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ORIGINAL ARTICLE

The effect of anti-TfR mouse/human chimeric antibody on anti-transplant rejection

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Keywords
complement-dependent cytotoxicity, graft-versus-host reaction, lymphocyte proliferation, mouse–human chimeric antibody, transferrin receptor.

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Summary

The expression of TfR/CD71 in T-cell surface plays a pivotal role in T-cell activation and proliferation. Anti-human-TfR monoclonal antibody could be used as an immunosuppressant in transplant therapy because of their potential to suppress T-cell responses to alloantigens. We therefore examined the feasibility of an anti-human-TfR chimeric antibody (D2C) in suppression of T-cell activation *in vitro* and graft-versus-host reaction (GVHR) in animals. D2C is a chimeric antibody produced by introducing the human Fc fragment. This antibody showed low antigenicity but high suppressive effect manifested by high potency to block the activation and proliferation of lymphocytes in response to alloantigens. D2C also showed capability to mediate complement-dependent cytotoxicity, which could be correlated with TfR expression in peripheral blood mononuclear cells (PBMCs). Importantly, administration of D2C significantly prolonged survival time of nude mice transplanted with human PBMCs when compared with that of control IgG-treated animals (61.2 ± 4.46 vs. 22.1 ± 5.5 days), which is associated with inhibited GVHR characterized by decreased interleukin-1 and tumor necrosis factor α production derived from transplanted PBMCs. Human-TfR chimeric antibody such as D2C could be a valuable option for the treatment of acute form of graft-versus-host disease.

Introduction

Previous studies have consistently demonstrated that the iron-binding protein transferrin and its receptor, TfR, are implicated in iron uptake, which is essential for cell proliferation [1]. As a result, TfR is more abundantly expressed in rapidly dividing cells and in those nondividing cells that require high levels of iron [2–6]. Other than its role in iron transport, TfR has also been found to be implicated in T-cell activation as manifested by the significant upregulation in T cells following the interaction between the major histocompatibility complex (MHC)-antigen complex and T-cell receptor (TCR). TfR forms a complex with TCR/CD3ζ chain on the cell surface to promote the transduction of activation signals into the cells. Iron has also been implicated in antigen processing by affecting oxidation to influence immunoreactive development [7]. As a result, blockade of TfR by monoclonal antibodies (mAbs) has the potential to interfere with T-cell activation and antigen processing.

As T-cell activation and antigen processing play critical roles in the rejection of transplants, mAbs against TfR had been demonstrated with great potential to prolong allograft survival in a murine cardiac transplan-

tation model [8]. In this study, we investigated the feasibility of blockade of TfR expressed on active immune cells to prevent allograft rejection with minimum side effects. To prevent the development of specific immunity or an allergic reaction after the blocking antibody administration [9], we have constructed a human–mouse chimeric antibody against TfR (named D2C) [10]. Previously, we have demonstrated that D2C dose-dependently inhibits phytohemagglutinin-induced peripheral blood mononuclear cell (PBMC) proliferation [11]. We now sought to characterize its role in prevention of transplant rejection. We employed a graft-versus-host reaction (GVHR) model by engrafting human PBMCs into peripheral blood of nude mice (Hu-PBMCs-nude mice) to address the issue.

Materials and methods

Mice

Homozygous Balb/c (nu/nu) nude mice and Balb/c mice were purchased from the Laboratory Animal Center, Tongji Medical College, Huazhong University of Science and Technology. All experimental animals were housed in the specific-pathogen-free and P3 animal facilities of the Laboratory Animal Center. The protocols for the care and use of the mice were approved by the Committee on Animal Research before the initiation of this study [10]. Four to 8-week-old mice were used to induce ascites, while 3- to 4-week-old mice were used for the generation of Hu-PBMCs-nude mouse model.

Reagents and antibodies

The transfectoma cells expressing the chimeric antibody D2C and Transfectoma D4 cells were inoculated intraperitoneally into a Balb/c (nu/nu) nude mouse to induce ascites formation for the generation of human–mouse chimeric antibody against TfR [12] and human–mouse chimeric antibody against CD4 [13], respectively. Similarly, the hybridoma 7579 cells which produce a mouse mAb against TfR (kindly provided by the Institute of Immunology, University of Duisburg–Essen, Germany) were inoculated into a Balb/c mouse as above. The quality and content of the antibodies were analyzed using enzyme-linked immunosorbent assay (ELISA). All antibodies were purified from the ascites by DEAE–Sephadex A-50 chromatography and analyzed using SDS-PAGE.

Mixed lymphocyte reaction

Mixed lymphocyte reaction (MLR) was carried out using the colorimetric methyl thiazolyl tetrazolium (MTT) tetrazolium salt cell viability/proliferation assay as described previously [11]. In brief, PBMCs derived from subject A were treated with or without mitomycin C (25 μg/ml) at 37 °C for 30 min to serve as stimulators (2×10^6/ml). The responsive PBMCs (2×10^6/ml, without mitomycin C treatment) were derived from subject B with a different MHC genotype. The stimulators (100 μl/well) and reactors (100 μl/well) were aliquoted into a 96-well plate, and incubated at 37 °C for 12 h. D2C (with the final concentration of 0, 1, 10 or 100 μg/ml, respectively), or an isotype-matched human control IgG (final concentration 10 μg/ml) was then added into the cultures, followed by additional 5-day culture at 37 °C. Cell proliferation was estimated as $\delta = (A-B)/A \times 100\%$ (A and B represent the absorbance for isotype-matched IgG and DC2, respectively). For analysis of PBMC proliferation after Epstein–Barr virus (EBV)-transformed B lymphocyte stimulation, B lymphocytes (2×10^6/ml) transformed with EBV were incubated with mitomycin C (25 μg/ml) at 37 °C for 30 min to serve as stimulators, while normal PBMCs (2×10^6/ml) were used as reactors, and the assays were carried out as detailed above.

Quantitative analysis of TfR expression

Fluorescence-activated cell sorting (FACS) was employed for quantitative analysis of mitogen-induced TfR expression in T cells [14]. PBMCs from five human subjects were isolated by the standard Ficoll–Paque method as described previously [14]. Informed consents were obtained from all human subjects. PBMCs were adjusted to 2×10^6 cells/ml. The expression of TfR in T cells (CD3+) after mitogen induction (25 μg/ml) [15] for 12, 36 or 60 h were analyzed by indirect immunofluorescence using a FACScan cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) with Cellquest software.

Complement-dependent cytotoxicity

Peripheral blood mononuclear cells cultured in the presence of mitogen (25 μg/ml) for 12, 36 or 60 h were aliquoted in triplicate into a 96-well plate (100 μl/well). D2C (0, 1, 10 or 100 μg/ml) or an isotype-matched control IgG (10 μg/ml) was then added into the cultures, followed by mixing the samples by vibration for 5 s. After 1 h of incubation, 100 μl/ml fresh rabbit serum was added, and the cells were cultured for another 1 h. Heat-inactivated serum was used as a control to ensure the measurement of complement-dependent lysis. The total number of cells in each well was calculated by trypan blue staining using an optical microscope. Antibody-mediated complement-dependent cytotoxicity (CDC) was analyzed by subtracting the percentage of PBMC lysis that was attributable to complement alone.

In vivo experiments

Engraftment of human PBMCs into peripheral blood of nude mice (Hu-PBMCs-nude mice) was performed as described previously with minor modifications [11,12]. Before day 7 of Hu-PBMC injection, the nude mice (3–4 weeks old) were pretreated daily with cyclosporin A (50 mg/kg; Sandoz, Basel, Switzerland) by gastric intubation [16]. Before the day of Hu-PBMC injection, 50 mg/kg anti-asialoganglioside GM1 antibodies were administered via i.p. to deplete the activity of natural killer and cytolytic T lymphocyte. On the day of transplantation, Hu-PBMCs from healthy subjects were isolated by Ficoll–Hypaque centrifugation. The cells were then injected intravenously (1×10^8 PBMCs/mouse) into nude mice via the vena caudalis under sterile conditions. For GVHR experiments, the mice (15 mice/group) were treated with the following therapeutic agents: (i) D2C antibody (D2C group); (ii) mouse–human chimeric antibody against CD4 (CD4 antibody group); or (iii) isotype-matched control IgG (negative control group). Each therapeutic agent was administered at a dose of 100 μg/mouse via i.v. on the day of grafting and day 1 after grafting. Ten mice were used to monitor survival time, while the rest of five mice for each study group were used for histological analysis. Mice showing cardiac arrest or respiratory failure were presumed to be dead. Statistical significance was determined using the Wilcoxon rank-sum test.

Histological examination

Animals were killed at day 20 after grafting. Heart, liver, spleen, lung, kidney, brain, stomach, intestine, skin, and muscular tissue were collected for histological examination. Tissues were fixed with 10% formalin, embedded in paraffin, and 4-μm sections were cut. The sections were stained with hematoxylin and eosin (H&E) by standard methods and assessed in a blinded fashion. Criteria used for elevation of pathomorphological changes were described as our previous report [12].

Splenocyte proliferation assay for Hu-PBMCs-nude mice

Single splenic cells were prepared from the Hu-PBMCs-nude mice (2×10^5 cells/ml). The cells were then aliquoted into a 96-well plate (100 μl/well) with or without Bacillus Calmette-Guerin (BCG) (80 μg/ml) followed by a 5-day culture at 37 °C. Cell proliferation was determined as described earlier.

Assay for human cytokines in Hu-PBMCs-nude mice

Human interleukin-1 (IL-1) and tumor necrosis factor α (TNF-α) production were measured in Hu-PBMCs-nude mice treated with D2C, the mouse–human chimeric antibody against CD4, or isotype-matched control IgG or untreated nude mice using ELISA kits (R&D Systems, Minneapolis, MN, USA) as instructed.

Statistical analyses

All experiments were performed in triplicates. Data are shown as means ± SD. Statistical analysis was performed using SPSS 13.3 for Windows. Individual comparisons were made with a two-tailed, unpaired Student's *t*-test. Comparisons of multiple samples were performed using the chi-squared test. A value of $P < 0.05$ was considered statistically significance.

Results

D2C represses PBMC proliferation after antigen stimulation

Colorimetric MTT cell viability/proliferation assays were performed to determine the effect of D2C on PBMC proliferation. We first performed assays with allogenic stimulation, in which both stimulators and reactors were human PBMCs but with different MHC genotypes. Both D2C and the mouse mAb against TfR dose-dependently suppressed PBMC proliferation when compared with that of control IgG ($P < 0.05$). However, D2C showed much stronger inhibitory effect ($P < 0.05$) when compared with the mouse mAb against TfR at the same concentration (Fig. 1a). These results suggest that D2C has a higher potency in terms of suppression of T-cell proliferation stimulated by an allogeneic antigen than that of the mouse mAb against TfR. We next examined their effect on PBMC proliferation induced by EBV-transformed B lymphocytes. Similarly, both D2C and the mouse mAb against TfR suppressed PBMC proliferation (Fig. 1b). Unexpectedly, we failed to detect a significant difference in terms of suppressive potency between D2C and the mouse mAb against TfR. These results demonstrate that D2C does not have higher inhibitory potency than that of the mouse mAb against TfR when cells were stimulated by a xenogeneic antigen.

TfR expression on activated T cells after mitogen induction

It was found that T cells were induced to express TfR upon mitogen induction. After 12, 36 or 60 h of induction, the proportion of T cells that expressed TfR was 38.6 ± 8.3%, 89.6 ± 12.4%, and 90.8 ± 6.6%, respectively. These findings indicate that mitogen can induce the expression of TfR on T cells, and the activation and proliferation of T cells were in a time-dependent manner.

D2C-mediated CDC

To determine whether D2C was able to mediate CDC, we performed CDC assays using sera from rabbits as the source of complement. For this purpose, cultured TfR-expressing PBMCs were used as target cells. The results indicated that complement-dependent lysis was mediated by D2C in a dose-dependent manner (Fig. 2). In sharp contrast, the mouse mAb against TfR, the isotype-matched control IgG (10 μg/ml), or complement alone, induced <10% cell death without a significant morphology change. However, obvious cell morphological and structural changes were observed in cells treated both with varying concentrations of D2C and with complement after exposure of the PBMCs to mitogen for 12, 36 or 60 h. These changes were dependent on the time frame of exposure to mitogen. For example, the most significant morphological and structural changes in the cells were observed after 60 h exposure to mitogen. Both the expression of TfR following PBMC exposure to mitogen and D2C-mediated CDC dependent on the length of time exposed to mitogen suggest that D2C-mediated CDC could be related to surface expression of TfR in PBMCs.

D2C prolongs the survival of Hu-PBMCs-nude mice

Previous experiments have demonstrated that D2C can inhibit the activation and proliferation of PBMCs by blocking the expression of TfR on T cells *in vitro*. We therefore sought to confirm the *in vivo* immunosuppressive properties of D2C in a Hu-PBMCs-nude mouse model. To determine whether D2C would be useful in treating ongoing graft rejection, Hu-PBMCs-nude mice were i.v. injected with 100 μg D2C/mouse on day 0 (the day of transplantation) and day 1 after grafting. Previously, we demonstrated that the administration of anti-CD4 mouse–human chimeric antibody *in vivo* at the time of transplantation could significantly prolong graft survival in a mouse graft model [14]. As a result, we used anti-CD4 as a positive control for the study. Graft survival was prolonged significantly in animals treated with either D2C or the anti-CD4 mouse–human chimeric antibody (60.3 ± 2.04 and 77.6 ± 2.04 days, respectively), while animals treated with an isotype-matched IgG only survived 22.2 ± 1.38 days ($P < 0.05$; Fig. 3).

Histological evidence for the inhibition of rejection

To investigate whether D2C suppresses GVHR *in vivo* and prolongs the survival time of Hu-PBMCs-nude mice by inhibiting the activation and proliferation of graft inflammatory cells and their infiltration into the host, we checked graft inflammatory cell infiltration in various organs and tissues of the hosts. We observed a significant inhibition of graft inflammatory cell infiltration at day 20 after grafting in the recipient mice treated with either D2C or an anti-CD4 mouse–human chimeric antibody compared with that of control animals. A typical characteristic change mediated by GVHR is manifested by the marked small lymphocytic infiltration in all organs and tissues of the animals in the negative control group. The most severe small lymphocytic inflammation was noticed in the lung, while moderate infiltration was noticed in the liver, stomach, intestine, skin (Fig. 4A-a), and brain (Fig. 4A-c). GVHR-associated partial organ necrosis was observed most severely in the liver with scattered hepatocytic apoptosis, and spotty cellular, piecemeal, and bridging necrosis. There was an extensive monocytic infiltration in the spleen associated with an increase in the sizes of the white pulp and small lymphocytic

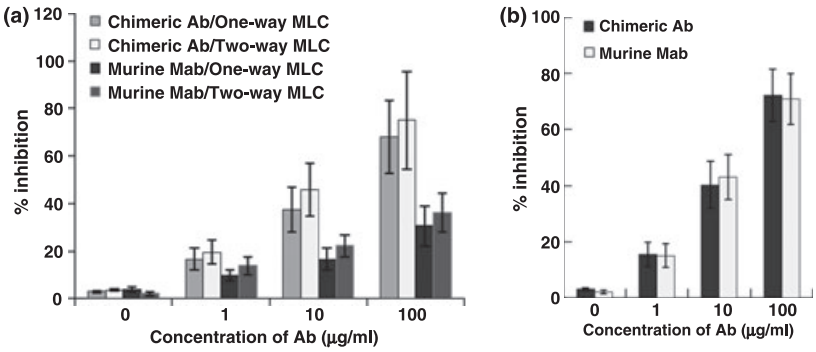

Figure 1 Suppressive effect of anti-TfR antibody on PBMC proliferation. (a) Human PBMCs with different MHC genotypes were used as stimulators or reactors. Stimulators were treated with mitomycin for one-way MLR, while stimulators without mitomycin treatment were used for two-way MLR. (b) EBV-transformed B lymphocytes treated with mitomycin C were used as stimulators, and the same human PBMCs were used as reactors. The ratio between stimulators and reactors was 1:1. Different concentration of chimeric anti-TfR antibody was applied in all treatment and results show a strong dose-dependent inhibitory effect. Data are shown as mean ± SD of three independent experiments.

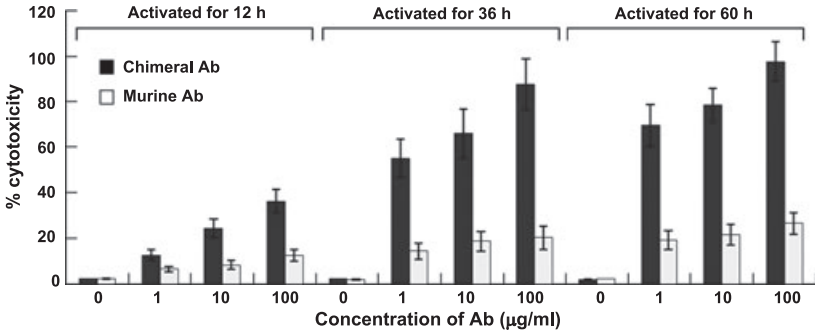

Figure 2 The anti-TfR chimeric antibody mediates strong complement-dependent cell death (CDC). PBMCs were stimulated by mitogen (25 μg/ml) for 12, 36, and 60 h, and then treated with various concentrations of anti-TfR chimeric antobody or mouse mAbs for 1 h, fresh serum (100 μl/ml) from rabbit was applied in all groups as complement source. The anti-TfR antibody showed high potency to mediate a dose-dependent CDC. Data presented here are mean ± SD of three independent experiments.

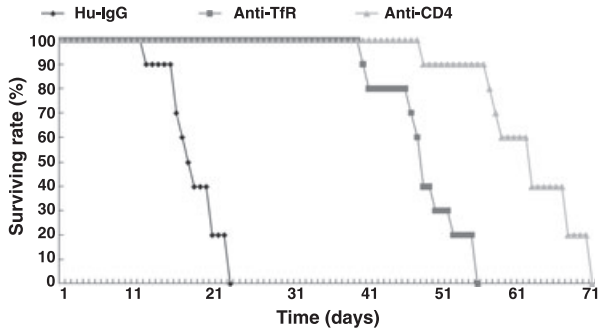

Figure 3 Administration of anti-TfR antibody prolongs mice survival after Hu-PBMC transplantation in nude mice. Nude mice were transplanted with human PBMC through i.v., anti-TfR chimeric antibody, anti-CD4 chimeric antibody and Human normal IgG were administered by i.v at day 0 (grafting day) and day 1 after grafting. Hu-PBMC-nude mice survival time of the experiment group prolonged from 22.5 ± 6.36 to 61.2 ± 4.46 days compared with control group. Each group contains 10 mice. ※, $P < 0.05$ compared with the control group.

proliferation (Fig. 4A-g). By contrast, animals treated with D2C, the extent of small lymphocytic infiltration was significantly decreased in the heart, liver, lung, kidney, skin (Fig. 4A-b), brain (Fig. 4A-d), stomach, intestine, and intestinal mesentery. No significant change was observed in the muscle. Moreover, hepatic necrosis was reduced significantly (Fig. 4A-f) and splenic monocytic infiltration was alleviated (Fig. 4A-h). Using a standard scoring system (Fig. 4B) for graft rejection, we found that the rejection scores were decreased significantly in animals treated with either D2C or anti-CD4 antibody (Fig. 4C). These results strongly suggest that D2C or anti-CD4 mouse–human chimeric antibody was able to suppress graft rejection in the GVHR model.

Inhibition of splenocyte proliferation in Hu-PBMCs-nude mice

To examine whether any other synergistic effect contributes to the suppression of GVHR in the presence of sera, we investigated the suppression of splenocyte proliferation in Hu-PBMCs-nude mice induced by their own serum *in vitro*. It was found that after BCG stimulation, the proliferation of splenocytes was suppressed by serum from Hu-PBMCs-nude mice treated with either D2C or the anti-CD4 mouse–human chimeric antibody when compared with that of control mouse IgG (Fig. 5 a). Furthermore, much stronger suppression was observed in mice treated with the anti-CD4 mouse–human chimeric antibody than that of mice treated with D2C ($P = 0.016$). On the basis of these results, we presumed that both D2C and the anti-CD4 mouse–human chimeric antibody could suppress splenocyte proliferation through affecting some substance in the serum to relieve the GVHR.

Change in concentration of human inflammatory factors

To check whether reduced GVHR is associated with decreased production of inflammatory cytokines, we assayed the concentrations of human IL-1 and TNF-α in all experimental animals. Human IL-1 and TNF-α (Fig. 5 b) were virtually undetectable in nude mice injected with human PBMCs, whereas they were present at high concentrations in Hu-PBMCs-nude mice treated with isotype-matched control IgG. However, D2C or anti-CD4 mouse–human chimeric antibody treatment reduced IL-1 and TNF-α in the Hu-PBMCs-nude mice when compared with that of control IgG-treated mice. Interestingly, the production of human IL-1 in D2C-treated Hu-PBMCs-nude mice was lower than that of anti-CD4 mouse–

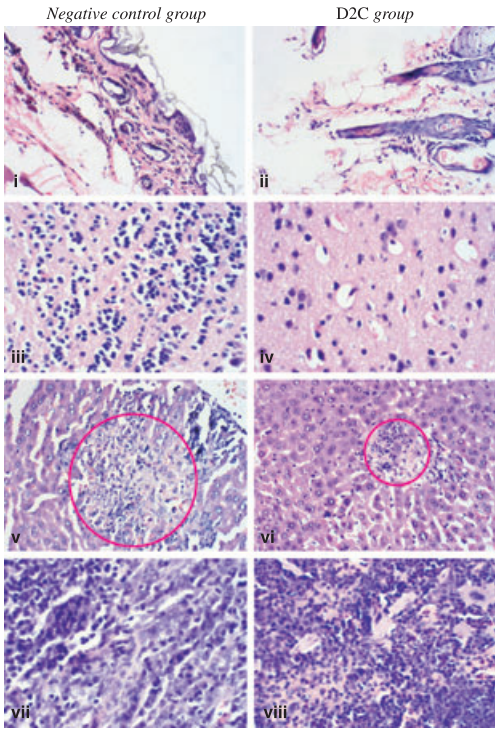

Figure 4 Administration of anti-TfR antibody suppresses xenogenic GVHR on nude mice. Nude mice were transplanted with Hu-PBMC along with i.v. administration of anti-TfR chimeric antibody (D2C group), or anti-CD4 chimeric antibody (CD4 antibody group), or Human normal IgG (negative control group). Mice were killed at day 20 after grafting and tissue samples were collected. (a) All sections were subjected to H&E staining, and were evaluated under a light microscope (a and b: skin × 200, c and d: brain × 400, e and f: liver × 400, g and h: spleen × 400). (b) The scoring system for GVHR in Hu-PBMC-nude mice. (c) Scores for rejection in main tissues and organs collected from recipients. All results were expressed as mean ± SD of five mice. ※, $P < 0.05$ compared with the negative control group.

(b) The scoring system for GVHR in Hu-PBMC-SCID

Degree	Lymphocytic infiltration	Splenic macrophages and lymphocytic around artery	Hepatic necrosis
0	No or seldom lymphocytic infiltration, <10 lymphocytes/hpf	No or seldom lymphocytic hyperplasia around artery, <1 macrophages/hpf	Non-necrosis
1	Small amounts of lymphocytic infiltration, <50 lymphocytes/hpf	Small amounts of lymphocytic hyperplasia around artery, <2 macrophages/hpf	Light punctiform necrosis or spotty necrosis
2	Medium amounts of lymphocytic infiltration, <90 lymphocytes/hpf or lymphocytes infiltration in fragmentis	Medium amounts of lymphocytic hyperplasia around artery, < 3 macrophages/hpf	Medium necrosis or piecemeal necrosis or bridging necrosis
3	Heavy amounts of lymphocytic infiltration, >90 lymphocytes infiltration/hpf or lymphocytes infiltration in flakiness	Heavy amounts of lymphocytic hyperplasia around artery, > 3 macrophages/hpf	Grave necrosis

hpf: high-power field

(c) **The scores for rejection in main tissues and organs (n = 5 mice/group)**

		CD4 antibody group	D2C group	Negative control group
Lymphocytic infiltration	Heart	0.4 ± 0.548※	0.4 ± 0.490※	1.4 ± 0.548
	Liver	1 ± 0※	1.6 ± 0.800※	2.2 ± 0.837
	Lung	2.4 ± 0.548※	2.4 ± 0.490※	3 ± 0
	Kidney	1.4 ± 0.8941※	1 ± 0※	1.6 ± 0.548
	Brain	1.2 ± 0.447	1 ± 0※	1.4 ± 0.894
	Skin	1 ± 0※	1.6 ± 0.490※	2 ± 0.707
	Muscle	0.2 ± 0.447※	1.6 ± 0.490	1.4 ± 0.548
	Stomach	1.2 ± 0.447※	1 ± 0※	2.4 ± 0.548
	Intestines	1 ± 0※	1 ± 0.632※	2 ± 1.225
Splenic hyperplasia		1.6 ± 0.548	1.6 ± 0.490	2.2 ± 0.447
Hepatic necrosis		0.8 ± 0.447	1.4 ± 0.548	2.2 ± 0.447

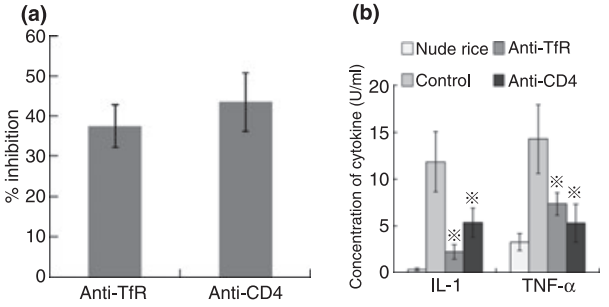

Figure 5 Other synergistic effect of anti-TfR antibody in suppression of Xeno GVHR in Hu-PBMC transplanted nude. (a) Anti-TfR antibody affects the proliferation ability of splenocytes. Single splenic cells treated with BCG were cultured for 5 days. Proliferation of splenocytes after BCG treatment was suppressed by serum from either D2C or anti-CD4 mouse–human chimeric antibody-treated Hu-PBMC-nude mice. (b) Anti-TfR antibody decreases transplanted human PBMC secretion of cytokines. Human IL-1 and TNF-α in the sera of recipient mice were determined using ELISA. All results are expressed as means ± SD from five mice. ※, $P < 0.05$ compared with the control group.

human chimeric antibody-treated mice, although the production of human TNF-α did not differ between these mice.

Discussion

During T-cell activation, the expression of TfR is necessary to initiate proliferation in quiescent T lymphocytes. In addition, the intracellular iron transported by the Tf-TfR complex is implicated in antigen processing by its involvement in oxidation. Therefore, mAbs specific for TfR would have great potential to inhibit T-cell activation and antigen presentation. In line with this assumption, treatment of recipient mice with these antibodies significantly prolonged cardiac allograft survival [8].

As a result of the restriction of clinical applications for the murine mAb, we first studied the effect of an anti-TfR human–mouse chimeric antibody (D2C) and a murine mAb on the activation of lymphocytes using mitogen stimulation *in vitro*. We next established a graft-versus-host animal model to evaluate the feasibility of D2C in the prevention of GVHR *in vivo*. We also examined the biological effect of this chimeric antibody on activated PBMCs and compared its functionality with a murine mAb. We have demonstrated that both D2C and the murine mAb have the capability to inhibit PBMC proliferation induced by EBV-transformed cells. Similarly, they can also inhibit donor cell-induced immunocyte proliferation *in vitro*. However, in the mixed lymphocyte culture system, D2C showed much stronger inhibitory effect than the murine mAb ($P < 0.05$). Furthermore, in the presence of fresh complement, D2C displayed a CDC effect on proliferating PBMCs activated by mitogen. By contrast, the murine mAb only showed a relatively weak CDC effect. The above results suggest that introduction of the human Fc fragment into the chimeric antibody could enhance its ability to prevent graft rejection which is consistent with those of previous reports [16–18]. Maximum of TfR expression was observed in PBMCs along with 60 h of mitogen stimulation. A large number of TfR molecules were then available to be bound by the chimeric antibody which led to pronounced PBMC apoptosis.

CD4+ T-cell-mediated immune response is important for allograft rejection, and CD4 plays an important role in the activation of CD4+ T cells. Therefore, anti-CD4 antibody can inhibit the activation of CD4+ T cells to prevent graft rejection. Since anti-CD4 antibodies can block all CD4 molecules, administration of these antibodies could lead to the functional disturbance of all CD4+ T cells which would impact host defenses. As a result of the significant effect of anti-CD4 antibodies on anti-graft rejection, we did use such an antibody as a positive control for our study.

The anti-TfR human–mouse chimeric antibody (D2C) and the anti-CD4 human–mouse chimeric antibody were administered into recipient mice on days 0 and 1 posthuman PBMC transplantation. Our results showed that both D2C and the anti-CD4 human–mouse chimeric antibody prolonged the survival time of Hu-PBMCs-nude mice from 22.5 ± 6.36 to 61.2 ± 4.46 and 78.3 ± 15.13 days, respectively. By a proliferation assay using BCG-induced spleen cells, we examined the reactivity of transplanted human immunocytes in the Hu-PBMCs-nude mice to antigen stimulation. Using the plasma of each individual animal to re-suspend the corresponding splenic cells, we imitated the *in vivo* microenvironment. The results showed that the anti-TfR and anti-CD4 human–mouse chimeric antibodies inhibited the proliferation of transplanted human immunocytes in response to antigen stimulation, while anti-CD4 human–mouse chimeric antibody showed much stronger inhibitory effect than that of the anti-TfR human–mouse chimeric antibody ($P < 0.05$). As IL-1 is secreted by activated monocytes and macrophages, the concentration of IL-1 can, therefore, reflect the activation level of monocytes and macrophages *in vivo*, and can be correlated positively with the severity of acute rejection [19,20]. TNF-α is a pro-inflammatory cytokine, and the concentration of TNF-α can reflect the severity of rejection *in vivo* [21,22]. Along with the progression of rejection, the concentrations for both IL-1 and TNF-α would increase [23,24]. We examined the concentrations of human IL-1 and TNF-α in the plasma of nude mice in each group using ELISA. PBMCs injected into Hu-PBMCs-nude mice had been activated abundantly and showed strong graft anti-host activity. When the anti-TfR or anti-CD4 human–mouse chimeric antibody was administered, lymphocyte activation and graft anti-host activity were inhibited. The pathological examination of different organs further confirmed these results as manifested by more severe inflammatory infiltration in control animals.

As D2C is an anti-human-TfR chimeric antibody, the effect of this antibody against TfR on iron metabolism must be considered. Many molecular regulators such as transferrin, transferrin receptor, ferritin, and hepcidin are involved in iron metabolism of erythropoiesis and other organs, and therefore, the impact of this antibody against the TfR on erythropoiesis and other organs by affecting iron metabolism is yet to be addressed.

In summary, the anti-TfR human–mouse chimeric antibody is able to specifically bind to TfR, which is highly expressed on the surface of activated immunocytes. As a result, they showed high capacity to inhibit cell proliferation and induce CDC. Therefore, administration of an anti-TfR human–mouse chimeric antibody to recipient mice prolonged the survival time by decreasing the proliferation of human immunocytes in response to antigen stimulation and by inhibiting the activation of lymphocytes associated with impaired graft anti-host reaction. Although its effect is not as strong as the anti-CD4 human–mouse chimeric antibody, the anti-TfR human–mouse chimeric antibody has significant less side effects, and therefore, it could serve as a new immunosuppressive agent in the clinical treatment of graft rejection.

Authorship

QY, ZW, GS and SW: research design. ZW, YL, HZ and HZ: acquisition of data. QY, YL and PL: data analysis and result interpretation. GS: administrative, technical, or material support.

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