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The Efficiency of Adoptive Immunotherapy for Ehrlich Tumors in Mice and Fate of the Transferred Spleen Cells in the Recipient

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Summary. **It has been shown that adoptive immune transfer in mice for Ehrlich ascites tumors (EAT) is successful in a genetically defined combination such as B6 as donor and B6-nu/nu as recipient. Among allogeneic mouse strains, the adoptive immune transfer was rarely successful with a few exceptions. To observe the mechanism, the fate of intraperitoneally transferred donor spleen cells in the recipients was monitored by staining the cells with fluorescent PKH-26 dye, and using some typical donor and recipient combinations. The results are as follows.**

Spleen cells from B6-Ly-2^a,3^a mice were very effective in suppressing subcutaneous EAT outgrowth in B6-nu/nu mice but not in DBA/1 or in ddY-drm. Spleen cells of B6-Ly-2^a, 3^a mice were alive and infiltrated everywhere in B6-nu/nu mice. Some of the effector cells both reached and made contact with EAT cells and lethally affected the tumor cells. Accordingly, EAT outgrowth in B6-nu/nu mice was remarkably suppressed and no solid tumor formation was recognized.

On the other hand, spleen cells from B6-Ly-2^a, 3^a mice were killed at an early stage by peritoneal macrophages in the recipients in DBA/1 or in ddY-prg mice, and they did no reach the EAT site. As a result, subcutaneous EAT progressed gradually and formed a solid tumor. The spleen cells of B6-Ly-2^a, 3^a mice which were subcutaneously inoculated into the recipients in a mixture form with EAT cells were sufficiently active for suppressing EAT outgrowth both in DBA/1 and ddY-prg mice.

From the results, it is concluded that the contact of donor spleen cells with EAT cells in the recipient without any disturbance is highly important in producing a suppressive effect on EAT *in vivo*. At an early stage in the spleen cell transfer, recipient macrophage activity is the first barrier to the effective donor cells transferred.

Key words—spleen cell transfer, success or failure in adoptive immunity, Ehrlich tumor.

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INTRODUCTION

In an earlier study, spleen cells from EAT-regressive mice were adoptively transferred to EAT-progressive ones and the immunotherapeutic effect on Ehrlich ascites tumor (EAT) in the recipients was evaluated. We demonstrated some successful cases of adoptive immune transfer acting on the tumor, but these was rather restricted by the genetic compatibility between donors and recipients[1].

Adoptive immune transfer was quite successful in such combinations as B6 mice as donor and its athymic mice as recipient. B6-Ly congenic mice such as B6-Ly-1^a, B6-Ly-2^a and B6-Ly-2^a, 3^a were similarly available as donors in this system. Adoptive immune transfer between inbred mouse strains with different H-2 haplotypes is usually not successful. Donor and recipient combinations such as ddY-drm (H-2^s) and ddY-prg (H-2^q), or B6-Tla^a (H-2K^k • D^b) and B6-nu/nu (H-2^b) seemed to be exceptional cases which achieved success in immune transfer in spite of the allogeneic combinations. The reason for such success or failure, however, is still not fully understood.

In the present series of experiments, spleen cells from B6-Ly-2^a, 3^a mice were stained with fluorescent PKH-26 dye and intraperitoneally transferred to the recipient. The differences in the fate of the donor effector cells in the recipients were compared to

clarify the reason for such success or failure *in vivo*. It will be necessary to know how to carry out further genetic modification for histocompatibility in order to obtain greater effectiveness in adoptive immunotherapy with allogeneic donor-recipient combination.

MATERIALS AND METHODS

Laboratory animals used

B6-Ly-2^a,3^a(H-2^b) mice, which are EAT-regressive, were used as donors. They were donated by Aichi Cancer Center Research Institute (Nagoya, Japan). B6-nu/nu (H-2^b), which is EAT-progressive, was used as the recipient in an example of successful immune transfer. B6-nu/nu mice were from the Jackson Lab. (Maine, USA.), which have been maintained and bred in our Laboratory. DBA/1(H-2^q), which is EAT-progressive, was used as a recipient in an example of failure. DBA/1 was purchased from SEAC Inc. (Fukuoka, Japan). ddY-prg (H-2^q)[2] was chosen as another example of failure.

All the mice were used in specific pathogen-free conditions. Three to four mice were housed in plastic cages (14.3×29.3×14.8 cm, Charles River Japan Inc. (Atsugi, Japan) with bedding (cedar shavings) and fed a cube diet (CE-2, CLEA Japan Inc., Tokyo, Japan) and water ad libitum. All cages and bedding were autoclaved before use and stored in a separated room. The environmental conditions of the animal room were controlled at a constant temperature (23 ± 1℃) and humidity (45 to 75%). The room was ventilated 18 times per hour and was illuminated at 300 lx by daylight fluorescent lamps in a 12/12-hour light/dark cycle.

All animal procedures conformed to established guidelines (ILAR)[3] and the Guidelines for the Regulation of the Animal Experimentation (JALAS, 1987)[4]. The mice were killed by cervical dislocation.

Tumors

EAT, maintained by the intraperitoneal transfer of 10^7 cells to ddY mice (closed colony, H-$2^{s/q}$, 5 to 8 weeks old), were harvested on days 7 to 10 post transfer, and washed thoroughly in phosphate-buffered saline (pH7.4) before inoculation.

Passive immune transfer from donor to recipient

B6-Ly-2^a, 3^a mice, which are EAT-regressive, were immunized two times (at 0 and 20～30 days) with subcutaneous injections of 2×10^7 EAT cells. On days 4—7 after the final immunization, the spleen cells ("activated") were collected immediately after cervical dislocation. After filtration through a nylon mesh, the spleen cells were washed and collected by centrifugation at 250×g for 5 min. After staining the cells with Z-PKH-26-GL fluorescent dye, 10^8 cells/mouse were transferred intraperitoneally into EAT-progressive mice. Immediately after that, EAT cells (2×10^7) were subcutaneously inoculated into the central portion of the back skin of the recipients. EAT outgrowth was then monitored by measuring the length and width of the developing solid tumors.

PKH-26 staining of activated spleen cells

A Z-PKH-26-GL fluorescent staining kit for general cell membrane labeling (Zynaxis Cell Science Inc.) was purchased from Dainippon Pharmaceutical Co., Ltd. (Laboratory Products Division, Osaka, Japan). The method staining the activated spleen cells with the dye was according to the protocols indicated[5-7]. Briefly, activated spleen cells (2×10^7) were washed in phosphate-buffered saline (PBS) and stained for 3 min at 25℃ with 2×10^{-5} M fluorescent dye. After staining, the cells were treated with bovine serum to block residual dye and finally washed with PBS without bovine serum 3～5 times. The stained spleen cells (10^8/mouse) were intraperitoneally injected. At several intervals, PKH-26 labeled cells which had remained in the peritoneal cavity were collected and observed under a fluorescence microscope. Simultaneously, the liver, lung, spleen, and the dorsal skin of the recipient were sectioned at 6μ cryostatically and observed under a fluorescence microscope.

Acid-phosphatase activity

Donor spleen cells and EAT cells recovered from recipient peritoneal cavity and dorsal skin were stained with naphtol AS-BI dye to determine the acid phosphatase activity according to Yam's method[8].

RESULTS

Fate of intraperitoneally transferred spleen cells in an effective donor-recipient combination

Activated spleen cells (PKH-26 labeled) from B6-Ly-2^a, 3^a mice were transferred intraperitoneally to B6-nu/nu mice. The activated spleen cells transferred by this route were very effective in suppressing EAT outgrowth in B6-nu/nu mice. PKH-26 labeled

spleen cells in the peritoneal cavity, liver, spleen, and dorsal skin were observed at intervals.

As shown in Table 1, the spleen cell population decreased in number to some extent by day 3 but gradually increased due to cell division in the peritoneal cavity. The appearance of PKH-26 positive B6-Ly-2^a, 3^a spleen cells in the peritoneal cavity of B6-nu/nu mice is shown in Fig. 1A in comparison with that in another recipient, DBA/1 (cf. Fig. 1B). The number of acid phosphatase-positive lymphocytes increased in the cell population in B6-nu/nu mice; they were conspicuous on 7 to 16 days after transfer (Fig. 1C). The population of B-cells also increased in the peritoneal cavity. A few PKH-26 labeled B6-Ly-2^a, 3^a spleen cells appeared in the dorsal skin tissue from one day after the intraperitoneal transfer. They were usually seen in the dermis, comparatively near the base membrane (Fig. 1D). The number of PKH-26 labeled cells, however, was not so numerous in the dermis during observations. Gradually, EAT outgrowth in B6-nu/nu mice was remarkably suppressed and no solid tumor formation was recognized. Microscopical observation of a day 16 specimen showed that subcutaneous EAT cells had degenerated and grouped together (Fig. 1E). In those areas, large aggregates of PKH-26 fluorescent material were often observed (Fig. 1F) around the EAT cell population.

Fate of intraperitoneally transferred spleen cells in ineffective donor-recipient combination

Activated spleen cells (PKH-26 labeled) from B6-Ly-2^a, 3^a mice were transferred intraperitoneally to DBA/1 mice. In this combination, the transferred spleen cells were ineffective in suppressing subcutaneous EAT outgrowth in DBA/1. At intervals, PKH-26 labeled spleen cells were observed in the peritoneal cavity, liver, spleen, and dorsal skin.

As shown in Table 2, PKH-26 positive B6 spleen cells in the peritoneal cavity remarkably decreased at an early stage, accompanying the appearance of host (recipient) macrophages (Fig. 1B). Some fluorescent lymphoid cells were detected within the macro-

Table 1. Fate of B6-Ly-2^a, 3^a spleen cells in B6-nu/nu mice

	PKH-26 positive spleen cells			
Days after i.p. inoculation	1	3	7	16
Peritoneal cavity	+++	++	+++	++++
Dorsal skin site	+	++	++	+++
EAT outgrowth in B6-nu/nu				
+B6-spleen cells (i.p.)	−	−	−	−
−	−	−	++	+++

PKH-26 positive spleen cells: −, nothing; +, PKH-26-positive cells 1~2 / 6μ thin, 1cm long section; ++, PKH-26-positive cells 3~4 / ibid; +++, PKH-26-positive cells 5~6 / ibid; EAT outgrowth: −, completely regressive, +, solid tumor (~1 cm diameter), ++, solid tumor (1~2 cm diameter); +++, solid tumor (2~3 cm diameter).

Table 2. Fate of B6-Ly-2^a, 3^a spleen cells in DBA/1 mice

	PKH-26 positive spleen cells			
Days after i.p. inoculation	1	3	7	16
Peritoneal cavity	+++	++	+	−
Dorsal skin site	−	−	−	−
EAT outgrowth in DBA/1				
+B6-spleen cells (i.p.)	−	−	++	+++
−	−	−	++	+++

PKH-26 positive spleen cells: −, nothing; +, PKH-26-positive cells 1~2 / 6μ thin, 1cm long section; ++, PKH-26-positive cells 3~4 / ibid; +++, PKH-26-positive cells 5~6 / ibid; EAT outgrowth: −, completely regressive; +, solid tumor (~1 cm diameter); ++, solid tumor (1~2cm diameter); +++, solid tumor (2~3 cm diameter).

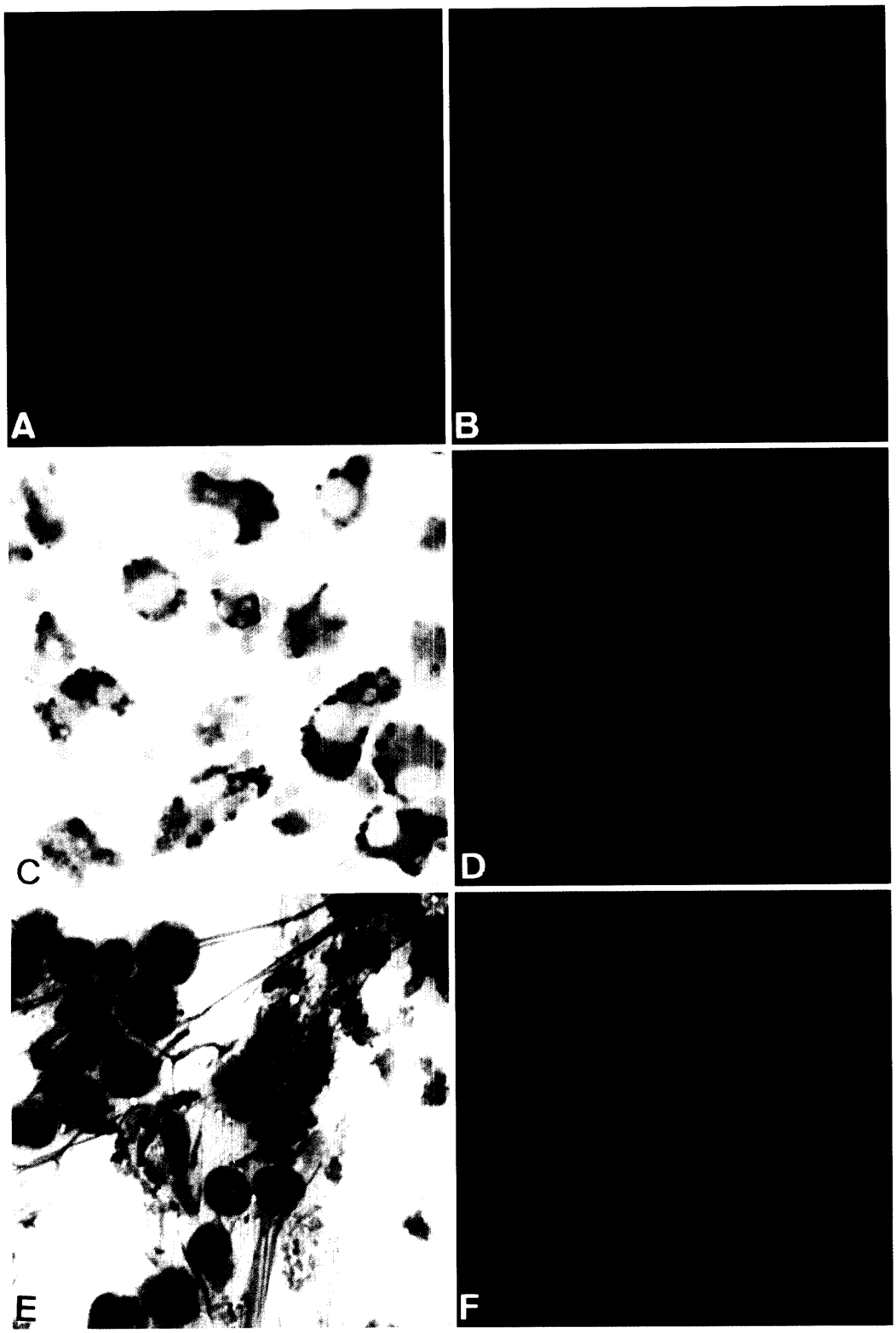

Fig. 1. Legend on the following page.

Table 3. Fate of B6-Ly-2^a, 3^a spleen cells in ddY-prg mice

	PKH-26 positive spleen cells			
Days after i.p. inoculation	1	3	7	16
Peritoneal cavity	+++	++	+	−
Dorsal skin site	−	−	−	−
EAT outgrowth in ddY-prg				
+B6-spleen cells (i.p.)	−	−	+	++
−	−	−	+	++

PKH-26 positive spleen cells: −, nothing; +, PKH-26-positive cells 1~2 / 6μ thin, 1cm long section; ++, PKH-26-positive cells 3~4 / ibid; +++, PKH-26-positive cells 5~6 / ibid; EAT outgrowth: −, completely regressive, +, solid tumor (~1 cm diameter); ++, solid tumor (1~2 cm diameter).

phages due to their phagocytotic activity. Some PKH-26 positive B6-Ly-2^a,3^a spleen cells could infiltrate into the circulation, but they did not appear in the dorsal skin at all. PKH-26 fluorescent materials were apparent in liver and spleen tissues in the scattered image, showing that some of the transferred spleen cells might be caught and also cleared by those tissues. PKH-26 labeled cells were absent from the dorsal skin of DBA/1 throughout the observation period. Finally, subcutaneously inoculated EAT progressed gradually and formed a solid tumor. In the day 16 specimen after EAT cell inoculation, it reached about 1~2 cm in diameter.

Activated spleen cells (PKH-26 labeled) from B6-Ly-2^a,3^a mice were transferred intraperitoneally to ddY-drm mice. In this combination, the transferred spleen cells were ineffective in suppressing subcutaneous EAT outgrowth in the recipient. As shown in Table 3, a process similar to that in DBA/1 mice also took place in this recipient. Namely, flourescent spleen cells were first attacked by macrophages in the recipient peritoneal cavity, and no effector cells appeared in the tumor site of the dorsal skin throughout the observation period. Finally, subcutaneously inoculated EAT cells progressed gradually in ddY-prg mice and formed a solid tumor. In the day 16 specimen, it reached about 2 cm in diameter.

Anti-EAT activity of B6-Ly-2^a, 3^a mice spleen cells

As shown in Tables 4 and 5, activated spleen cells of donor mice (B6-Ly-2^a,3^a), which were subcutaneously inoculated into the recipients in mixture form with EAT cells, were sufficiently active in suppressing EAT outgrowth both in DBA/1 and ddY-prg mice. This confirms that contact between effector cells and target cells is essential in killing the tumor cells. In the ineffective donor-recipient combination, in turn, effector cells were disintegrated by host defense mechanisms such as the macrophage phagocytotic function before they made contact with the target cells in the allogeneic recipients.

DISCUSSION

The effector cell population from donor mice is thought to comprise various types of cells such as T, B lymphocyte, natural killer (NK) cells, lymphokine activated killer cells (LAK), dendritic cells /macrophages, and others[9]. The mechanisms of tumor destruction by the cellular immune functions are rather complex. Cytotoxic T (Tc) lymphocytes have been reported to play an important role in tumor destruction[10-15]. The majority of Tc cells are $CD8^+$ and therefore recognize the antigen in the context of

Fig.1. Fate of transferred spleen cells and degradation of EAT in the recipient.
A. Activated spleen cells transferred from B6 mice to B6-nu/nu on day 3 after transfer. Z-PKH-26-GL fluorescent staining, ×200. **B.** Activated spleen cells transferred from B6 mice to DBA/1 mice, on day 3 after transfer. Z-PKH-26-GL fluorescent staining, ×200 **C.** Activated spleen cells transferred from B6 mice to B6-nu/nu on day 3 after transfer. *In vitro* culture (3 days) and acid phosphatase staining, ×400 **D.** Z-PKH-26-GL fluorescent positive cells migrated to the dorsal dermis of B6-nu/nu on day 7 after transfer, ×200 **E.** Degenerating EAT cells in B6-nu/nu mice with B6 spleen cells-transfer. Stamped specimen and Gimsa'staining, ×400 **F.** Fluorescent aggregates often appearing around EAT cells population in B6-nu/nu mice with B6 spleen cells-transfer, ×200

Table 4. Anti-tumor activity of B6-Ly-2^a, 3^a spleen cells in the mixture form with EAT in DBA/1 mice

	EAT outgrowth			
Days after inoculation	1	3	7	16
EAT (2×10^7, s.c.) only	−	−	++	+++
EAT (2×10^7, s.c.) +B6-Ly-2^a, 3^a spleen cells (10^8, s.c.)	−	−	−	+

EAT outgrowth: −, completely regressive; +, solid tumor (~1 cm diameter); ++, solid tumor (1~2 cm diameter); +++, solid tumor (2~3 cm diameter).

Table 5. Anti-tumor activity of B6-Ly-2^a, 3^a spleen cells in the mixture form with EAT in ddY-prg mice

	EAT outgrowth			
Days after inoculation	1	3	7	16
EAT (2×10^7, s.c.) only	−	−	++	+++
EAT (2×10^7, s.c.) +B6-Ly-2^a, 3^a spleen cells (10^8, s.c.)	−	−	−	+

EAT outgrowth: −, completely regressive; +, solid tumor (~1 cm diameter); ++, solid tumor (1~2 cm diameter); +++, solid tumor (2~3 cm diameter).

self-MHC I molecules. A small percentage of Tc cells bearing the $CD4^+$ phenotype recognize antigens in association with class II molecules. Since EAT cells lack almost any MHC-class I antigens on their cell surface, minor H gene products on EAT cells may be recognized by the donor effector cells in the context of self-MHC molecules associated with regulatory functions.

The participation of LAK cells would also be expected in the present series of experiments because activated spleen cells were proliferative in *in vitro* culture, suggesting the enhanced release of interleukins. On the other hand, the participation of delayed type hypersensitivity on tumor destruction was also postulated to some extent because chronic inflammation was usually produced by the subcutaneous inoculation of EAT cells[16].

NK cells, which have acid phosphatase activity in the cytoplasm[17-20], are of importance during the early stages of tumor development because of their non-specific cytotoxic action on tumor cells[21-23]. It has been shown that the activities of NK cells in mice are related to their spontaneous resistance to the transplanted lymphoma cells[24,25]. In the present series of experiments, however, the relationship between mouse strain susceptibility to EAT and NK activity was not clear. NK activity is not MHC-restricted.

Macrophages would rather ingest degenerated EAT cells than kill them. Macrophages are active in B6-nu/nu mice but EAT easily progresses in athymic mice.

In general terms, peritoneal macrophages in B6-nu/nu mice survey the spleen cells from donor B6 mice but they do not vigorously attack them because of their syngeneic character, except for nu-gene. Out of the macrophage's surveillance, the donor effector cells can infiltrate everywhere in the recipient body.

Previously we demonstrated that activated spleen cells from BALB/c mice were ineffective in suppressing EAT outgrowth in DBA/2 mice even though they have the same H-2 haplotype d[26]. Phagocytotic cells such as macrophages in DBA/2 seemed to recognize some unknown allogeneic antigens on the lymphocytes of donor BALB/c and probably ingest them.

In the present series of experiments, it is evident from the successful cases of immune transfer that effective spleen cells from donor mice infiltrate everywhere in the recipient and some of them reach the dorsal skin where EAT is inoculated. Some effector cells made contact with EAT cells, recognized antigens, and had a lethal effect on the tumor cells. Degenerated cells involve the surrounding tumor cells and form large fluorescent aggregates. On the other

hand, for ineffective donor-recipient combinations, donor spleen cells are strongly attacked by host (recipient) macrophages at an early stage and cleared from the recipient body. They also seemed to be cleared from liver and spleen tissues if they had infiltrated into the circulation. In those cases, no GVHD was produced.

In the previous series of experiments, we demonstrated that activated spleen cells from parent mice (B6 & C3H) are ineffective in suppressing EAT outgrowth in the hybrid; [B6×C3H] F_1[1]. In this donor-recipient combination, recipient macrophages could not recognize the surface antigens on the donor spleen cells but the donor spleen cells attacked the allogeneic molecules of the recipient normal cells and resulted in developing GVHD (graft versus host disease)[1]. The phenomenon of GVHD greatly disturbs their own anti-tumor activity *in vivo*.

In our experience, recognition and killing of the tumor cells by allogeneic lymphocytes is most effective. A way for activated allogeneic lymphocytes to break through the surveillance by the host (recipient) defense mechanism would be significant in providing a powerful tool in adoptive immunotherapy for cancer.

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