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Original Article

Comparative study of human and cynomolgus T-cell depletion with rabbit anti-thymocyte globulin (rATG) treatment-for dose adjustment in a non-human primate kidney transplantation model

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Abstract: Rabbit-antithymocyte globulin (rATG) is commonly used in kidney transplantation (KT) as an induction agent and is also commonly used in non-human primate (NHP) KT models. However, the optimal dose has not been reported. In this study, we evaluated which cumulative dose of rATG was most appropriate for transplantation in NHPs. Cynomolgus monkeys were treated with intravenous 5 mg/kg rATG (Thymoglobulin®, Genzyme Ltd., UK) twice, on days 0 and 2 (a total of 10 mg/kg, n=2), or 4 times, on days 0, 1, 2, and 3 (a total of 20 mg/kg, n=6). In addition, we performed allo-KT in cynomolgus monkeys (n=4) with a cumulative 20 mg/kg dose of rATG with optimized dosing for induction therapy. We further compared immune cells, including naïve, central memory, and effector memory T cells, in reconstituted distributions in human KT patients (n=22). The kinetics of lymphocytes showed a rapid decrease at day 1 that was maintained for 2 weeks in the 20 mg/kg rATG group, while lymphocyte depletion was not maintained for more than 1 week in the 10 mg/kg rATG group. During the early period of rATG treatment in the NHP-KT model, the frequency of total T cells in the 20 mg/kg group showed a pattern of depletion similar with that of KT patients treated with rATG (1.5 mg/kg, 3 days). However, the pattern of reconstituted T cell subpopulations was different, as the number of effector memory cells rebounded in the NHP-KT model. These data indicate that lymphocyte-depletion induced by rATG was influenced by cumulative dose, and that an rATG dose of 20 mg/kg is suitable for induction therapy in renal transplantation in cynomolgus monkeys compared to human KT.

Keywords: Rabbit-antithymocyte globulin, immunosuppressants drug, induction therapy, kidney transplantation, cynomolgus monkey

Introduction

Rabbit-antithymocyte globulin (rATG) is purified polyclonal immunoglobulin used as an induction immunosuppressant in various organ transplantation fields. rATG induces T cell depletion by prompting complement-dependent cell lysis and affects the cell surface and adhesion molecules that regulate T cell function and leukocyte endothelial interactions [1-3]. rATG is also commonly used in preclinical animal studies utilizing non-human primates (NHPs) [4-10]. However, the optimal dose of rATG has not been determined and the dose, duration and frequency of rATG injection varied in among studies. Preville *et al* demonstrated that rATG treatment of cynomolgus monkeys induced dose-dependent lymphopenia in the blood and, to a lesser extent, in the spleen and lymph nodes, but not in the thymus [4]. In the same study, low-dose rATG (1 mg/kg×8 doses) induced significant T cell depletion, while high-dose rATG (5 mg/kg×8 doses) induced major T cell depletion. A very high dosage (20 mg/kg×8 doses) induced almost complete T cell depletion in the lymph nodes and spleen. These data

suggest that the magnitude of T cell depletion in peripheral tissues may be related to the peak concentration of rATG rather than the cumulative dose. However, in clinical transplantation, the cumulative dose of rATG has remained an issue in kidney transplantation (KT) recipients [11, 12].

Therefore, we compared the effect of two cumulative doses (Group 1; 5 mg/kg×2 days, Group 2; 5 mg/kg×4 days) of rATG in NHP (cynomolgus monkey) model by analyzing the effects on white blood cell (WBC) subpopulations. WBC subpopulation analysis data from human KT recipients treated with rATG were used as the standard. We attempted to identify the cumulative dose that would produce a similar WBC subpopulation to use in humans. In addition, we looked for doses that could produce effective and sustained T cell clearance, since higher lymphocyte counts after treatment with a polyclonal preparation are reportedly associated with higher rejection rates and may decrease graft survival [13].

Materials and methods

Experimental animals

Male and female 2- to 3-year-old cynomolgus monkeys (*Macaca fascicularis,* Cambodia) weighing between 2.5 and 5 kg were used in this study. Our detailed husbandry strategy is described in a previous report [14]. Eight monkeys were used to test rATG and four allo-KT recipient monkeys were used to confirm the results of rATG induction. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act in the animal facility of the Primate Organ Transplantation Research Center at Genia (Sung-nam City, Korea). This experiment was also approved by the Institutional Animal Care and Use Committee (IACUC) at Genia (IACUC number; ORIENT-IACUC-14194).

ATG preparation, doses injected, and treatment schedule

rATG (Thymoglobulin®, Genzyme Ltd., UK) diluted in 50 ml of normal saline was injected intravenously at a rate of 5 ml/hr via the internal jugular vein using a catheter and automatic syringe pump. Before procedures, monkeys were sedated with intramuscular ketamine hydrochloride (10 mg/kg, Yuhan, Seoul, Korea). In Group 1, cynomolgus monkeys were treated with 5 mg/kg rATG twice on days 0 and 2 (totally 10 mg/kg, n=2); in Group 2, they were treated four times on days 0, 1, 2, and 3 (totally 20 mg/kg, n=6). Twenty mg/kg solu-cortef, 4 mg peniramine and 200 mg propacetamol were injected to prevent possible side effects. During the experiment, we assessed the animals at least twice a day for any symptoms related to infection. However, there were no instances of cough, diarrhea, loss of appetite, or lethargy. C-reactive protein (CRP) was also measured twice a week for any sign of inflammatory reactions.

Immune suppression protocol in cynomolgus monkey kidney transplantation

We performed MHC-mismatched allo-KT in cynomolgus monkeys (n=4). The basic immunosuppressive protocol consisted of rATG (5 mg/kg, intravenous), tacrolimus (2 mg/kg/day twice daily with a target trough level of 10-15 ng/mL, oral) and mycophenolate (500 mg twice daily, oral). rATG was administered on day 0 and postoperative days 1, 2 and 3. Tacrolimus and mycophenolate were started on day 1. A protocol biopsy was performed on day 14 and recipients with biopsy proven rejection (BPR) were treated with 20 mg/kg methylprednisolone once daily for 3 days. Ganciclovir (5 mg/kg) was administered daily until day 14 in all animals. Four KT recipients who did not experience clinical/subclinical rejection were selected for analysis of the kinetics of WBC subpopulations.

Immune suppression protocol in human kidney transplantation

From June 2014 to September 2016, 22 human patients without immunological risk, such as ABO incompatibility, positive crossmatch, or presence of a donor-specific HLA antigen, underwent KT with low-dose rATG induction (1.5 mg/kg, 3 days) at Samsung Medical Center. Among them, seven patients who did not experience a BPR episode were enrolled in this study. rATG was administered at a dose of 1.5 mg/kg/day on day 0 and postoperative days 1 and 2 as induction immunosuppression. Patients were initiated on tacrolimus (0.1 mg/

kg/day with a target trough level of 8-10 ng/mL) and mycophenolate (1080 mg twice daily) on postoperative day 3. All transplant recipients were given 500 mg of intravenous methylprednisolone during the operation until postoperative day 2, followed by a tapered dose of 60 mg per day for a period of five days and 8 mg prednisolone, twice per day, for one month thereafter starting on postoperative day eight. After that, recipients received 4 mg of MPD twice a day for 2 months. Data on WBC subpopulations of patients were collected 1 week, 2 weeks, 1 month, 3 months, and 6 months after KT. This study protocol was reviewed and approved by the Institutional Review Board of Samsung Medical Center, Sungkyunkwan University School of Medicine (IRB No. SMC 2012-09-019).

Complete blood analysis and flow cytometric analysis

Peripheral blood samples were periodically obtained from the saphenous vein and collected in an EDTA-coated tube. These samples were used to analyze complete blood counts and for flow cytometry. Chemistry analysis included alanine aminotransferase (ALT), aspartate aminotransferase (AST) blood urea nitrogen (BUN), and creatinine. Clinical hematology information included whole blood cell counts, neutrophil counts and lymphocyte counts.

For flow cytometric analysis in cynomolgus monkeys, we used the following monoclonal antibodies that had cross-reactivity with NHPs; FITC CD20 (2H7), PE CD56 (MY31), PE CD28 (CD28.2), PerCP-cy5.5 CD4 (L200), PerCP5 CD21 (B-ly4) PE-cy7 CD3 (SP34-2), PE-cy7 CD20 (2H7), PE-cy7 CD14 (M5E2), APC CD95 (DX2), APC CD14 (M5E2) and APC-H7 CD8 (SK1) from BD Pharmingen™ (San Diego, CA, USA), and V450 CD27 (M-T271), V450 CD16 (3G8) and BV510 NHP-CD45 (D058-1283) from BD Biosciences (San Jose, CA, USA). T-lymphocyte subsets in macaque peripheral blood were analyzed based on surface expression of NHP-CD45, CD3, CD4, and CD8. Both $CD4^+$ helper T cells and $CD8^+$ cytotoxic T cells can be divided into two major subsets, naïve and memory cells. We used surface expression of CD28 and CD95 to delineate the naïve ($CD28^+CD95^-$), central memory (CM; $CD28^+CD95^+$) and effector memory (EM; $CD28^-CD95^+$) T cell subpopulations [15]. We analyzed the B cell subset in cynomolgus monkeys based on CD20 expression as the definitive B cell marker, because commercial CD19 monoclonal antibodies were weakly cross-reactive with macaque B-lymphocytes [16]. For immune monitoring in human kidney transplantation, we analyzed data based on previously reported human immunophenotyping [17].

Peripheral blood mononuclear cells (PBMCs) isolated using Ficoll-Plaque PLUS (GE Healthcare Life Sciences) were stained with relevant antibodies in PBS for 30 min at 4°C in the dark. Stained cells were washed several times with PBS and analyzed on the LSR Fortessa (Beckton Dickinson, USA) with FlowJo software (Treestar). The percentages of cell subpopulations on flow cytometric analysis were converted to absolute numbers using lymphocyte count per microliter of whole blood.

Histological and immunohistochemical analysis

Spleen fragments and mesenteric lymph nodes were obtained from two monkeys in Group 2 to evaluate the suppressive effects of rATG at totally 20 mg/kg. These tissues were collected before and after rATG induction at the indicated time points and were fixed in 10% neutral buffered formalin solution and embedded in paraffin. The paraffin-embedded tissue samples were created from 4 μm tissue sections processed for hematoxylin and eosin (H&E) staining. For immunohistochemical analysis, serial paraffin sections were single stained using anti-human CD3 and anti-human CD20cy antibodies (DAKO) and the Dako EnVision™ detection system horseradish peroxidase (HRP)/DAB kit. The slides were counterstained with hematoxylin. All anti-human antibodies used in this experiment had confirmed cross-reactivity in cynomolgus monkeys.

Statistical methods

Absolute numbers of cells in each WBC subpopulation after rATG injection in each group were compared by Mann-Whitney test. Statistical analysis was performed with SPSS version 22.0 (IBM, Armonk, NY, USA) and statistical significance was defined as a P value <0.05.

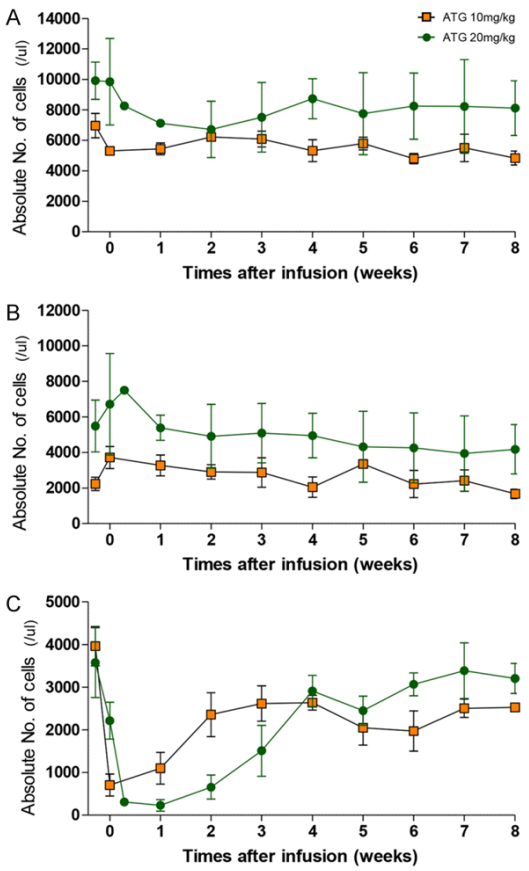

Figure 1. The absolute number of white blood cells, neutrophils and lymphocytes in peripheral blood of cynomolgus monkeys with rATG induction. Absolute numbers of white blood cells (A), neutrophils (B), and lymphocytes (C) were counted after rATG induction at a different dose. Orange squares represent total dosage of 10 mg/kg rATG, and green circles represent total dosage of 20 mg/kg rATG. This data is shown average ±SEM.

Results

Influence of different dosages of rATG on blood components in NHP

Total WBC count and absolute neutrophil count (ANC) suppression were similar between the two groups (**Figure 1A** and **1B**). However, absolute lymphocyte count (ALC) suppression was stronger and more prolonged in Group 2 (**Figure 1C**).

In both groups, rapid induction of T cells by rATG was shown in the periphery (**Figure 2A**). Although rATG induction is commonly used for depletion of T cells, peripheral B cells were also suppressed together with T cells (**Figure 2B**). In the early period of rATG induction, the total frequency of T cells in Group 2 was almost nonexistent. In the recovery state, $CD8^+$ cells reconstituted earlier than $CD4^+$ cells and were maintained at a higher proportion (**Figure 2C** and **2D**).

In newly produced $CD4^+$ cells after rATG induction, naïve cells comprised most of Group 1 and effector memory phenotype T cells comprised most of Group 2 (**Figure 3A-C**) In newly recovered $CD8^+$ cells, similar suppression patterns were observed in the two groups (**Figure 3D-F**).

In these monkeys, hepatic and renal parameters were monitored to determine toxic response to high doses of rATG induction. There was no liver or kidney toxicity during the follow-up period although both groups received relatively high doses of rATG (**Figure 4**).

Based on the induction results of rATG treatment, rATG at 20 mg/kg was more effective at suppression in the periphery without evidence of toxicity. In addition, in the case of KT, induction therapy with 20 mg/kg rATG was appropriate for maintenance of peripheral T cell count with a target trough level of 500 cells/μl or less.

Influence of rATG on peripheral lymphoid tissues

We performed immunohistochemical analysis on peripheral lymphoid organs to evaluate the suppressive effects of 20 mg/kg rATG treatment. In the lymph nodes, rATG had a weak suppressive effect on $CD3^+$ T cells for the two weeks after drug injection (**Figure 2E**). However, $CD20^+$ B cells in the lymph nodes gradually declined up to three weeks after induction (**Figure 2E**). These results are inconsistent with peripheral blood results which showed rapid depletion of B cells. In the spleen, $CD3^+$ cells and $CD20^+$ cells were suppressed until two weeks after injection and started to recover at three weeks (**Figure 2F**).

Influence of rATG on WBC subpopulations in NHP compared to human KT

We observed that a cumulative dose of 20 mg/kg rATG achieved the target lymphocyte count of 500 cells/μl or less after more than 2 weeks. The use of 20 mg/kg rATG in monkeys is considered an appropriate dose for renal trans-

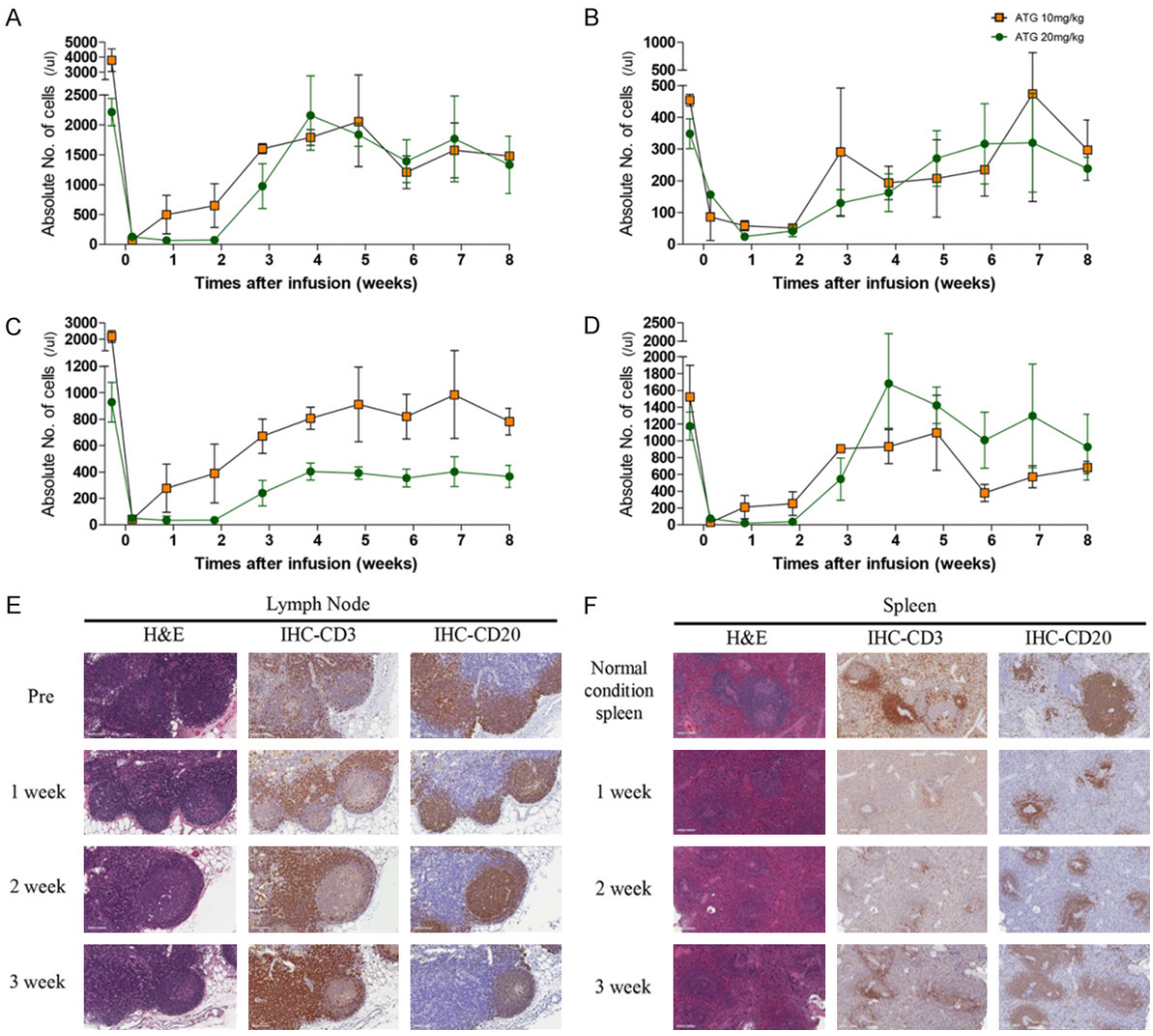

Figure 2. Comparison of lymphocyte subsets between cynomolgus monkeys that received immunosuppressants at different doses of rATG in the periphery and secondary lymphoid organs. Counts of pan-CD3$^+$ T cells (A), pan-CD20$^+$ B cells (B), helper CD4$^+$ T cells (C), and cytotoxic CD8$^+$ T cells (D) were measured by flow cytometry after rATG treatment. These graphs are shown that totally 10 mg/kg of rATG treatment is orange square, and totally 20 mg/kg of rATG treatment is green circle. This data is shown average ±SEM. Histological analysis of the lymph note (E) and spleen (F) after induction of rATG with 20 mg/kg. Immunohistochemistry was performed with anti-CD3 and anti-CD20cy antibodies, and analyzed every week for 3 weeks after rATG treatment with 20 mg/kg.

plantation conditioning compared with human KT. Therefore, we attempted renal transplantation in monkeys with this initial conditioning regimen.

After rATG induction and kidney transplantation, neither humans nor monkeys showed a significant change in neutrophil numbers (**Figure 5B**). In human KT, lymphocytes were depleted to nearly 500 cells/μl after rATG treatment (1.5 mg/kg×3 days) and suppression was maintained. Cynomolgus KT led to more suppressed lymphocyte counts at one week (92.3% reduction compared to pre-operative counts) than in human KT (69.0% reduction) (**Figure 5C**). However, within total lymphocyte count, there was no significant difference between the two groups within 1 month post-KT (**Figure 5C**). Even though there was no difference in lymphocyte counts, the absolute number of T cells was significantly different until 3 months post-KT. The number of CD8$^+$ T cells in monkeys was greatly increased, resulting in a higher total T cells than in humans (**Figure 5D-F**).

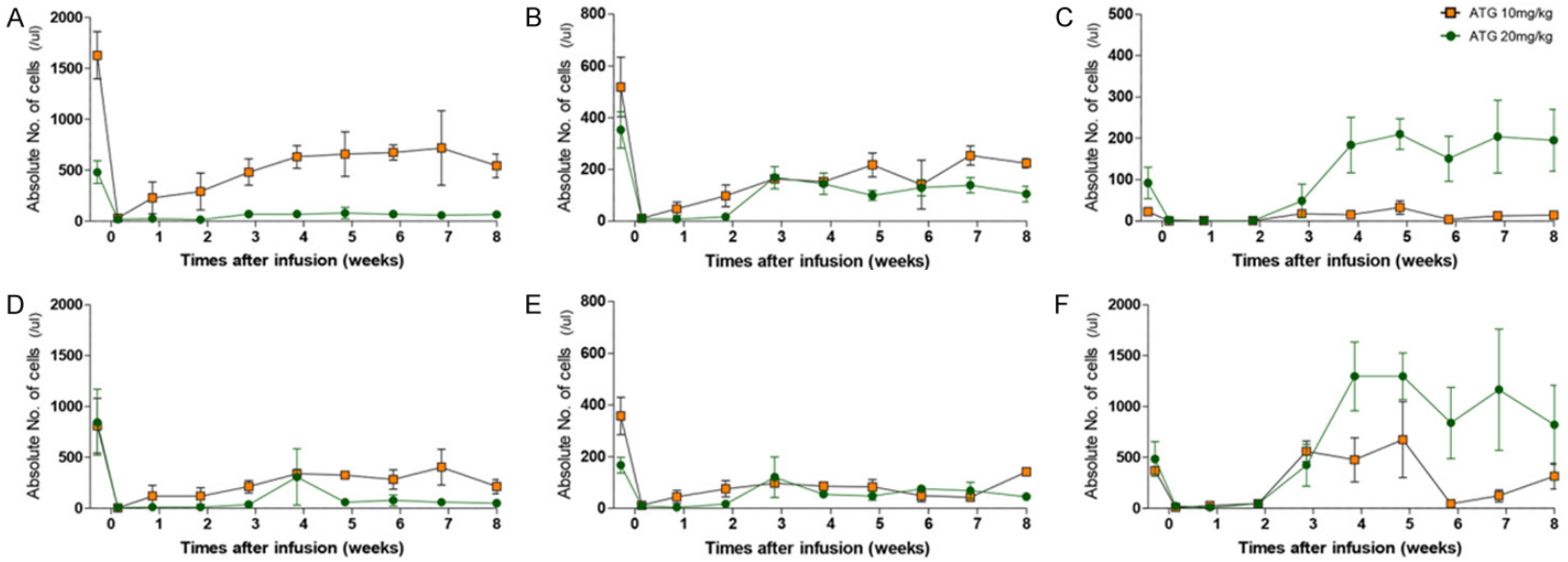

Figure 3. The comparison of lymphocyte subpopulations between cynomolgus monkeys that received immunosuppressants at a different dose of rATG. The frequency of naïve ($CD28^+CD95^-$), central memory (CM; $CD28^+CD95^-$) and effector memory (EM; $CD28^-CD95^+$) in two major subset of T cells was calculated based on ALC data. The frequencies of $CD4^+$ naïve (A), $CD4^+$ CM (B), $CD4^+$ EM (C), $CD8^+$ Naïve (D), $CD8^+$ CM (E), and $CD8^+$ EM (F) were measured by flow cytometry. These graphs are shown that totally 10 mg/kg of rATG treatment is open orange square, and totally 20 mg/kg of rATG treatment is closed green circle. This data is shown average ±SEM.

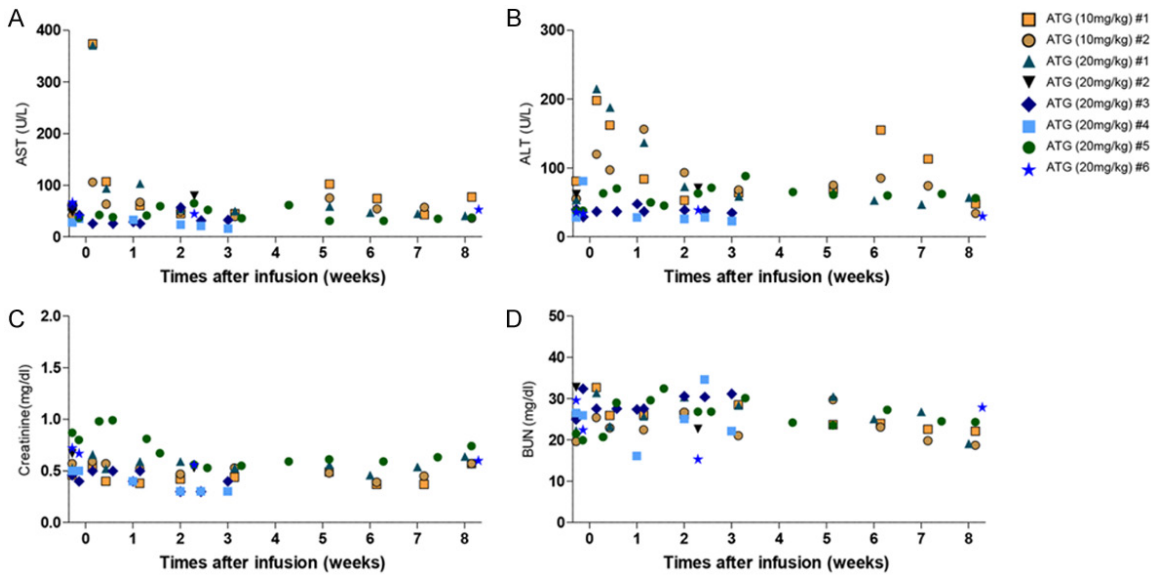

Figure 4. Serum chemistry in the all experimental regimens. Kinetics of Serum level of AST (A), ALT (B), Creatinine (C) and blood urea nitrogen (D) were serially monitored after rATG induction.

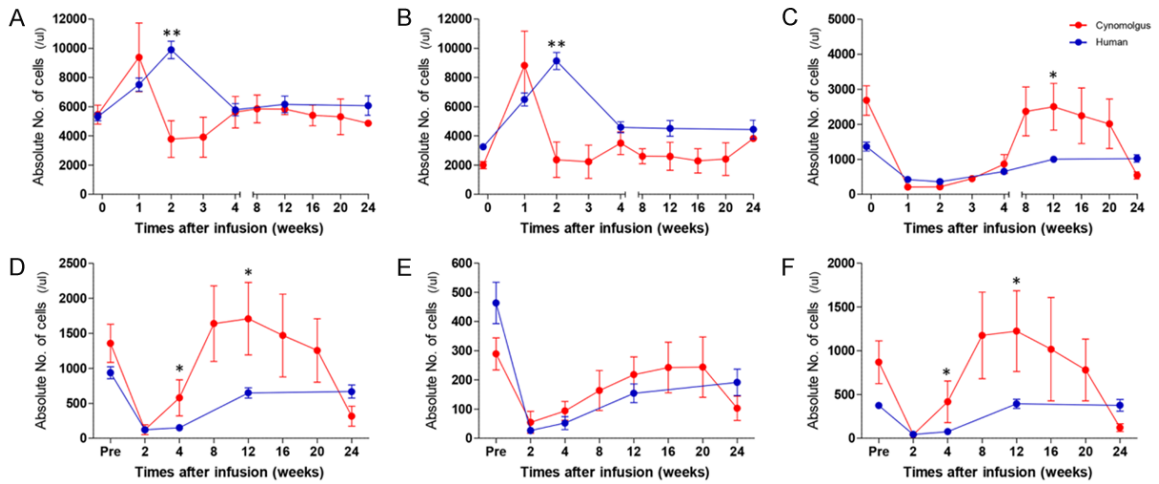

Figure 5. Comparison of Human KT versus Cynomolgus monkey KT. The absolute number of white blood cells (A), neutrophils (B) and lymphocytes (C) was serially monitored in NHP KT and human KT. The counts of pan-CD3$^+$ T cells (D), helper CD4$^+$ T cells (E) and cytotoxic CD8$^+$ T cells (F) were measured by flow cytometry after KT. These graphs are shown that red circle is the result of cynomolgus monkey post-transplantation (totally 20 mg/kg of rATG) and blue circle is the result of human renal kidney transplantation (totally 4.5 mg/kg of rATG). This data is shown average ±SEM. $^*P<0.05$ and $^{**}P<0.005$ versus the two groups at same time point.

Reconstituted compartments of CD4$^+$ T cells and CD8$^+$ T cells were different in the two groups (**Figure 6**). Lymphopenia-induced expansion of memory phenotype CD8$^+$ T cells occurred more rapidly than that of memory CD4$^+$ T cells in all regimens. Especially in NHPs, memory-phenotype cells started to recover first and made up a large portion of the newly reconstituted T cells (**Figure 6**). Further, within the CD4$^+$ T cell compartments, central memory T cells made up majority of cells in primates (**Figure 6B**), while effector memory T cells comprised a large part of human's CD4$^+$ T cells (**Figure 6C**). During NHP-KT, naïve T cells were much more susceptible to rATG induction compared to human KT. Each subset of human T cells by percentage was almost unchanged throughout the early period post-KT compared

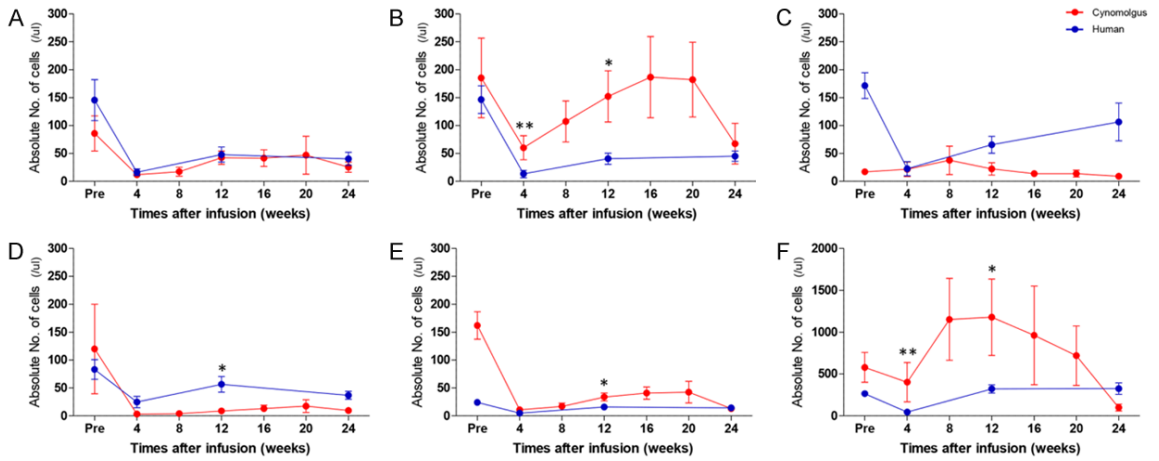

Figure 6. Comparison of Kinetics of T cell subpopulations in human KT and cynomolgus monkey KT. The T cell subsets were measured by flow cytometry. These graphs are kinetics of $CD4^+$ naïve (A), $CD4^+$ CM (B), $CD4^+$ EM (C), $CD8^+$ Naïve (D), $CD8^+$ CM (E), and $CD8^+$ EM (F) after KT in both species. These graphs are shown that red circle is the result of cynomolgus monkey KT (totally 20 mg/kg of rATG) and blue circle is the result of human KT (totally 4.5 mg/kg of rATG). This data is shown average ±SEM. $^*P<0.05$ and $^{**}P<0.005$ versus the two groups at same time point.

to pre-KT. These findings demonstrate that the pattern of recovery of T cell subpopulations in primates differ from that of human KT patients.

Discussion

NHPs have unique advantages as a transplant animal model. The immune system of NHPs is more similar to human systems than other laboratory animals, including with regard to endothelium-expressed MHC class II, innate immunity, and memory T cells [18]. To achieve reproducibility, an animal model with an immune system very similar to that of humans is needed in tolerance induction research.

In this study, we attempted to determine the cumulative dose of rATG that most effectively suppressed lymphocytes in NHPs. We also compared immunological responses between an NHP KT model (treated with a total of 20 mg/kg rATG) and human KT recipients (treated with a total of 4.5 mg/kg rATG). The rATG dosage in cynomolgus monkeys required to achieve suppressed lymphocyte count similar to that in humans was more than four-fold of human dosage. These results are possibly associated with species differences in the epitopes of rATG. After rATG infusion in cynomolgus monkeys, clearance of rATG results from a strong anti-rabbit IgG response within 2 weeks, regardless of dosing [4]. Serum level of active rATG in hematopoietic stem cell transplant recipients who received 10 mg/kg of rATG was undetectable within 40 days [19]. Because of the species differences in rATG clearance rates between humans and NHPs, cynomolgus monkey transplant models need to be treated with relatively high doses of rATG.

In Group 2 (rATG induction of 5 mg/kg×4 days in monkeys), lymphocyte counts decreased below 500 cells/μl and were suppressed for about two weeks. However, in Group 1 (5 mg/kg×2 days), lymphocyte counts did not decrease below 500 cells/μl and were suppressed for only one week. In human KT recipients with rATG induction (1.5 mg/kg×3 days), lymphocytes were depleted to nearly 500 cells/μl, and this level was maintained for two weeks. In addition, only Group 2 reached a depleted T cell count similar to that seen in human KT. We did not observe any symptoms associated with side effects of high-dose rATG in these regimens. Therefore, we conclude that rATG induction in Group 2 was more similar to human induction and is suitable for use in an NHP solid organ transplantation model.

T cell reconstitution after induction therapy occurred mainly through effector memory T cells, which is consistent with previous studies. Haanstra *et al* demonstrated that naïve cells comprised only 38% of $CD4^+$ T cells and 23% of $CD8^+$ T cells 21 days after rATG injection (20 mg/kg×2 doses) in rhesus monkeys [20].

Gurkan *et al* reported that rATG-induced T cell depletion is followed by immune reconstitution, with both new thymic emigration of naïve T cells and homeostatic proliferation of depletion-resistant memory T cells [5, 21]. In human KT recipients in this study, effector memory T cells comprised a major portion of newly produced T cells. In our clinical and NHP study, recovering T cells after lymphopenia represented similar patterns of reconstituted memory-phenotype T cells, although the kinds of memory-phenotypes were different. However, Group 1 receiving rATG at total of 10 mg/kg showed insufficient suppression. Because of that, they might not show the same expansion of memory-phenotype cells after lymphopenia [22-24].

In NHP-KT, $CD4^+$ cells and $CD8^+$ cells were suppressed for more than four weeks. However, in Group 2, $CD4^+$ cells and $CD8^+$ cells started to recover two weeks after KT. When used as a maintenance immunosuppressive agent, tacrolimus suppresses cytokine production from memory T cells and proliferation of memory T cells [25]. Therefore, using of tacrolimus explains this prolonged T cell suppression and is essential for the NHP-KT model.

B cells were suppressed in NHPs after rATG treatment even in Group 1 (total of 10 mg/kg). Zand *et al* demonstrated that B cell suppression by rATG is dependent on the serum concentration of rATG [26]. rATG contains antibodies against numerous B cell surface proteins such as CD19, CD20, and CD38 [27]. Therefore, this result is possibly explained by lower serum rATG concentration in humans than in NHPs, or there may be more surface proteins that can be the targets of rATG expressed on NHP B cells. Although the depletion mechanism of rATG is well known [28], but further experiments to clarify the cause are necessary in monkeys.

rATG treatment is effective not only for reducing acute cellular rejection, but also for achieving tolerance induction. Previous studies reported that conditioning transplant recipients with rATG induces suppressive allogeneic regulatory T cells (Tregs) [29] and NK T cells ($CD3^+CD161^+$) [30]. At low doses of rATG, Tregs are maintained through the immunological impact [27, 29-31]. Relatively high doses of rATG decrease frequency of total Tregs in NHP-KT. Therefore, in the field of organ transplantation, pre-conditioning with rATG has the likelihood to achieve induction of tolerance by modification of the post-transplant immune system.

The lymphocyte depletion induced by rATG was influenced by cumulative dose. A rATG dose of 20 mg/kg (5 mg/kg×4 days) effectively depleted lymphocytes and is suitable for induction therapy in an NHP organ transplantation model.

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Disclosure of conflict of interest

None.

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