ORIGINAL ARTICLE

Rabbit antithymocyte globulin induces human lymphocyte activation, proliferation, and apoptosis in the absence of complement: an experimental study

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SUMMARY

Rabbit antithymocyte globulin (rATG) has become the first choice for induction therapy in HLA-presensitized patients undergoing organ transplantation. Meanwhile, complement inhibitors have been approved for preventing or treating antibody-mediated rejection in these patients. The biological effects of rATG on lymphocytes in cases of complement deficiency or significant inhibition are not yet clear. We measured lymphocyte activation, proliferation, and apoptosis in response to rATG treatment in the absence of complement. T-cell subsets were analyzed transcriptomically features to rATG stimulation. Activation-related phenotypes on T cells were determined in patients after rATG administration. We found that rATG treatment led to lymphocyte activation and proliferation *in vitro* without the addition of complement. A dose-dependent apoptosis in rATG-treated lymphocytes was detected, which was partially caspase-3-dependent but Fas/FasL-independent. T cells were more sensitive to rATG stimulation than were non-T cells. Both $CD4^+$ T cells and $CD8^+$ T cells upregulated a series of genes related to cell activation, cytokine production and apoptosis to rATG stimulation. CD69 and CD25 levels in surviving T cells were increased in patients after rATG administration. These findings indicate that rATG can stimulate lymphocyte activation, proliferation, and apoptosis in the absence of complement. Biologic effects of rATG other than complement-dependent cytotoxicity need to be concerned.

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Introduction

Over the past 40 years, rabbit antithymocyte globulin (rATG) has been one of the most widely used agents in clinical induction therapy to suppress allograft rejection and/or prevent graft-versus-host disease in solid organ transplantation and allogeneic stem cell transplantation [1–3]. Given the large amounts of specific cluster of differentiation (CD) antibodies present in polyclonal rATG [4], treatment with rATG primarily results in a rapid depletion of T lymphocytes from peripheral blood as a result of complement-dependent cell lysis [5]. rATG is advantageous in preventing acute rejection among transplant recipients at high risk [6], especially in human lymphocyte antigen (HLA)-presensitized patients [7]. Therefore, it is the first choice for induction therapy in HLA-presensitized patients undergoing organ transplantation [8,9].

Increasing evidence indicates an integral role for the complement system in the deleterious inflammatory reactions that occur during critical phases of the transplantation process [10]. In recent decades, the complement system has been shown to play a prominent role in antibody-mediated rejection (AMR), which leads to devastating damage to the transplanted organ [11]. HLA sensitization in transplant recipients is a critical risk factor for, and correlates strongly with, post-transplant AMR [12–15]. Despite the fact that the standard of care for desensitization is extensively applied, AMR remains a significant problem [16,17]. Therefore, other more effective strategies, such as complement inhibition, are needed to facilitate kidney transplantation in sensitized individuals [18].

A number of complement-inhibiting drugs have been used to block the activation of C1, C3, or C5 in clinical trials for the prevention or treatment of AMR [10]. Clinical evidence has confirmed the efficiency of complement inhibitors in the prevention of early and late AMR, thus improving allograft function in sensitized patients [19–23]. However, these sensitized patients received rATG induction therapy at the same time. Given the pivotal contribution of the complement system to the cytotoxic effect of rATG, it is reasonable to speculate that T-cell depletion in patients treated with complement inhibitors is significantly affected. The biological effects of rATG on lymphocytes in cases of complement deficiency or significant inhibition of complement activation are not completely clear. In addition, it is noteworthy that rATG-induced T-cell depletion is different in the peripheral blood and lymphoid organs [24]. rATG can deplete T cells in the blood much more effectively than T cells in lymphoid tissues. Therefore, it is also important to pay attention to the biological effects of rATG stimulation on residual surviving T cells.

It has been reported that OKT3, an anti-human CD3 antibody, is a potent T-cell activator and can induce T-cell mitogenesis and cytokine release *in vitro* [25]. However, little is known about whether rATG also has activating effects on lymphocytes in the absence of the complement system. Here, we have designed *in vitro* experiments to investigate whether the polyclonal antibodies contained in rATG could lead to lymphocyte activation, proliferation, cytokine release, and the complement-independent cytotoxicity in the absence of complement system activity.

Materials and methods

Populations

Blood samples from renal transplant recipients ($n = 8$) and healthy donors ($n = 20$) were collected at Tongji Hospital and Wuhan Blood Center, respectively. Recipient characteristics and medical reasons for brain death of the donors in this study are listed in Table 1 and Table 2, respectively. The ethics committee of Tongji Medicine College, Huazhong University of Science and Technology granted approval for all aspects of this study. All patients gave written informed consent. All human subjects were adults. Immunosuppression protocols for renal transplant recipients were defined according to the immunologic risk, determined according to the current system based on lymphocytotoxic panel-reactive antibodies, donor-specific antibodies, and T- and B-cell-based assays. Patients received induction therapy consisting of rATG (Thymoglobulin, Sanofi) or anti-CD25 antibody (Basiliximab, Novartis).

Reagents and antibodies

CellTrace™ Violet was purchased from Thermo Fisher Scientific. Fluorescence-conjugated antibodies were purchased from BD Biosciences and Biolegend: fluorescent antibodies against CD3 (UCHT1), CD4 (RPA-4), CD8 (SK1), CD25 (M-A251), CD69 (FN50), Fas (DX2), active caspase-3 (92–605), and rabbit IgG (Poly4064). Annexin V and propidium iodide (PI) were from BD Biosciences. Blocking antibodies against Fas (ZB4) and FasL (10F2) were purchased from GeneTex. The

Table 1. Clinical characteristics of patients enrolled.

Induction	rATG (n = 6)	Basiliximab (n = 2)
Gender (M/F)	4/2	1/1
Age, years (year)*	41 (21–58)	47.5 (41–54)
Serum creatinine (μmol/L)*	1043 (653–1371)	872 (757-987)
Blood urea nitrogen (μmol/L)*	22.18 (13.6–34.02)	27.88 (26.61–29.15)

* Data were shown as median (range).

Table 2. Cause of death for deceased donors enrolled.

Causes	Stoke	Trauma	Anoxia
Number	5	2	1
Specification	–	Car accident	–

caspase-3 inhibitor Z-DEVD-FMK was purchased from MCE.

Isolation and in vitro stimulation of peripheral blood mononuclear cells (PBMCs) and untouched T cells

Human PBMCs from patients and healthy donors were isolated by Ficoll density gradient centrifugation as described [26]. Untouched T cells were purified with a $CD4^+$ T Cell Isolation Kit and $CD8^+$ T Cell Isolation Kit (Miltenyi Biotec) by removing the magnetically labeled non-$CD8^+$/non-$CD4^+$ cells. Cells were seeded at 2.5×10^5 cells/well in 96-well round-bottom plates (Costar) containing 200 μl of RPMI-1640 medium (Gibco) with 10% heat-inactivated fetal bovine serum (Gibco) per well. rATG was added at various concentrations as indicated. Blocking antibodies against Fas and FasL (5 μg/ml), or the inhibitor Z-DEVD-FMK (50 μM) were added 2 h before the rATG stimulus. The plates were incubated in a humidified chamber at 37°C and 5% CO_2. Cells and supernatants were harvested at the times indicated.

Complement-dependent cytotoxicity (CDC) assays

PBMCs (5×10^5) were incubated with rATG at the concentrations indicated for 15 min at room temperature. Then, after two washes with phosphate-buffered saline, 50 μl of standard rabbit complement (Cedarlane) was added to each well. The mixtures were incubated for 30 min at room temperature, and supernatants were collected for the detection of cytokine production. Cell killing was measured by PI staining according to the manufacturer's instructions.

RNA-seq and bioinformatic data analysis

Total RNA was extracted from untreated $CD4^+$ or $CD8^+$ T cells with TRIzol (Invitrogen). Total RNAs (2 μg) were used for stranded RNA sequencing library preparation by means of a KC Stranded mRNA Library Prep Kit from Illumina (DR08502, Seqhealth) according to the manufacturer's instructions. The library products corresponding to 200–500 bp were enriched, quantified, and finally sequenced on an Hiseq X 10 sequencer (Illumina). The gene expression profiles of rATG-treated T-cell subsets and the corresponding control cells were determined by RNA-Seq data analysis (SeqHealth). In brief, raw sequencing data were first filtered by Trimmomatic (version: 0.36); low-quality reads were discarded, and adaptor sequences were trimmed. After quality filtering, each sample had ~40.4–54.8 million clean reads. Clean reads from each sample were mapped to the Homo sapiens GRCh38 reference genome using the Star program (2.5.3a). Significantly, differentially expressed transcripts were screened by applying the criteria FC ≥ 2 or ≤ -2 and P-value ≤ 0.05. The RNA-seq data, entitled "Transcriptome RNA-seq analysis for rATG-treated PBMCs," were deposited in the Sequence Read Archive (SRA) with BioProject number PRJNA659618. Gene Ontology (GO) analysis of differentially expressed genes was conducted using the GOseq R package, with a corrected P-value cutoff of 0.05 to determine statistically significant enrichment.

Flow cytometry

For surface staining and Annexin V/PI staining, harvested cells were stained with the appropriate antibodies and/or dyes according to the manufacturer's instructions. For proliferation assays, purified PBMCs were resuspended in phosphate-buffered saline with 0.1% bovine serum albumin and labeled with CellTrace™ Violet (CTV) at a density of 1×10^7 cells/ml according to the manufacturer's instructions; the dye dilution was analyzed at 72 h. Active caspase-3 was detected by

intracellular staining by using a Fixation/Permeabilization Solution Kit (BD Biosciences). Cytokines in culture supernatants were quantitated by using a Cytometric Beads Array kit for human Th1/Th2 cytokines (BD Biosciences) according to the manufacturer's protocols. Data were collected using FACSCelesta (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Statistics

Graphs were generated and analyzed by using GraphPad Prism 7.0 (GraphPad Software, San Diego, CA, USA). Data were analyzed by Student's *t*-test or paired *t*-test for comparisons of groups with normal distribution and equal variance. For non-normally distributed variables, a Mann–Whitney U test or Wilcoxon matched-pairs signed rank test was used. *P*-values less than 0.05 were considered statistically significant.

Results

rATG rapidly eliminates lymphocytes in the presence of complement

To verify the activity of the polyclonal depletion antibodies, we used flow cytometry to detect rabbit IgG binding to human lymphocytes as well as complement-dependent cytotoxicity (CDC) after co-culture of the lymphocytes with rATG at various concentrations. We observed that the majority of the lymphocytes rapidly bound rATG (Figure S1); after the addition of exogenous rabbit complement, we saw a significant dose-dependent killing effect of rATG on the lymphocytes (Figure 1a). At the same time, both Th1 and Th2 cytokines were almost undetectable in the supernatants (Figure 1b), indicating that the lymphocyte death caused by CDC did not cause the passive release of the cytokines.

rATG treatment leads to lymphocyte activation and proliferation in the absence of complement

To investigate whether rATG has activating effects on lymphocytes in the absence of the complement system, we measured lymphocyte proliferation, activation-related surface antigen expression, and cytokine production in response to stimulation with various concentrations of rATG. Lymphocytes showed strong proliferation *in vitro* after a 3-day co-culture with rATG (50 to 200 μg/ml) (Figure 2a). In addition, rATG treatment at >=50 μg/ml for 24 h produced markedly elevated transmembrane CD69 and CD25 expression on lymphocytes (Figure 2b, c). The increase in the expression of the leukocyte early activation antigen CD69 in response to rATG stimulation was much higher in T cells than in non-T cells (Figure 1b). We further examined the levels of IL-6, IL-10, IL-4, IFN-γ, TNF-α, and IL-2 in the culture supernatants by using a cytometric bead array kit. rATG treatment led to a substantial release of these Th1/Th2 cytokines in a dose-dependent manner (Figure 2d). Together, these results indicate that treatment with rATG induced a rapid activation of quiescent lymphocytes, coinciding with robust cytokine production.

rATG induces lymphocyte apoptosis in the absence of complement

Activated lymphocytes are subjected to an immunological elimination known as activation-induced cell death (AICD) [27]. To determine whether rATG treatment can trigger AICD in the absence of complement, we examined apoptosis *in vitro* in rATG-treated lymphocytes by Annexin V staining. In the 50, 100, or 200 μg/ml treatment groups, rATG induced a significantly increased apoptosis of lymphocytes at 24 h that was sustained up to 72 h (Figure 3a,b). The lymphocyte apoptosis appeared to be strongly dose-dependent but only weakly time-dependent (Figure 3b). When the dynamic effects of rATG on lymphocytes were observed, we noted that rATG treatment activated quiescent lymphocytes and simultaneously induced apoptosis. Notably, T-cell apoptosis began as early as 24 h, whereas non-T-cell apoptosis was detected after 48 h (Figure 3c). Thus, T cells were more sensitive to rATG stimulation than were non-T cells.

The expression of activation- and apoptosis-related genes in both $CD4^+$ and $CD8^+$ T cells is elevated in response to rATG stimulation

After confirming that T cells were highly sensitive to rATG stimulation, we were interested in exploring the expression of activation- and apoptosis-related genes in T-cell subsets. To this end, we purified $CD4^+$ T cells and $CD8^+$ T cells. After stimulation by rATG *in vitro*, the cells were used for transcriptome RNA-seq analysis or characterization. Cell sorting was performed by depletion of magnetically labeled non-$CD4^+$/non-$CD8^+$ cells to avoid target cell activation. The purity of both the sorted $CD4^+$ T cells and $CD8^+$ T cells exceeded 95% (Figure S2). According to the values for fragments per kilobase of exon per million mapped reads (FRKM), 3087 and 2250 transcripts at a fold change > 1 and false discovery rate

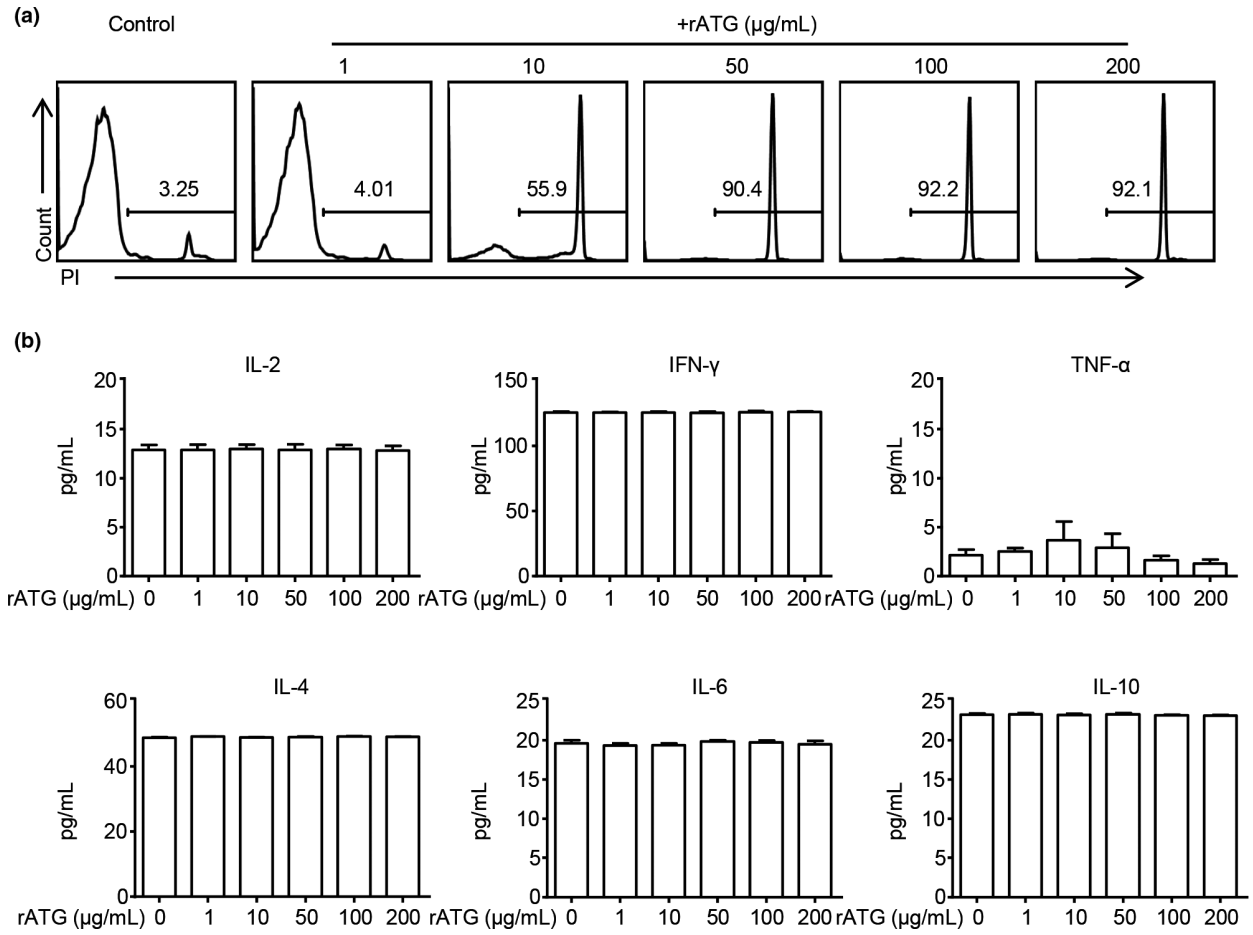

Figure 1 rATG treatment induces a rapid lymphocyte depletion coinciding with undetectable cytokine production in the presence of complement. Flow cytometric analysis of isolated PBMCs (5×10^5) in 96-well plates treated with rATG at the various concentrations indicated, or untreated, then incubated for 30 min with 50 μl of standard rabbit complement. (a) Representative graphs showing complement-dependent cell lysis of rATG-treated lymphocytes, as indicated by PI staining. The percentage of PI positive cells is shown. (b) Bar graphs summarizing the concentrations of Th1 (IL-2, IFN-γ, and TNF-α) and Th2 (IL-4, IL-4, and IL-10) cytokines in supernatants. All the values shown are means ± SEM and pooled from four independent experiments.

(FDR) of < 0.05 were upregulated in $CD4^+$ T cells and $CD8^+$ T cells, respectively, when compared to the corresponding control groups (Figure 4a,b). As highlighted on the heatmap, the upregulated genes were mainly involved in immune response and apoptosis (Figure 4a and Table S1). More than half of these elevated transcripts were common to both T-cell subsets (Figure 4c). When we analyzed the commonly upregulated genes using Gene Ontology (GO) enrichment analysis, we identified a series of terms related to apoptosis, T-cell activation, and cytokine production (Figure 4d).

rATG-induced apoptosis is partially caspase-3-dependent

In the periphery, caspase-dependent Fas/FasL interactions may contribute to lymphocyte AICD [28]. To investigate whether these apoptotic pathways are involved in rATG-induced lymphocyte apoptosis, we evaluated intracellular caspase-3 and Fas expression in the cell membranes of the lymphocytes. Treatment with rATG caused an increase in the expression of Fas on lymphocytes, but an upregulation of caspase-3 was only observed at 100 μg/ml, and not 50 μg/ml, rATG (Figure 5a). To further identify which pathway plays a role in rATG-induced apoptosis, we tested several apoptotic pathway inhibitors to see if they would prevent rATG-induced lymphocyte death. We found that the caspase-3 inhibitor Z-DEVD-FMK partially blocked apoptosis in the high-dose (100 μg/ml) rATG-treated group (Figure 5b). However, pretreatment with either anti-Fas or anti-FasL antibody had no anti-apoptotic effect. Taken together, these results suggest that rATG-induced lymphocyte apoptosis is partially caspase-3-dependent but Fas/FasL-independent.

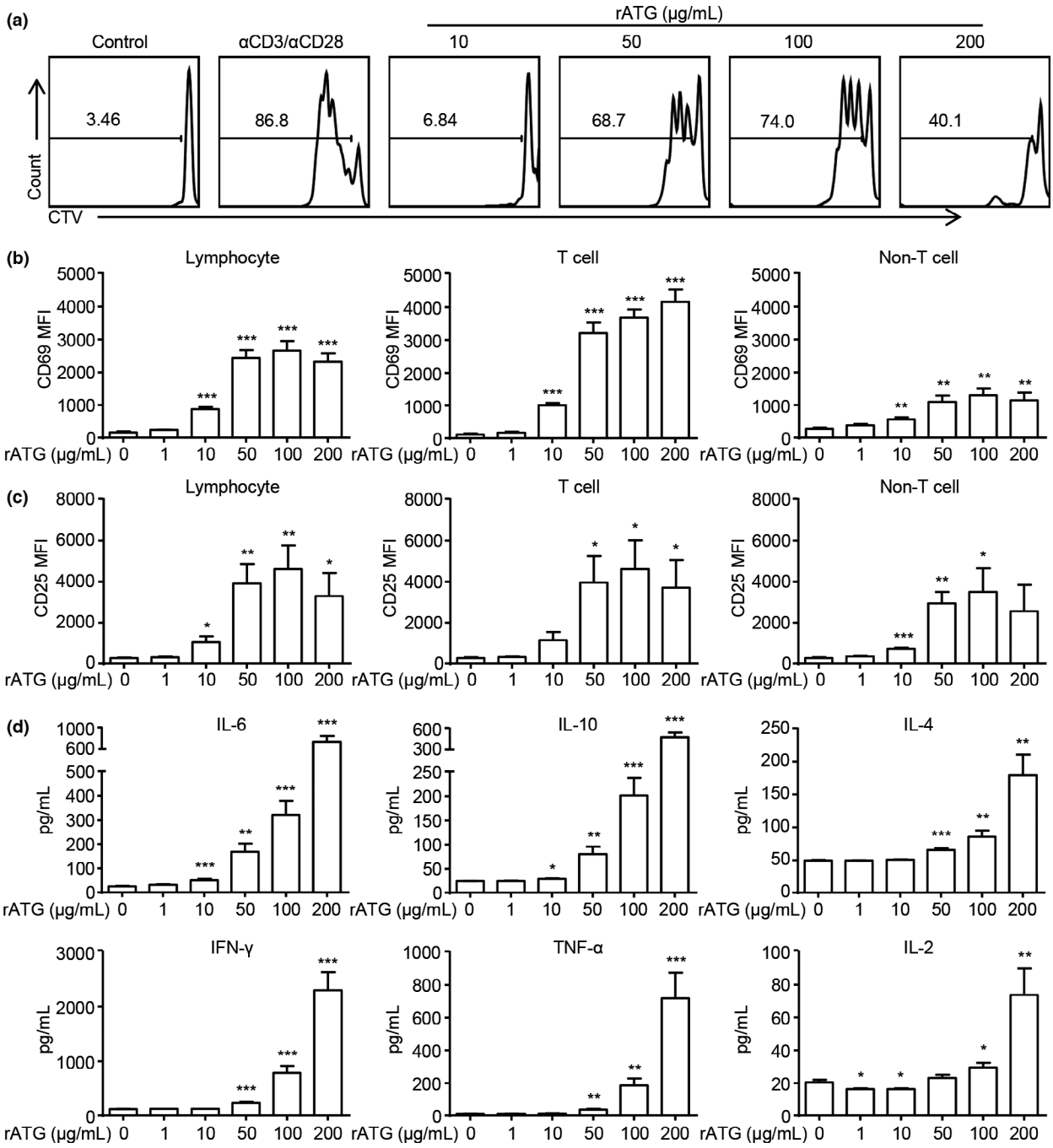

Figure 2 rATG treatment leads to lymphocyte activation, proliferation, and cytokine production in the absence of complement. PBMCs were treated with various concentrations of rATG as indicated. The control group was not treated with rATG. (a) PBMCs were stained with CTV and stimulated with rATG at the indicated concentration for 72 h. The αCD3/αCD28-stimulated group served as a positive control. Representative curves for lymphocyte proliferation are shown. Numbers within the graph denote the percentage of proliferated cells. (b and c) Summary bar graphs displaying CD69 (a) and CD25 (b) levels on lymphocytes, T cells, and non-T cells after rATG treatment for 24 h (n = 4 individuals per group). (d) Levels of IL-6, IL-10, IL-4, IFN-γ, TNF-α, and IL-2 in culture supernatants after rATG treatment for 24 h (n = 8 individuals per group). Bar graphs shown means ± SEM. *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the control group.

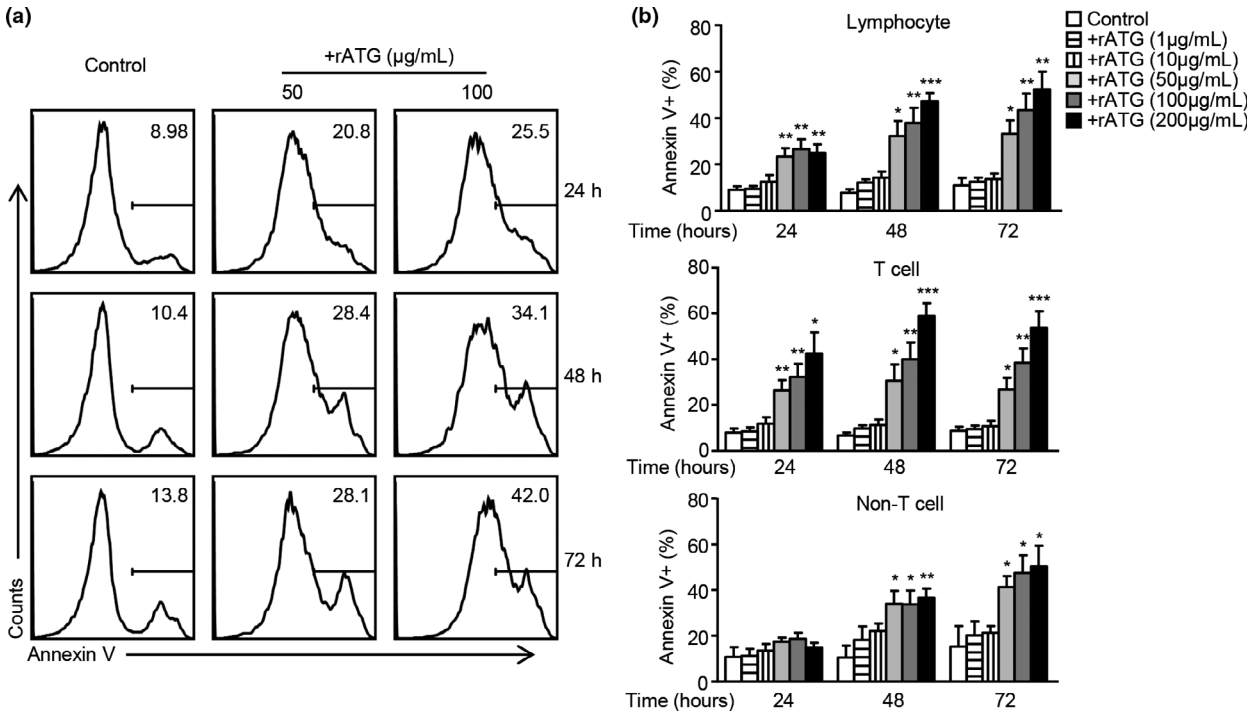

Figure 3 rATG directly induces lymphocyte apoptosis in the absence of complement. (a) PBMCs were treated with various concentrations of rATG for 24, 48, or 72 h, and apoptotic cells were measured by Annexin V staining. Representative curves for lymphocyte apoptosis are shown. Numbers within the graph indicate the percentage of Annexin V positive cell. (b) Bar graphs depicting apoptosis of lymphocytes (upper panel), T cells (middle panel), and non-T cells (lower panel) at 24, 48, and 72 h after treatment with various concentrations of rATG as indicated. Data represent the means ± SEM of three independent experiments (n = 4 individuals per group). *P < 0.05, **P < 0.01, and ***P < 0.001 vs. the control group.

Surface markers related to lymphocyte activation are elevated in transplant recipients after rATG administration

Given that rATG can induce rapid activation of T cells *in vitro*, we further examined whether transplant recipients display activated phenotypes in their T-cell subsets after rATG administration *in vivo*. To this end, using patients' lymphocytes, we measured rabbit antibody binding to T-cell subsets as well as CD69 and CD25 expression on the same subsets before and after rATG induction therapy. We found that rabbit IgG antibody was extensively bound to the surviving T cells obtained 24 h after rATG administration (Figure 6a). Furthermore, both CD69 and CD25 expression were remarkably elevated on residual $CD4^+$ and $CD8^+$ subsets (Figure 6a,b) after rATG adminstration. In contrast, the CD69 expression did not change on T cells from control patients receiving Basiliximab induction therapy (Figure 6a). Taken together, this evidence indicates that rATG has an immune-stimulating role beyond T-cell depletion.

Discussion

As the most widely used lymphocyte-depleting preparation in solid organ transplantation, rATG has been recommended as the first choice for induction therapy in recipients at high immunologic risk [29]. High peak serum levels (135 μg/ml average maximum) and a long circulating half-life have been reported for rATG, indicating its powerful function and usefulness for long-time maintenance [30]. However, the possibility that rATG has complex effects beyond T-cell depletion is not yet resolved. In the present study, we have obtained *in vitro* evidence to show that rATG treatment directly induces a rapid lymphocyte activation and proliferation, cytokine production, and complement-independent apoptosis in the absence of complement. T cells appear to be the key effector cells of these biologic effects. Furthermore, we found that patients treated with rATG show activated phenotypes on surviving T cells. The activation of T cells may be attributed to the T-cell target antibody pool present in rATG, including stimulators (such as anti-CD2 and anti-CD3) and co-

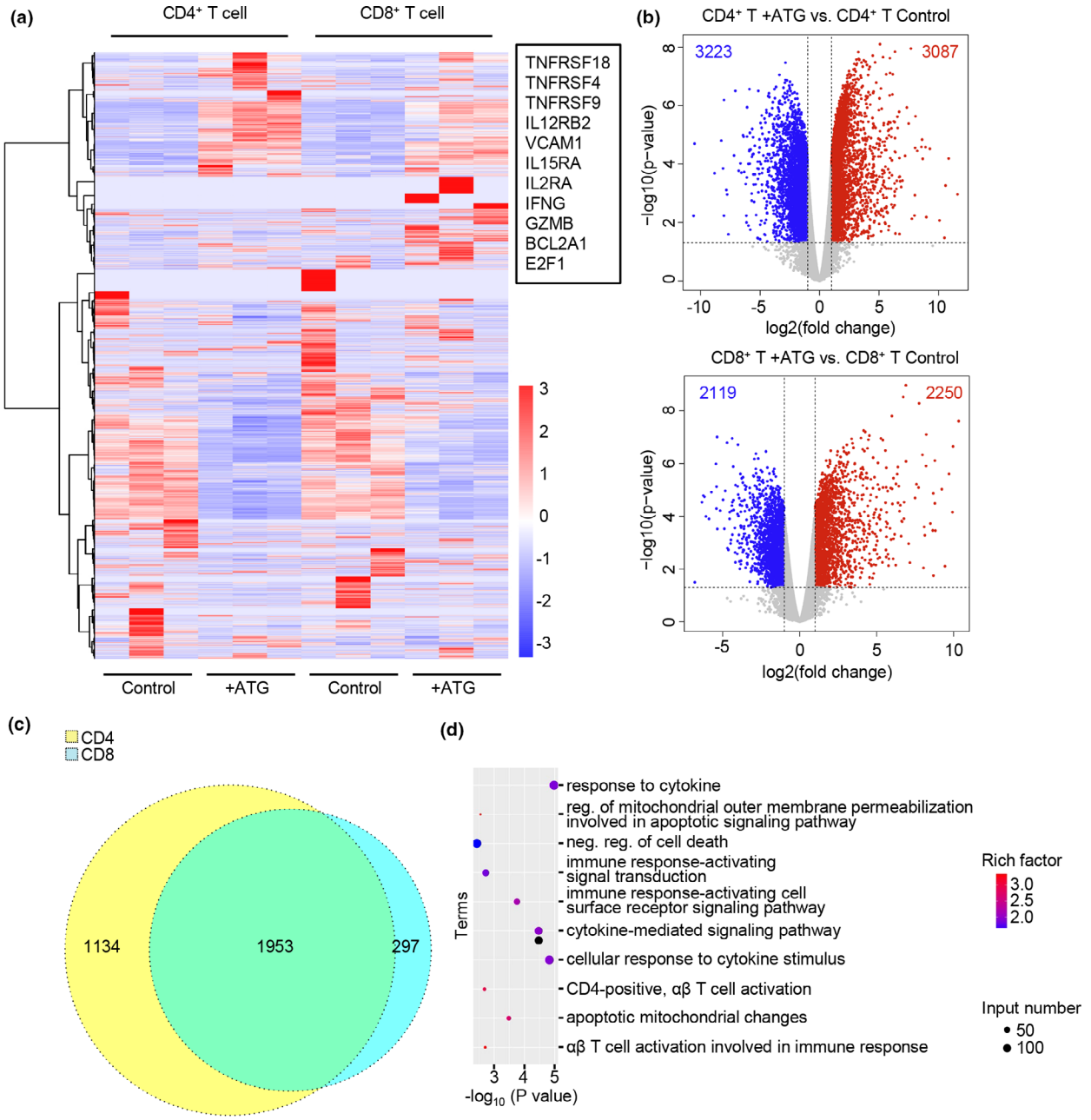

Figure 4 Transcriptome analysis reveals upregulation of apoptotic-, activated-, and cytokine-related genes in rATG-treated $CD4^+$ and $CD8^+$ T cells. Untouched $CD4^+$ and $CD8^+$ T cells were purified from three healthy individuals. (a) Clustered heatmap of significantly differentially expressed mRNAs in control and rATG (100 μg/ml)-treated $CD4^+$/$CD8^+$ T cells. Some representative genes related to immune response and apoptosis are shown below. (b) Volcano plots indicating the significance of upregulated genes (red) and downregulated genes (blue) between control and rATG (100 μg/ml)-treated $CD4^+$ (upper) /$CD8^+$ (bottom) T cells with numbers of differentially expressed genes. (c) Schematic flowchart showing the overlapping of the upregulated mRNA in $CD4^+$ and $CD8^+$ T cells after rATG treatment. (d) Scatter plot showing the Gene Ontology (GO) enrichment results in the upregulated genes. The dot size indicates the relative number of differentially expressed genes contained in the GO terms, and the shade of the dots indicates the extent of the enrichment.

stimulators (such as anti-CD28 and anti-CD80) [31]. The other biologic activities of rATG beyond T-cell depletion suggest that the antibodies contained in rATG can not only bind to the membrane antigens of target cells to activate the complement system, but also have other specific functionality. The antigen-nonspecific T-cell activation, proliferation, and cytokine production induced by rATG may have certain adverse effects on the recipient and allograft. In contrast, significant cell apoptosis following T-cell activation may favor

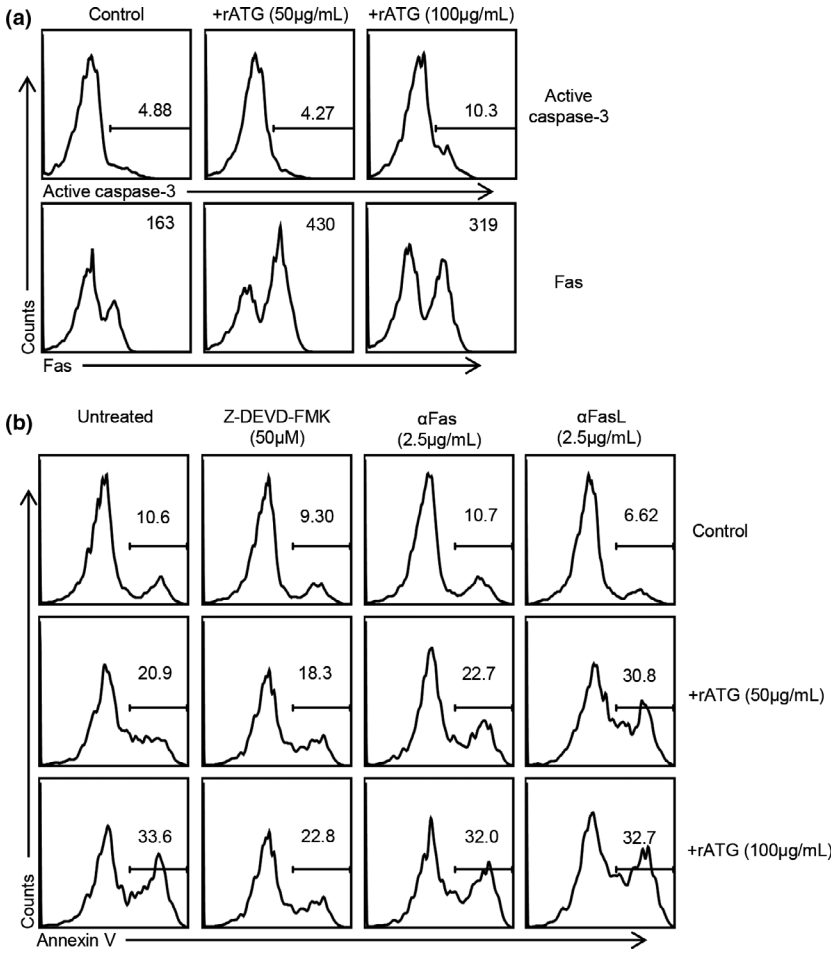

Figure 5 rATG-induced apoptosis is partially caspase-3-dependent but Fas/FasL-independent. (a) Active caspase-3 levels in the cytoplasm (upper panel) and transmembrane Fas expression (lower panel) in rATG-treated PBMCs were measured by flow cytometry. The percentage of active caspase-3-positive cell and the mean values of Fas expression are shown, respectively. (b) Caspase-3 inhibitor (Z-DEVD-FMK) or blocking antibody against Fas/FasL was added 2 h before rATG treatment. rATG induced lymphocyte apoptosis was measured at 24 h. Numbers indicate the ratio of Annexin V positive cell. One representative experiment out of two independent experiments is shown.

allograft survival. How will these biological effects of rATG ultimately affect transplantation deserves further attention.

There is a broad consensus that complement-dependent cell lysis is the primary underlying mechanism for the T-cell depletion caused by rATG in the blood compartment [5]. Thus, it is reasonable to speculate that there is an inadequate lymphocyte depletion in patients receiving rATG induction during complement inhibition therapy. However, only minimal impairment of rATG-induced T-cell depletion has been reported in patients with concomitant terminal complement blockade [32], an observation that may be explained by the careful timing of the use of complement inhibitors and rATG. Complement-dependent cytotoxicity begins within minutes [33]. Once rATG is injected before complement inhibition therapy, the majority of the peripheral T cells are rapidly depleted. In addition, it is noteworthy that rATG-induced T-cell depletion is different in the peripheral blood and lymphoid organs [24]. rATG administration leads to a rapid depletion of almost all T cells in the blood, but only partial depletion of T cells in lymphoid tissues. Therefore, it is important to pay attention to the biological effects of rATG stimulation on residual T cells. These data indicate that when complement inhibitors and rATG induction are used in combination, the timing of the administration of the two drugs and the immunological monitoring after treatment should be both deliberate and meticulous. Once the complement inhibition therapy has been applied to patients, using rATG subsequently may induce lymphocyte activation and cytokine production, which is worthy of consideration and attention in clinical practice.

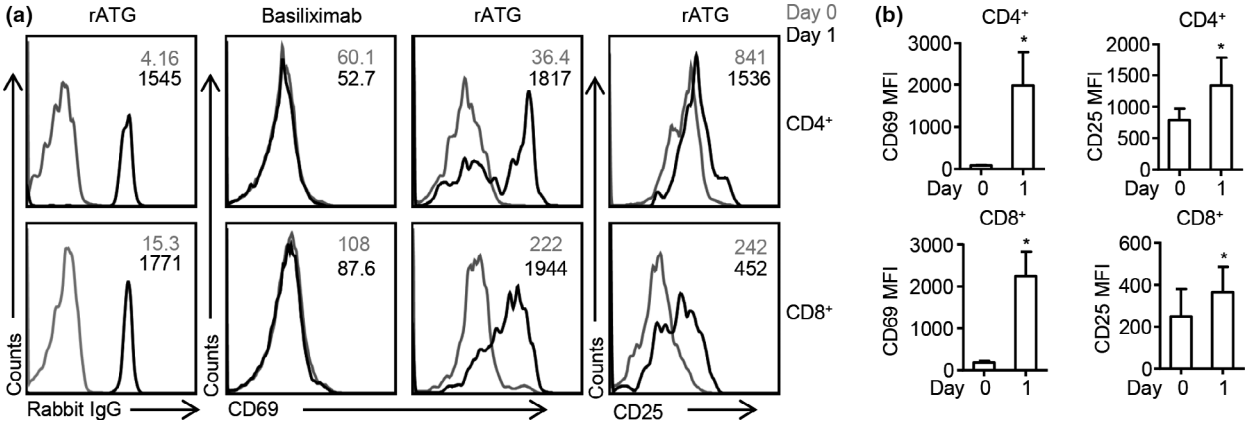

Figure 6 T-cell activation also occurs in patients receiving rATG patients. PBMCs were isolated from kidney transplant recipients before (Day 0) and 24 h (Day 1) after rATG administration. (a) Representative graphs showing rATG binding and CD69 and CD25 expression on $CD4^+$ and $CD8^+$ T cells from patients who had been treated with Basiliximab or rATG. Mean values are shown. (b) Summary bar graphs depicting CD69 and CD25 levels on $CD4^+$ and $CD8^+$ T cells before (Day 0) and 24 h (Day 1) after rATG injection. Data shown are means ± SEM and are pooled from three independent experiments (six individuals per group). $*P < 0.05$, vs. Day 0.

In addition to data from complement-dependent lysis, evidence from *in vitro* studies has suggested that antibody-dependent cell-mediated cytotoxicity (ADCC) is involved in the lymphocyte death caused by rATG [34]. Low concentrations of rATG in the range of 0.1 to 1 μg/ml have been shown to induce the lysis of PHA-preactivated T cells, but not resting T cells, through a Fas/FasL interaction. Our results here have shown that rATG treatment directly induces resting T-cell activation and proliferation as well as complement-independent cell death at higher concentrations (50–200 μg/ml). This cell death is most likely apoptotic, since it was documented via the externalization of membrane phosphatidylserine as measured by the binding of Annexin V in FACS analysis. Nevertheless, the apoptosis was Fas/FasL-independent, indicating that in these cells there is an underlying apoptotic pathway different from ADCC.

Cathepsin-B-dependent apoptosis has been reported as another important pathway in rATG-induced apoptosis [35]. There is strong evidence to indicate that cathepsin B plays a critical role in triggering apoptosis at a high concentration (250 μg/ml) of ATG, and this apoptosis is associated with the release of cathepsin B from the lysosomes into the cytoplasm in the T cells. However, in our *in vitro* experiments, lymphocyte apoptosis occurred at 50 μg/ml of rATG, a concentration that is unlikely to initiate the translocation of cathepsin B [30]. In addition, concentrations of rATG achieved during standard clinical use are generally far below the trigger level for cathepsin B activation. Activation-induced cell death (AICD) is another mechanism that can lead to T-cell apoptosis [27]. Caspase-3 has been reported to be a key effector molecule for AICD [36]. In the present study, we found that the use of a caspase-3 inhibitor partially blocked rATG-triggered apoptosis, suggesting that AICD is partially involved in the cell apoptosis we saw here. The underlying mechanism (s) by which rATG initiates complement-independent T-cell apoptosis still needs to be further explored.

Conclusions

Overall, we have highlighted the immune-stimulating effect of rATG on lymphocytes. At the same time, we detected complement-independent apoptosis of peripheral lymphocytes, especially T cells, after rATG treatment. Our results offer novel insights regarding the active biologic effects of rATG, underscoring the importance of the effects of rATG in the clinical situation.

Authorship

XT, LZ, and GC: conceived and designed the experiments. XT, HF, ZG, LW, CF, LS, CL, YL, QX, and LH: performed the experiments. XT, CL, LZ, and GC: analyzed the data and wrote the manuscript.

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Conflict of Interest

The authors have declared no conflicts of interest.

Acknowledgement

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Proportion of rATG bound lymphocyte at different rATG concentrations.

Figure S2. Purity of T cell subsets before and after magnetic cell sorting.

Table S1. Overlapping upregulated DEGs (FDR < 0.05) involved in immune response and apoptosis in $CD4^+$ and $CD8^+$ T cell groups.

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