the centrifugal is clear. The upper layer has a small amount of suspended flocculation light yellow foam dead cells, and the width of the lymphocyte layer is significantly reduced. Do the cleaning of dead cells

Dead cells Taipanlan stain count, grass turtle NK cells account for 43%

2) On the 8th day, after cleaning up the dead cells, take out another bag of 35ml of Beg dog lymphocyte plasma from the blood storage refrigerator, warm up, tilt the centrifugal tube 30 degrees, absorb the lymphocyte layer in the tube with a pipette gun, carefully transfer it into the new blood bag, dilute 35ml 0.9% saline, and put it flat into the incubator.

Dead cells Taipanlan stain count, grass turtle NK cells account for 23%

3) On the 9th day, the plasma color of mixed lymphocytes is clearer and the liquid temperature is normal. Take two 50ml centrifugal tubes. If operated above, first inject 10ml Percoll isolate, then tilt the centrifugal tube 30 degrees, absorb mixed lymphocyte plasma with a straw, carefully lay on the Percoll isolate, low-speed centrifugal, 300g centrifugal for 20 minutes, the inner layer of the centrifugal tube is clear, and there is only a small amount of remaining dead cell flocculation foam at the top layer, which can be absorbed.

Dead cells Taipanlan stain count, grass turtle NK cells account for 5%

5. Natural repair of grass turtle NK cells without any growth factors

1) On the 10th day, the plasma color of the mixed lymphocytes in the centrifugal tube completely returned to the clear and transparent color seen before immune rejection. The liquid temperature was normal, indicating that the immune rejection response in the centrifugal was completely stationary. Please take a look at the sample picture below. Compared with the lymphocyte plasma color before the immune rejection, the transparency of the liquid is slightly less transparent, but not turbid.

Inject 10ml of PBS into the centrifugal tube, add an appropriate amount of penicillin + streptomycin + butamine kanamycin heavy suspension cells, mix well, 300 to centrifugal for 10 minutes, discard, repeat the above steps, repeatedly clean the centrifugal three times, after discarding the supernatant, carefully spread the liquid on the mixed lymphocyte layer in the centrifuge tube into the T175 culture bottle with a straw., add KPMI 1640 basic medium 25ml, 5% Big dog plasma, 1% double-hang P/S, and rest culture in the incubator.

Sampling, staining, flow cytometry detection, and the rejection reaction is completely static, in the mixed lymphocyte fluid, grass turtle NK cells accounted for 40%, and Beagle dog NK cells accounted for 60%. The survival rate of grass turtle NK cells is 28%, and the cell activity is acceptable, but it is not as good as before the reaction. Please refer to the table below:

Survival rate and activity of grass turtle NK cells before and after immune rejection in vitro

Experimental stage	Survival rate	Activity (natural killing rate)
Before the immune rejection in vitro	>95%	47.6%-76.8%
After the immune rejection in vitro	28%	53%

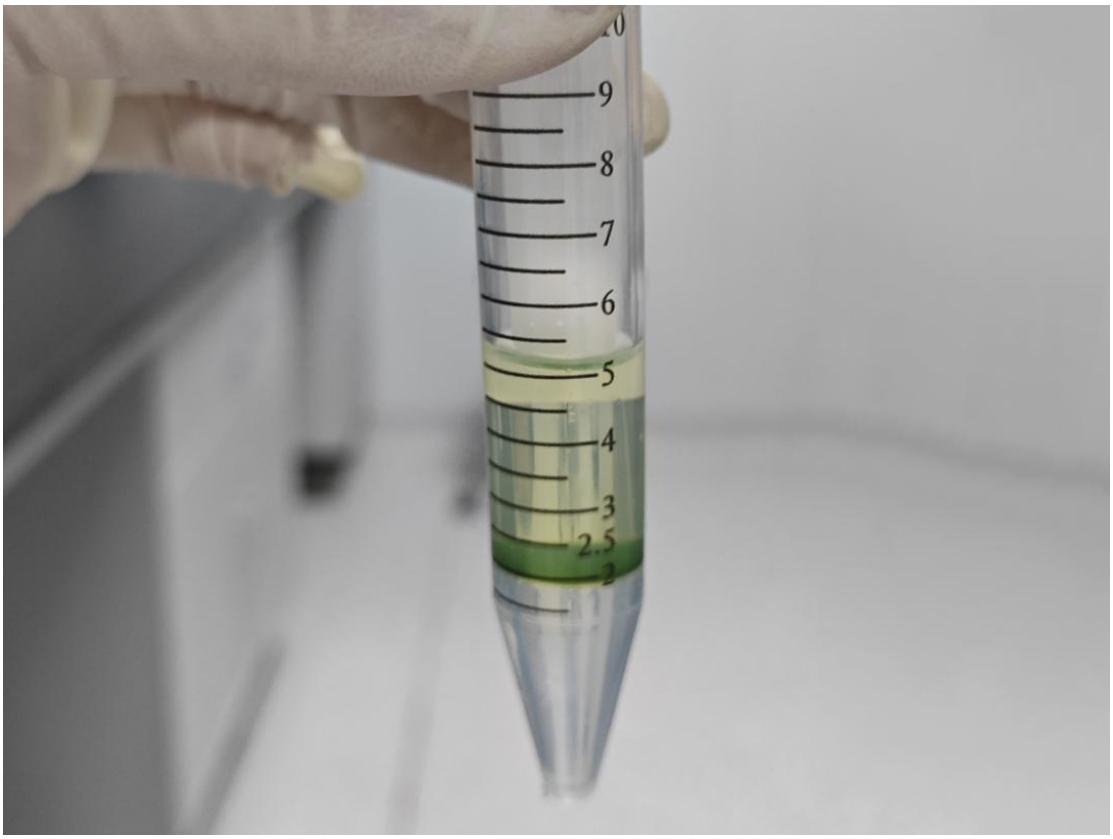
2) On the 12th day, add KPMI 1640 basic medium 25ml, 5% Big Dog plasma, 1% Shuanghang P/S, continue to cultivate

3) On the 14th day, add KPMI 1640 basic medium 25ml, 5% Big Dog plasma, 1% Shuanghang P/S, continue to cultivate

Sampling, flow cytometry detection, grass turtle NK cells account for 48% of mixed lymphocytes, and (Big dog NK cells account for 52%) ,cell activity is still 53%..

Add KPMI 1640 basic medium 25ml, 5% Big Dog plasma, 1% Shuanghang P/S, continue to cultivate

Please see the figure below. This is the 14th day, a sample of mixed lymphocyte fluid. The surviving grass turtle NK cells reached a consensus with the receptor Beagle immune cells to maintain a state of peacekeeping and coexistence that does not exclude each other.



4) On the 15th day, about 85 ml of the mixture of surviving grass turtle NK cells coexisting with Beagle lymphocytes was reinfused into beagle dog intravenously, and the process went smoothly.

5) For 3 consecutive days, 0.9% saline 100ml + ceftriaxone sodium 1.0g static droplet. One week later, the Big dog has a physical examination, and the indicators are normal.

3. Analysis and Discussion

1. The clinical significance of establishing an in vitro immune rejection for the receptor (Sun Wensheng, & Wang Fuqing, 2004; Zhang Qiang, Liu Xiaohong, & Zhao Min, 2016; Wang Jun, Li Li, & Zhang Lijun, 2017; Sun Lifei, 2009)

1) Immune rejection is a process in which the body destroys the graft (allogenic cell, tissue or organ) through specific immune response. Generally speaking, after transplantation, the recipient can identify the graft antigen and produce a response, and the immune cells in the graft can also identify the recipient's antigen tissue and produce a response.

There are two basic types of immune rejection: hostversusgraftreaction (HVGR) and graftversushostreaction (GVHR), and the former is the most common one in clinic. According to the mechanism, time, speed and clinical manifestations of occurrence, HVGR can be divided into 3 types.

A. Ultra-acute rejection occurs within minutes to a few hours after the graft enters the host. It occurs quickly, strongly and irreversibly. The basic reason for the occurrence is that there are anti-donor HLA antibodies in the circulation of the recipient's body, which is common in the following situations: 1.

ABO blood type does not match; 2. the receptor has multiple pregnancies, repeated blood transfusions, past history such as cells, tissue or organ transplantation; 3. improper preservation or handling of grafts. If ABO blood type Rh blood type examination and cross-test are carefully carried out in advance, or seriously inquire about the patient's past history, this phenomenon can be avoided.

B Acute rejection is the most common type, mostly occurring a few days after transplantation, or within a few weeks and a year. The early or late occurrence of acute rejection and the severity of the reaction are directly related to the degree of HLA matching between donor and receptor. The higher the matching degree, the later the reaction will occur and the symptoms will be mild. In some cases, symptoms may even appear 2 years after transplantation. Acute rejection can be alleviated by timely and appropriate immunosuppressive treatment.

C. Chronic rejection is a delayed allergic reaction, which occurs months or even years after transplantation. Although the progress is slow, there is no obvious clinical effect of immunosuppressive treatment.

D. Graft-to-host reaction mostly occurs in the same kind of bone marrow transplantation and is also seen in spleen, thymus and small intestine transplantation. At this time, the patient's immune state is low, and the rich immune active cells in the graft treat the recipient's immune cells as non-self-antigens and have an immune response.; the graft's T cells proliferate in the recipient's lymphatic tissue and produce a series of damaging effects. GVHR is divided into acute and chronic types. The acute type is common, mostly occurring within 3 months after transplantation. Although it is a reversible change, the mortality rate is high; the chronic type is transferred from the acute type, and patients have severe immune disorders, often dying from severe infection or malignant fluid.

It can be seen that no matter what type of immune rejection is in clinic, it should occur in the recipient. All adverse blows and various degrees of tissue damage caused by immune rejection are unilaterally borne by the receptor. From the conventional logic point of view, this is a natural thing. The immune rejection should have occurred in the receptor. If you want to transfer it to any place, it is unrealistic. The establishment of the in vitro immune rejection of the recipient broke the framework of traditional understanding, and replaced the immune rejections that should have occurred inside the recipient's body with those outside the recipient body as expected, completely avoiding the possible harm caused by the immune rejection to the receptor.

2) Of course, before cell transplantation, we will carefully match the HLA between the donor and the receptor, and also use immunosuppressants, but in many cases, the immune rejection reaction still occurs. Are these immune rejection ultra-acute or acute? in the process of immune rejection, what reversible or irreversible pathological changes have occurred in the tissues of the receptor, and what targeted measures should be taken to avoid or reduce the adverse effects of immune rejection on cell transplantation; After the allogeneic immune cells are implanted in the receptor, the rejection reaction experienced together by the immune cells on both the donor and the receptor, from occurrence, development to stationary, what is the status of the whole clinical evolution process? What is the final

survival rate of allogeneic immune cells after being hit by the immune rejection of recipient, whether they are wiped out, or there are still a small number of survivors, and their cell activity How ? such problems cannot be seen by clinicians with the naked eye. The establishment of the in vitro immune rejection response for the receptor has replaced the immune rejection response that should have occurred inside the recipient's body with those outside the recipient body, allowing us to see for the first time the whole clinical process of immune cell rejection in front of us.

From the moment when the NK cells of the grass turtle were injected into the lymphocyte plasma of the beagle, after 60h, the plasma in the centrifuge tube was cloudy and not transparent, and the immune rejection reaction officially began. According to the definition of clinical immune rejection classification, the rejection reaction occurred on the third day after the immune cells of both the donor and the receptor were mixed. It can be seen that this immune rejection is a kind of acute rejection that is often seen in clinical practice. It is not the kind of superacute rejection that occurs quickly in a few minutes and a few hours and is extremely dangerous. The starting point of acute immune rejection should be the cellular immunity mediated by T cell toxicity of the recipient Beagle, not the anti-MHC antibody accumulated in the receptor Beagle. According to the routine operation, regularly observe and record the color changes and liquid temperature of mixed lymphocyte plasma every 12h. Regular centrifugal separation every day, cleaning up the dead cells suspended in the top of the centrifuge tube, Taipanlan staining count, and the proportion of grass turtle NK cells in the dead cells. Every 2 days, the lymphocyte plasma is changed to maintain the stability of the internal environment of immune rejection in vitro. On the 5th day, in the high peak period of the reaction, the plasma of mixed lymphocytes was grayish yellow, and the liquid temperature was 38.2c. The dead cells in the top layer of the centrifuge tube were cleaned with flocculent pale yellow blisters, and the proportion of grass turtle NK cells was as high as 92%. On the 7th day, the immune rejection reaction began to weaken, the color of the liquid in the centrifugal tube turns clear and slightly turbid. the liquid temperature dropped to 37.3 degrees, the dead cells were cleaned up, Taipanlan staining count, and the proportion of grass turtle NK cells accounted for 43%. After 2 days, on the 9th day, the color of the mixed liquid was clear and the temperature was normal. Clean up dead cells, Taipanlan staining count, grass turtle NK cells account for 5%. On the 10th day, the color of the mixed lymphocyte plasma in the centrifuge tube

completely recovered to the clear and transparent color seen before the rejection, and the liquid temperature was normal, indicating that the immune rejection reaction in the centrifuge tube was completely at rest. Centrifugation, the flocculation foam of dead cells cannot be seen at all in the top layer of the centrifuge tube. The lower monocyte layer and lymphocyte layer have clear boundaries, but the thickness of the lymphocyte layer is obviously narrowed. The flow cytometry detected that 28% of the grass turtle NK cells survived. After a few days of natural repair, this batch of surviving grass turtle NK cells, together with the lymphocytes of the receptor Beagle, were safely entered into the body of the Beagle.

3) Important revelations of experimental results

(1) Without any drug intervention, the immune rejection itself has a clinical process of natural occurrence, natural development and natural cessation. In this clinical process, with the occurrence and development of rejection, the mortality rate of grass turtle NK cells has soared. In the most intense stage of rejection, the daily death rate accounts for up to 92% of the dead cells. With the natural calming of the rejection reaction, the number of deaths of grass turtle NK cells gradually decreased. Finally, on the 10th day after the grass turtle NK cells were mixed with the receptor Beagle lymphocyte plasma, the immune rejection reaction naturally stopped, and 28% of the grass turtle NK cells survived and reached a consensus with the receptor Beagle immune cells to maintain a state of peacekeeping and coexistence that does not exclude each other. When this batch of grass turtle NK cells that have been tested for immune rejection of receptor Big dog in vitro, together with Beagle lymphocytes injected into the body of Big Dog, are no longer regarded as heterogeneous antigens by the receptor immune system and are not attacked by immune rejection. Analyzing, it may be related to the following factors:

A. In humans, the basic cause of super-acute rejection is mostly due to the inconsistency of ABO blood type between the donor and receptor, or the recipient has had multiple pregnancies, repeated blood transfusions, and a history of cell, tissue or organ transplantation, and anti-donor HLA antibodies are pre-preserved in the recipient's body. The early and late occurrence of acute rejection and the severity of the reaction degree are related to the degree of HLA matching between the donor and receptor. Animals may be the same as humans. The higher the matching of the **Major Histocompatibility Complex** (MHC) between the donor and receptor, the lower the incidence and response of transplant rejection. Perhaps, the Beagle we randomly selected has not participated in too many blood transfusions, dialysis and cell transplantation in past animal experiments, and there are not a large number of anti-MHC antibodies in its internal circulation. Therefore, when the surviving grass turtle NK cells enter the body of this Beagle, there is no super-acute immune rejection. In addition, this randomly selected Beagle may have a high degree of matching with the MHC of the grass turtle, the rejection reaction is later, the symptoms are mild, and even immune rejection is not felt (Sun Wensheng, & Wang Fuqing, 2004).

B. In the process of competition with Beagle immune cells in vitro, grass turtle NK cells may show extraordinary anti-immune rejection ability and amazing self-immune defense skills. After a large number of cell deaths on both sides of the donor and receptor, they finally reached a state of peacekeeping and coexistence that does not exclude each other, and Both the donor and the receptor have an immune tolerance (Bluestone, J. A., & Anderson, M., 2020).

C. In the process of resting and repairing in vitro, the specific markers on the surface of the surviving grass turtle NK cells may have changed, similar to the cell surface antigen modified by genetic engineering technology, which has "concealment" and can escape the attack of the receptor immune system (Liu Ying, Zhang Ruijun, Wu Zhiwei, et al., 2006).

(2) When the in vitro immune rejection has just stopped, the surviving grass turtle NK cells accounted for 40% of the mixed lymphocyte fluid, and the Beagle NK cells accounted for 60%. After that, no cell growth factors were added, and no special nutrients were added. The conventional KPMI 1640 basic culture medium was added with 5% Beagle dog autologous plasma. After a few days of recuperation and repair, the proportion of this surviving grass turtle NK cells in mixed lymphocytes unexpectedly climbed to 48%, while the proportion of Beagle NK cells dropped to 52%, suggesting that the vitality of the grass turtle is really strong (Zhou Weiguan, Qin Guosen, Li Yihuan, et al., 2007; Shi, H., & Parham, J. F., 2001; Guo, R., Ma, G., Zhai, X., et al., 2022; He Shengjie, Mao Xinliang, & Zhang Xuewu., 2014; Liu Feng, Qiu Ruixia, Shan Kai, & Shi Qiangui., 2016).

(3) So far in the experiment, we have transformed heterogeneous grass turtle NK cells into a component of lymphocytes in Beagle body, and the experimental plan of grafting heterogeneous grass turtle NK cells into the body of beagle dog has been completed. During the natural survival period of grass turtle NK cells, we can select the time to draw blood and prepare the peripheral blood mononuclear cell PBMC of Beagle. According to the principle of autologous NK cell retransfusion, mixed NK cells from grass turtles and beagle dog were isolated and cultured in vitro, and then reinfused into beagle dog. It can also be compared through staining, from the NK cells of a mixture of grass turtle and Beagle, specially selected grass turtle NK cells for isolation and culture in vitro, and re-infusion into the body of Beagle. Both methods belong to autologous NK cell retransfusion therapy (Tang, X., et al., 2022; Nickolas, C., Toai, T. N., & Rafael, G., 2022; Yue, Y., Zi, M., Feng, J., Wang, W., Ren, Z., Wu, C., & Yang, Z., 2024; Klingemann, H., 2023).

2. Establishing an in vitro immune rejection response for the receptor is a routine test that must be carried out for allogeneic immune cell transplantation

1) Working principle of the blood cell separator

The blood cell separator guides part of the whole blood of the blood donor for extracorporeal circulation through a special closed pipeline. Using the difference of specific gravity of blood components in whole blood, the separator separates and layers the blood components under the action of centrifugal force. Subsequently, the computer-controlled pipeline system directs different blood components to their own dedicated channels, so as to collect the blood cells to be collected into the product bag, and at the same time, return the unwanted components to the blood donors (Wen Baiping., 2007).

2) The working principle of blood cell separator components is the theoretical basis for establishing the receptor's immune rejection in vitro. At the beginning of this experiment, after we successfully isolated and extracted the NK cells of grass turtle, we applied a blood cell separator to perform a Lymphoplasmapheresis (LPE) in vitro on the receptor Beagle dog. The 200ml static blood of the Big dog was introduced out of the body, entered the blood cell separator, and extracted the plasma and lymphocyte components of the Big Dog, a total of 115ml (Liu Jinghan, & Wang Deqing, 2011). Among them, plasma component may contain a certain amount of anti-MHC antibodies that are enough to

cause ultra-acute immune rejection；In lymphocyte component, T cells can play a leading role in acute rejection, and B cells can participate in chronic rejection through humoral immunity. All these lymphocyte and plasma components closely related to ultra-acute, acute, chronic immune rejection are replaced to outside the receptor's body at the beginning of the experiment, providing a blood environment in vitro which is the same as the biochemical environment of the peripheral blood of the recipient Beagle dog for establishing the in vitro immune rejection of the recipient. resulting in a temporary stress adjustment state of Beagle's own immune system, reducing the ability of immune rejection against foreign antigens, and passively receiving a certain limit of allogeneic immune cell input. The surviving grass turtle NK cells were selected at this time node to safely enter the Big Dog. On this basis, we accidentally found that the time node of the autoimmune system stress adjustment that the receptor may occur during the immune rejection is the best time for allogeneic immune cells to enter the receptor. As for the size of the limit range of the input quantity, it should first be related to the degree of MHC matching between the donor and the receptor. The higher the degree of matching, the lower the strength of rejection, and the limit range of receptor limiting the input of allogeneic immune cells will also be relatively relaxed. Secondly, it is related to how much share of the receptor's lymphocyte plasma is replaced in vitro, causing the receptor autoimmune system to be under stress adjustment, reducing the ability to reject foreign antigens. In addition, the differences in the models of donor immune cells also have a certain impact on the strength of the rejection response. For example, when CAR-T cells are imported into the receptor as an allogeneic antigen, In addition to the immune rejection of the host against the graft, the CAR-T cell itself, as a graft, will also treat the immune cells of the receptor as allogeneic antigen and have an immune response. The double rejection response makes the limited range of receptor limiting the input of CAR-T cells much smaller than that of other types of immune cells. In the end, as a result of the in vitro immune rejection response, 28% of grass turtle NK cells survived and achieved a non-rejective peacekeeping coexistence with receptor immune cells. When the surviving grass turtle NK cells, which have been tested for immune rejection by the recipient Beagle in vitro, are injected into the body of the Beagle together with the immune cells of the recipient Beagle, they are no longer regarded as foreign antigens by the recipient's immune system and are not attacked by immune rejection. This batch of surviving grass turtle NK cells should be the allogeneic immune cells within a limited range that the recipient Beagle dog can passively accept at the time node of stress adjustment of the autoimmune system. In the future, all allogeneic immune cells, whether heterogeneous or homologous, such as NK cells, CAR-NK cells and CAR-T cells (Li, X., Li, W., Xu, L., & Song, Y., 2024; Teng, F. et al., 2024; Karlo Perica et al., 2025), before deciding to import these cells into the selected recipient, the samples should be taken without exception, just like the NK cells of grass turtle, accept the test of the receptor's immune rejection in vitro, identify three questions 1) Clinical classification of immune rejection reaction. 2) The final survival rate and cell activity of allogeneic immune cells after being hit by immune rejection in vitro. Based on the above-mentioned test results, the clinician decides whether to apply this type of immune cell retransfusion therapy to the

patient. 3) If you agree to implement this retransfusion therapy, then the number of allogeneic immune cells that survived after experiencing an immune rejection reaction in vitro should be the input of allogeneic immune cells within a limited range that the recipient can passively accept at the time node of stress adjustment of the autoimmune system. Then refer to the total amount of allogeneic immune cells prepared for transmission, and on the budget, how many milliliters of lymphocyte plasma should be prepared by the receptor to replace it outside the body at the cost. In field operations, we can realistically adjust the total replacement amount of lymphocyte plasma and the allogeneic immune cell input according to the actual situation of different receptors. For those weak receptors, bearing in mind the principle that deficiency of traditional Chinese medicine is not supplemented, it is planned to be injected in small quantities and in batches.

3. The problem of disparity between the in vivo and in vitro survival environments of immune cells

If we change the experimental plan, after the in vitro immune rejection is stationary, the surviving grass turtle NK cells will not be injected back into the Beagle, but adopt the culture and amplification in vitro, then, this kind of grass turtle NK cells cultured and amplified in vitro are still heterogeneic immune cells for receptor Big dog. Whether it is allogeneic immune cells or autologous immune cells, after being cultured and amplified in vitro, they leave the simple laboratory medium environment they are already familiar with, enter the receptor body, and come into contact with strange and complex biochemical environments in vivo, the huge drop in the living environment will inevitably directly affect the survival rate and cell activity of cells. As with immune rejection, the problem of disparity between the in vivo and in vitro survival environments of immune cells are all important topics that must be studied in depth in immune cell therapy today.

In the 1984, Professor Chambers et al in the University of London elaborated first of all a method of culture of isolated osteoclasts in vitro. When Chambers' masterpiece just came out, I happened to be studying in Europe, and I was lucky enough to learn this classic technology. The basic point of this technology is that cells are completely free from the complex environmental factors in the body. Cell characteristics are independently and helplessly displayed in a simple medium environment in vitro, which is more accurate and reliable than finding cell characteristics in a complex environment in the body, thus ending the long-standing academic dispute over the search for cell characteristics in vivo (Chambers, T. J. et al., 1984).

Today, the technology of NK cells being isolated, cultured and expanded in vitro, and returned to the body is generally on the rise. This NK cell immunotherapy for cancer prevention and treatment is definitely a promising attempt. More and more cutting-edge NK cell technologies and innovative theories bring new vitality to immune cell therapy (Abel, A. M., Yang, C., Thakar, M. S., & Malarkannan, S., 2018; Cooper, M. A., Fehniger, T. A., & Caligiuri, M. A., 2001; Crinier, A., Narni-Mancinelli, E., Ugolini, S., & Vivier, E., 2020; Huntington, N. D., Vosshenrich, C. A., & Di Santo, J. P., 2007; Loza, M. J., & Perussia, B., 2004; Scoville, S. D., Mundy-Bosse, B. L., Zhang, M. H., et al., 2016; Björkström, N. K., Ljunggren, H. G., & Michaëlsson, J., 2016; Quatrini, L., Della

Chiesa, M., Sivori, S., Mingari, M. C., Pende, D., & Moretta, L., 2021; Wu, S. Y., Fu, T., Jiang, Y. Z., & Shao, Z. M., 2020).

However, after NK cells are retransferred into the body, the cell survival environment becomes complicated compared to a simple culture medium in vitro, and its complexity varies from person to person, is constantly changing, as Chambers's work shows, the properties of the cells are much more diversified than those seen in a simple culture medium in vitro and survival is also an issue. Today, if we must remove NK cells from a calm and simple medium in vitro and return them back to the original complex and changing internal environment, what method can ensure that the survival and activity of NK cells will not be affected after entering the body?

Well, what we can do today is to try to create a basic culture medium in vitro that is the same as the biochemical environment of the peripheral blood in the receptor Beagle, so that the living environment of grass turtle NK cells cultured and amplified in vitro is basically not much different from the biochemical environment they encounter after entering the Beagle dog's body, so as to ensure the survival rate and activity of the cells. In today's advanced science and technology, it is not difficult to have AI technical support. The problem is that everyone has not fully understood the problem of disparity between the in vivo and in vitro survival environments of immune cells.

In clinical practice, the rapid acquisition and effectiveness of allogeneic NK cells are particularly important in the treatment of patients with impaired immune system or tumors. In order to reduce immune rejection, a healthy donor with a high degree of HLA matching with the patient is usually selected, and immune control agents are used before immunotherapy to reduce the possibility of immune rejection and the intensity of rejection of receptors, and then a qualified healthy donor NK cell is selected from the pre-prepared finished product to retransfer the patient. Healthy donor NK cells, the laboratory environment of culture and amplification in vitro is usually the basic medium of NK cells, plus cell growth factor, plus fetal bovine serum. Once they leave the culture medium environment of a unified pattern in vitro and come into contact with various complex and changing in vivo environments of different receptors, the huge difference in the survival environment will indeed affect the survival rate and cell activity of NK cells in the receptor body.

It is not difficult to create a basic culture medium in vitro for the grass turtle NK cell that is the same as the peripheral blood biochemical environment of the receptor Big Dog. But if we want to create a basic culture medium in vitro for every selected donor NK cell, which is the same as the peripheral blood biochemical environment of the receptor, it really needs to increase a lot of working hours and patience without fear of trouble. However, we must do this.

Like all allogeneic immune cells, as a new donor, the cultured and amplified grass turtle NK cells in vitro must collect samples, start the LPE technology again, and accept the test of immune rejection of the receptor Beagle dog in vitro before being imported into the Beagle body, determine the clinical classification of immune rejection response and the final survival rate and cell activity of grass NK turtle cells after being hit by immune rejection in vitro. Based on the above test results, a decision was

made whether to implement this type of grass turtle NK cell retransfusion plan for the Beagle Dog. If you agree, well, the number of new donor grass turtle NK cells that survived after immune rejection test in vitro is a passively acceptable input of allogeneic immune cells redefined by the receptor Big Dog at the time node of a new round of autoimmune system stress adjustment. In field operations, similarly, the total replacement volume of Beagle lymphocyte plasma and the input of new donor grass turtle NK cells should be realistically adjusted according to the recipient Beagle's weight, blood volume and physical condition.

By the way, CAR-T cells. The main mechanism of acute rejection is that allogeneic antigens (especially the Major Histocompatibility Antigens) on the surface of graft cells stimulate the recipient to produce a T-cell-mediated cellular immune response, where T lymphocytes are the main effector cells. At the same time, as a graft CAR-T cell itself, although genetically modified, it is originally a T cell. It will also regard the recipient's immune cells as heterologous antigens and have an immune response. Therefore, When CAR-T cells are transfused into the recipient, CAR-T cells will inevitably suffer from double different types of immune rejection from the host to the graft and graft-versus-host at the same time, the double rejection response may make the limited range of receptor limiting the input of CAR-T cells much smaller than that of other types of immune cells. It is recommended that before the CAR-T cell decides to enter the selected receptor, take a sample and undergo an immune rejection test of the receptor in vitro to verify what type of immune rejection will happen if the CAR-T cell is entered into this receptor, the degree of the reaction, the final survival rate and activity of the CAR-T cells, and then make the next experimental plan (Afonso, G. et al., 2010; Chenghao, G., Chen, Y., & Xiao, Y., 2025; Braun, T., et al., 2025).

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