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Rabbit Anti T-Lymphocyte Globulin Induces Apoptosis in Peripheral Blood Mononuclear Cell Compartments and Leukemia Cells, While Hematopoetic Stem Cells Are Apoptosis Resistant

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Polyclonal anti-T-lymphocyte globulins (ATG) are used in allogeneic stem cell transplantation (SCT) for the prophylaxis of graft versus host disease (GVHD) by in vivo T cell depletion. In this study we investigated the complement independent induction of apoptosis by rabbit ATG in peripheral blood mononuclear cell (PBMNC) compartments and hematopoetic stem cells (HSC). We also detected antileukemic activity of ATG by measuring apoptosis in myeloid and lymphatic leukemia cell lines and primary leukemia cells. We found ATG to induce apoptosis in T-lymphocytes ($CD4^+$, CD8+), B-lymphocytes (CD20+), natural killer (NK)-cells ($CD56^+$), and monocytes ($CD14^+$). HSC, in contrast, were apoptosis resistant and could be growth stimulated by low-dose ATG in the presence of bystander cells. The human leukemia cell lines Jurkat, Daudi, DG-75 (lymphoblastic), and K562, HL-60, KG1, and U937 (myeloblastic) underwent ATG-induced apoptosis, whereas the NK-cell line YT was resistant. Primary leukemia cells from 6 investigated patients with acute lymphoblastic leukemia, 9 of 10 patients with chronic lymphocytic leukemia, and 4 of 8 patients with acute myeloblastic leukemia underwent ATG-induced apoptosis. We conclude apoptosis induction in all PBMNC compartments contributes to GVHD prophylaxis. ATG might support engraftment. Finally, antileukemic activity of ATG could positively influence the transplantation outcome.

Biol Blood Marrow Transplant 15: 173-182 (2009) © 2009 American Society for Blood and Marrow Transplantation

KEY WORDS: ATG, Apoptosis, Periperal blood mononuclear cells, Hematopoetic stem cells, Leukemia

INTRODUCTION

The use of anti-T-lymphocyte globulins (ATG) in transplantation is established for the prevention of graft rejection in organ transplantation or graft-versus-host disease (GVHD) in allogeneic stem cell transplantation (SCT) [1,2]. A variety of ATGs differing in immunogen source and host species are currently in clinical use [3]. Their immunosuppressive action is thought to be mainly mediated by T-lymphocyte depletion. However, other peripheral blood mononuclear cell (PBMNC) compartments, especially B-lymphocytes and monocytes, are also involved in rejection and GVHD. ATG induces cell death by complement-dependent lysis and complement-independent surface protein crosslinking mediated apoptosis [4-7]. Because various surface antigens that act as epitopes for ATG have been identified to date including nonlineage-specific antigens, binding of ATG to cells of non-T-lymphocyte lineage could be demonstrated in several studies [8,9]. Also, apoptosis in B-lymphocyte cell lines was previously shown to be inducible by ATG [5]. In this study we provide data showing the induction of complement-independent apoptosis of rabbit ATG-Fresenius®, which is generated against Jurkat leukemia cells, in the T-lymphocyte, B-lymphocyte, monocyte, and natural killer (NK)-cell compartment of primary PBMNC.

In SCT, repopulation of the bone marrow is facilitated by hematopoetic stem cells (HSC). Hence, apoptosis of HSC would not be a desirable action of ATG. Binding of ATG to HSC has been demonstrated before [10] in patients with aplastic anemia. We investigated the sensitivity of healthy HSC to ATG and found HSC to be resistant to ATG-induced apoptosis. In contrast, ATG stimulated healthy HSC growth in methyl cellulose stem cell cultures at low concentrations as presumably maintained over longer periods in the bone marrow.

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Financial disclosure: See Acknowledgments on page 180.

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Received August 5, 2008; accepted November 9, 2008
1083-8791/09/152-0001$36.00/0
doi:10.1016/j.bbmt.2008.11.014

Finally, because rabbit ATG is generated by immunizing against the human leukemia cell line Jurkat, we studied the apoptosis-inducing activity of ATG against leukemia cells. In the lymphoblastic cell lines Jurkat, Daudi, DG-75, YT, and the myeloblastic cell lines K562, HL-60, KG1, and U937, a dose-dependent robust apoptotic response to ATG could be observed. In primary leukemia cells we found cells from all 6 patients with acute lymphoblastic leukemia (ALL), 9 of 10 patients with chronic lymphocytic leukemia (CLL), and 4 of 8 patients with acute myeloblastic leukemia (AML) to be responsive to apoptosis induction by ATG. In conclusion, we provide evidence that ATG has differing activity on all nonmalignant and malignant mononuclear cell populations of hematopoetic origin inducing apoptosis in the cell compartments involved in GVHD as well in many leukemia cells, whereas HSC are growth stimulated. Taken together, these data support the theory that ATG is beneficial in SCT for leukemia by more effects than mere T-lymphocyte depletion.

MATERIALS AND METHODS

Patients and Healthy Volunteer Donors

Cells were collected from patients and healthy donors who provided written consent for cell banking and in vitro studies. The study was approved by the internal review board. Included into this study were leukemic cells from 10 patients with CLL, 6 patients with ALL, 8 patients with AML, leukapheresis samples from 12 healthy stem cell donors, and PBMNC from 2 healthy volunteer donors.

Antibodies and Reagents

Rabbit ATG was from Fresenius, Graefelfing, Germany. All fluorescence conjugated antibodies were from BD Pharmingen, Franklin Lakes, NJ. Magnetic bead conjugated antibodies were from Miltenyi, Bergisch-Gladbach, Germany. The Annexin V, PI apoptosis kit was from R&D Systems, Wiesbaden, Germany; all other reagents, unless otherwise indicated, were from Sigma, St. Louis, MO.

Cell Culture

Cell lines were maintained in RPMI 1640 supplemented with 10% complement inactivated fetal calf serum (FCS) nonessential amino acids, L-glutamine, sodium pyruvate, 50 IU/mL penicillin, and 50 μg/mL streptomycin (all from Gibco Life Technology, Scotland, UK). Cells were kept in a humidified atmosphere containing 5% CO_2 at 37°C. All apoptosis experiments were performed in this media. All cell lines were from ATCC (Rockville, MD). Primary leukemic cells from peripheral blood were isolated using Ficoll hypaque density centrifugation. Their lineage specific surface markers were determined by routine immunofluorocytometry. Hematopoetic stem cells were from leukapheresis products from healthy stem cell donors.

PBMNC—Compartment and Stem Cell Separation

For magnetic cell separation T-lymphocytes, monocytes, B-lymphocytes, NK-cells from PBMNC, and HSC from leukapheresis products were purified by positive selection for $CD4^+$, $CD8^+$, $CD14^+$, $CD19^+$, $CD56^+$, and $CD34^+$, respectively, with the Mini-MACS column magnetic cell sorting system (Milteny, Bergisch-Gladbach, Germany) according to manufacturers protocol. Briefly, 1×10^7 cells were suspended in 80 μL of labeling buffer (PBS, EDTA 2 mM, BSA 0.5%). Magnetically labeled antibody was added 20 μL each. The cells were incubated for 15 minutes at 4°C. The cells were washed in labeling buffer. The resulting pellet was resuspended in 500 μL labeling buffer and transferred onto a MACS column placed in the MiniMACS magnet. For positive selection the column fraction was collected. To increase purity the separation was repeated once. Cell purity testing was performed with Phycoerythrin (PE)-labeled antibodies against the corresponding CD markers.

ATG Binding

For binding studies of ATG-Fresenius 0.5×10^6 magnetically selected cells were incubated with 20 μg/mL ATG for 20 minutes at room temperature, then washed twice with PBS and resuspended in 100 μL PBS with 1% BSA. Negative controls were treated with 20 μg/mL preimmune rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibody labeling was performed with 5 μL of FITC-conjugated goat antirabbit IgG (Cayman, Ann Arbor, MI) for 30 minutes at 4°C. Cells were washed twice and analyzed by fluorescence cytometry. Binding of ATG to unselected PBMNC, HSC, and leukemia cells was performed as above. Cells were double stained with lineage-specific PE-labeled antibody and FITC goat antirabbit IgG. Single dye-stained cells were used for double color compensation. Binding to leukemia cell lines was confirmed by single color staining with FITC antirabbit IgG. In all fluorescence cytometry assays 10,000 cells were counted.

Apoptosis in PBMNC, HSC, and Leukemia Cells

To measure apoptosis induction of ATG 1×10^6/mL cells were incubated with the indicated concentrations of ATG (20 μg/mL to 1000 μg/mL) or preimmune rabbit IgG for controls (Santa Cruz Biotechnology, Santa Cruz, CA) in cell culture media and analyzed at several time points (3-24 hours) by

flow cytometry. To determine the apoptosis rate in PBMNC compartments, HSC, and primary leukemic cells after incubation with ATG, cells were triple stained with PE-conjugated lineage-specific antibody as described and FITC-conjugated Annexin V and Propidium-iodide (PI) according to manufacturers instructions. Briefly, after incubation with ATG in complement-inactivated RPMI media cells were resuspended in 100 μL PBS stained with 5μL PE labeled lineage antibody and incubated for 20 minutes at room temperature. Then, cells were washed and resuspended in 100 μL AnnexinV binding buffer as provided by the manufacturer, 1 μL FITC-Annexin V and 10 ng/mL PI were added, then cells were analyzed by fluorescence cytometry. PE was detected in channel 2, FITC in channel 1, and PI in channel 3. Single colors were used for color compensation. For analysis cells were gated on their respective positive lineage marker. AnnexinV and PI positivity was then assessed according to the gate. Apoptotic cells were defined as Annexin V single positive (early apoptosis) and Annexin V/PI double postive (late apoptosis). Vital cells in HSC were defined as negative for AnnexinV/PI. In leukemic cell lines apoptosis was additionally analyzed by measuring loss of mitochondrial membrane potential (MMP) $\Delta\psi$ with the green fluorescent dye DiOC6 (3,3′dihexicyloxacarbocyanine iodide) as described [11]. DiOC6 was added at a final concentration of 0.5μmol to the cell suspension, incubated for 15 minutes at 37°C and analyzed flow cytometrically. In all fluorescence cytometry assays 10,000 cells were counted.

HSC—Colony Assay

To investigate the sensitivity of stem cells to ATG for a longer period of time we used a colony assay. Stem cells from 9 different leukapheresis products were plated on Petri dishes filled with methyl cellulose to allow growth of colonies of stem cells. Briefly, small Petri dishes were filled with 2.1 mL of a methyl cellulose-solution (21 g of methylcellulose [Methocel from Fluka, Buchs, Switzerland] solved in 500 mL distilled water and 500 mL IMDM-Medium), 0.9 mL BSA, and 1 μg/mL GM-CSF (Cellgenix, Freiburg, Germany). ATG or control rabbit IgG was added to dishes as indicated. Cells from leukapheresis products were plated at 1.5×10^5 cells/mL and incubated for 2 weeks at 37°C. Then, dishes were scanned for colony growth under a microscope; more than 30 cells on a defined point were counted as a colony. To investigate if cells other than HSC in the leukapheresis products have an influence on colony growth of HSC we performed the same experiment with stem cells magnetically sorted for CD34 from 2 different donors plated at 5×10^3 and 15×10^3 cells/mL each.

Data Analysis

All experiments were performed, unless otherwise noted, at least 2 times and in duplicate. To have comparable results of the apoptotis studies the specific cytotoxicity in % was calculated by following formula: specific cell death = (cell death[treated] − cell death[-control]/100 − cell death[control]) × 100. Statistical analysis was performed by 1-way analysis of variance (ANOVA).

RESULTS

ATG Binds to All PBMNC Compartments

Rabbit ATG binds to the surface of all PBMNC compartments. We first incubated unseparated PBMNC with ATG in increasing concentrations. We found ATG to bind to PBMNC in a dose-dependent manner. Figure 1A shows logarithmical increase of fluorescence intensity of antibody bound to PBMNC with increasing concentrations of ATG. To confirm the binding of ATG to blood cell compartments PBMNC were positively separated by magnetic activated cell sorting (MACS) for CD4, CD8, CD20, CD14, and CD56 to a purity of ≥90%, incubated with ATG 20 μg/mL for 20 minutes, and stained with FITC conjugated antirabbit IgG antibody. Figure 1B shows the binding of ATG to all purified PBMNC compartments. Alternatively, whole PBMNC were incubated with ATG, stained with FITC antirabbit IgG, and costained with the respective PE conjugated antihuman CD antibody. As shown in Figure 1C, the respective cell compartments were completely positive for ATG binding.

ATG Induces Apoptosis in PBMNC Compartments

We investigated apoptosis induced in cell compartments by triple-color fluorescence cytometry to avoid a possible influence of the MACS sorting process on apoptosis. The PBMNC were incubated with ATG at differing concentrations and incubation times. The cell compartments were stained with the lineage-specific PE conjugated antibodies. For apoptosis detection Annexin V/PI staining was performed and analyzed for the lineage specific marker gate. We found that apoptosis was induced in all cell compartments already at ATG concentrations of 20 μg/mL (not shown) with a dose- and time-dependent increase of apoptosis rate. Maximum apoptosis was seen at ATG 250 μg/mL. Figure 2 depicts the lineage specific apoptosis rate with ATG 250 μg/mL at 6 and 24 hours by Annexin V/PI staining. The time curve for apoptosis of PBMNC compartments treated with ATG 250 μg/mL from 3 to 24 hours is shown in Figure 3. Interestingly, B-lymphocytes and monocytes showed a higher

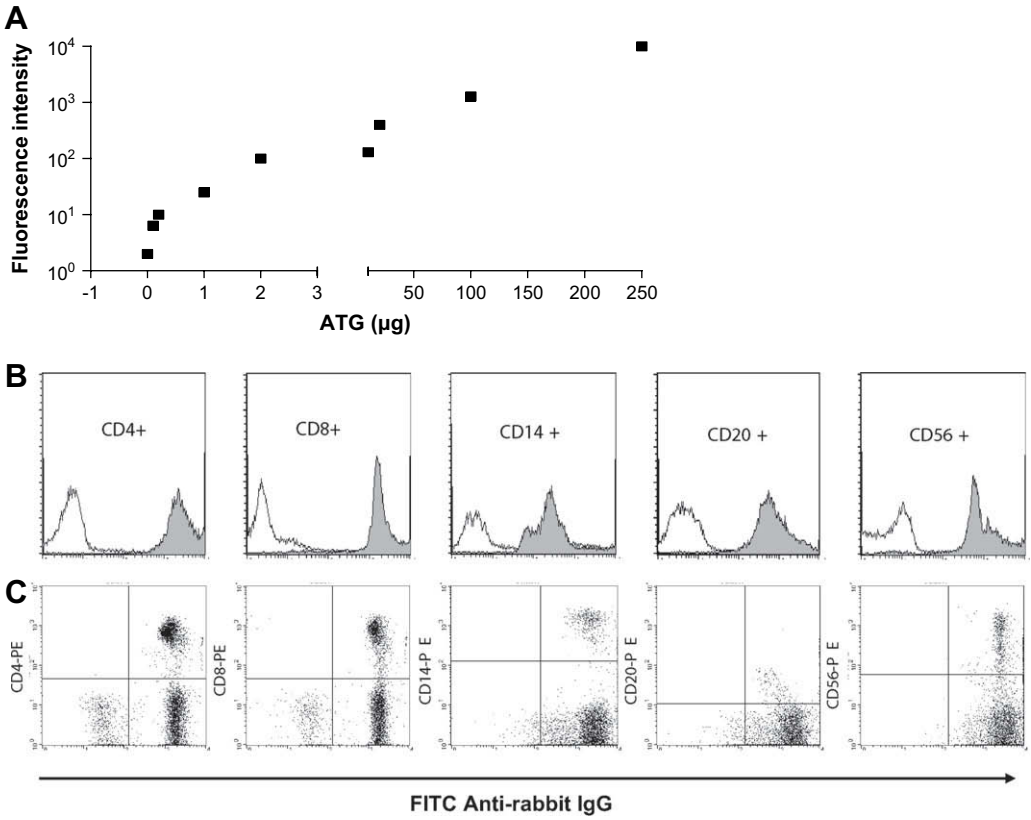

Figure 1. Binding of ATG to PBMNC compartments. Unseparated PBMNC were incubated with the indicated concentrations of ATG (X-axis is depicted in 2 sectors, left sector range: 0 to 3 µg/mL, right sector range: 10 to 250 µg/mL) for 20 minutes, stained with FITC-labeled antirabbit IgG, and analyzed by fluorocytometry. Binding is expressed as mean fluorescence intensity (A). CD4-, CD8-, CD20-, CD14-, and CD56-positive selected cells (purity ≥90%) were incubated with ATG 20 µg/mL for 20 minutes and stained with FITC antirabbit IgG (filled histograms) versus incubation with rabbit IgG isotype control (open histograms) (B). Double staining with PE-labeled anti-CD antibodies and FITC antirabbit IgG after incubation of unseparated PBMNC with ATG 20 µg/mL confirms the complete ATG binding of PBMNC compartments (C).

sensitivity to apoptosis than T-lymphocytes. Because the important role of antigen presenting cells in the pathogenesis of GVHD has become established and evidence for some role for B-lymphocytes in chronic GVHD (cGVHD) is also emerging, these data indicate that the activity of ATG for GVHD prevention is not limited to T-lymphocyte depletion, but might include depletion of B-lymphocytes and monocytes. NK-cells, in contrast, appear to be more apoptosis resistant.

HSC Are Resistant to ATG-Induced Apoptosis

Healthy HSC treated with pharmacologicaly relevant doses of ATG were higly resistant to apoptosis. Figure 4A confirms the binding of ATG to CD34-positive HSC. We investigated unseparated HSC from leukapheresis products of healthy human stem cell donors for their sensitivity to apoptosis. To induce significant apoptosis in $CD34^+$ HSC high concentrations of ATG above 500 µg/mL were required as shown in Figure 4B for a 24-hour incubation time. Figure 4C shows the rate of vital Annexin/PI negative $CD34^+$ HSC following incubation with the indicated concentrations of ATG for 6 and 24 hours. Again, a significant decline of vital cells could only be observed at concentrations above 500 µg/mL, that are not pharmacologically relevant [12]. This observation is in accordance with the clinical experience that ATG does not hamper HSC engraftment.

Low-Dose ATG Stimulates HSC Colony Growth

A growth stimulatory effect of horse ATG on HSC of aplastic anemia patients has been demonstrated before [10,13]. To investigate if rabbit ATG could stimulate the growth of healthy HSC, we performed methylcellulose colony growth assays with 9 unseparated leukapheresis products of healthy volunteer donors in the absence or presence of ATG at different concentrations. We found low-dose ATG to stimulate colony growth. In the presence of ATG 20 µg/mL significantly more colonies could be observed at 14 days (Figure 5A) compared to controls without ATG ($P < .0013$). However, a concentration of 250 µg/mL abrogated colony growth completely. We further found growth stimulation to be bystander cell dependent, because colony growth stimulation by low-dose ATG was not observed in CD34 positively selected cells from 2 donors seeded at 2 different cell concentrations (Figure 5B).

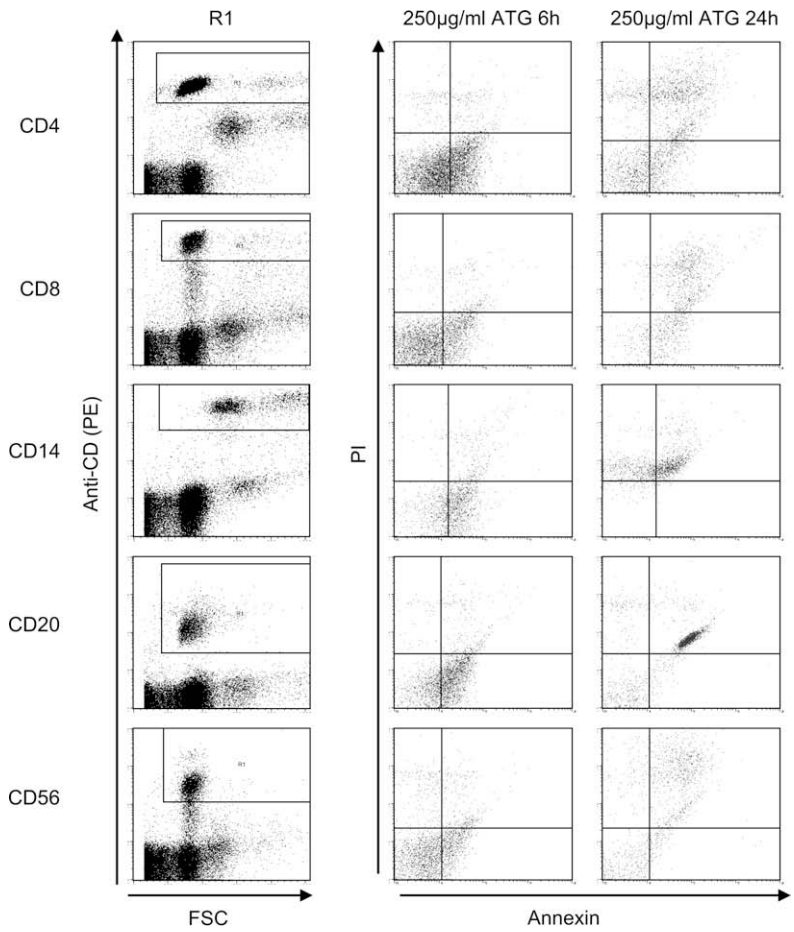

Figure 2. ATG induces apoptosis in PBMNC compartments. PBMNC incubated with ATG 250 µg/mL for 6 h and 24 h were analyzed by triple-color fluorescence for the lineage specific marker (PE anti CD) and Annexin V/PI staining for apoptosis detection. AnnexinV/PI plots represent the gate (R1) of the indicated CD marker.

ATG Induces Apoptosis in Lymphoblastic and Myeloblastic Leukemia Cell Lines

Because rabbit ATG is generated against Jurkat cells, we hypothesized a potential antileukemic activity of ATG. Apoptosis induction by ATG was investigated in human leukemia cell lines. To confirm the data obtained by Annexin V/PI doublestaining we also performed MMP assays monitoring the mitochondrial stage of apoptosis. Figure 6 shows apoptosis induced by ATG 250 µg/mL in HL-60 cells after 6 hours detected by Annexin V/PI (A) and MMP (B) assays. We found ATG to induce high levels of apoptosis in the human lymphoblastic cell lines Jurkat, Daudi, DG-75 (Figure 6C), and myeloblastic cell lines K562, HL-60, KG1, and U937 (Figure 6D) at pharmacologically relevant ATG concentrations. Only the human NK-cell leukemia line YT was resistant to ATG-mediated apoptosis. The results obtained by MMP closely matched those obtained by Annexin V/PI presented in the figure (data not shown). This indicates at the involvement of the mitochondrial pathway of apoptosis in ATG-mediated cell death. Although the response of cell lines of the lymphatic lineage was less surprising because ATG is generated against a lymphatic cell line, the high sensitivity of myeloblastic cell lines was much less expected by us.

ATG Induces Apoptosis in Primary Leukemia Cells

To address the question if ATG might be involved in the killing of primary leukemia cells in the course of conditioning therapy we investigated the binding and apoptosis inducing activity of ATG on leukemia cells of 10 patients with B-cell CLL, 6 patients with ALL, and 8 patients with AML. Following incubation with ATG 250 µg/mL for 24 hours, leukemia cells were stained for their lineage-specific marker with PE-coupled antibody (CD 19 for CLL and B-ALL; CD34 for 2 patients with precursor ALL; CD3 for 1 patient with T-ALL; CD33, CD34, or CD117 for AML), and binding was confirmed to all investigated cells with FITC antirabbit IgG (data not shown). Similarly, apoptosis was investigated by triple staining for lineage marker and Annexin V/PI for apoptosis. Figure 7 shows the apoptosis rates of all investigated primary leukemia cells treated with ATG. Except for 1 patient who did not respond,

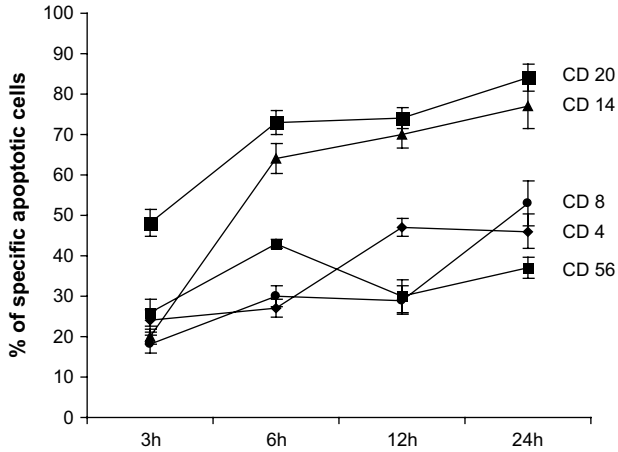

Figure 3. Time course of ATG-induced apoptosis in PBMNC compartments. Time-dependent apoptosis rates as analyzed by AnnexinV/PI positivity of PBMNC cell compartments incubated with ATG 250 μg/mL from 3 hours to 24 hours. Values represent mean ± SEM from 2 experiments.

leukemia cells of 9 of 10 CLL patients did undergo high rates of apoptosis upon ATG treatment (Figure 7A); apoptosis in leukemia cells was inducible in all 6 patients with ALL, albeit in 2 patients at a very low rate (Figure 7B). In AML leukemia cells of only 4 of 8 patients were sensitive to ATG-induced apoptosis (Figure 7C). This observation differs from the results with cell lines. It can be speculated that either cells in

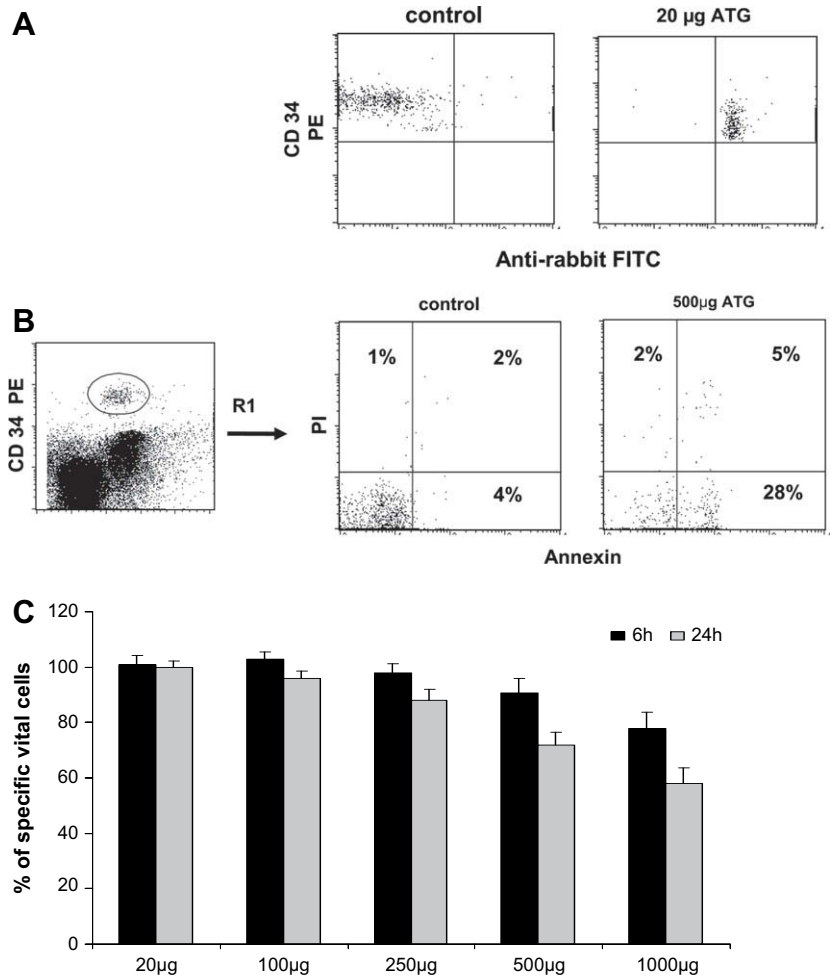

Figure 4. ATG binding to HSC and apoptosis induction. Incubation with ATG 20 μg/mL for 20 minutes results in complete binding to CD34-positive HSC (A). Annexin V/PI detection of apoptosis induced by incubation with ATG 500 μg/mL for 24h in the HSC gate ($CD34^+$) (B). Percentage of non-apoptotic $CD34^+$ HSC following incubation with the indicated concentrations of ATG for 6 hours and 24 hours defined by AnnexinV/PI negativity (mean ± SEM from 2 experiments) (C).

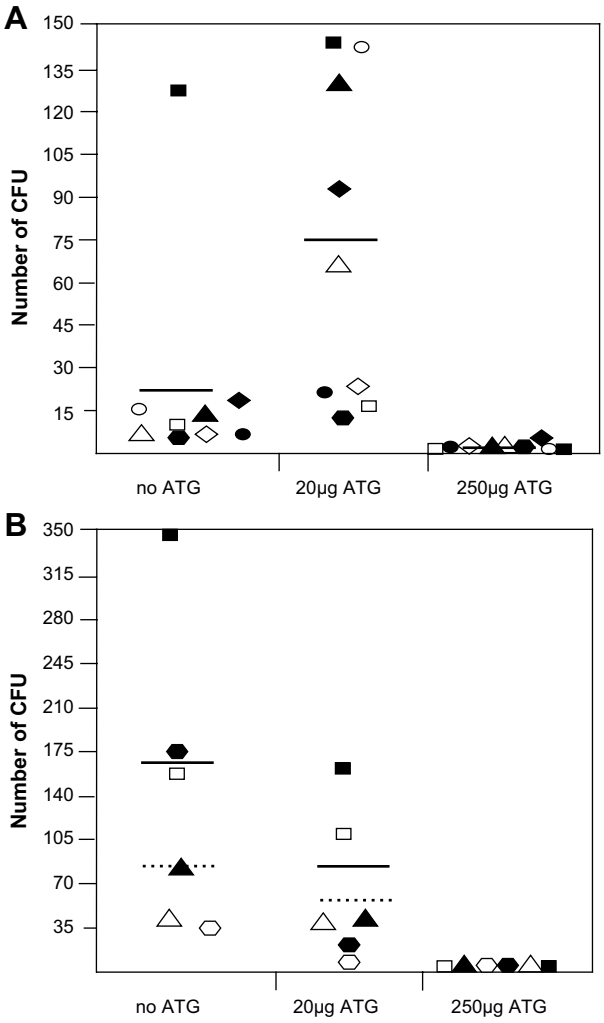

Figure 5. Colony growth of HSC in the presence of ATG. Unseparated leukapheresis products of 9 healthy HSC donors were plated at 1.5 × 10^5 cells/mL in methylcellulose as described. ATG was added at 20 μg/mL and 250 μg/mL. Significantly ($P < .0013$) more colonies (CFU) could be observed at 14 days in the culture with ATG 20 μg/mL, whereas ATG 250 μg/mL abrogated colony growth (mean: solid bars) (A). In HSC positively selected for CD34 no growth stimulation by ATG could be observed. CD34 selected HSC of 2 healthy donors were plated at 5 × 10^3 cells/mL (open symbols; mean: dotted bars) and 15 × 10^3 cells/mL (filled symbols; mean: solid bars) in the presence of the indicated ATG concentrations. At 14 days colony growth was reduced with both ATG concentrations (B).

some leukemia patients lost epitope subsets required for proper apoptosis transduction, lack downstream effectors required for apoptosis signal transduction, or third, do overexpress factors that directly facilitate resistance. However, ATG seems to have pro-apoptotic activity against a majority of primary leukemia cells, particularly those of lymphatic origin.

DISCUSSION

Our study investigated the induction of apoptosis in nonmalignant and malignant mononuclear cell populations by rabbit ATG generated against Jurkat lymphoblastic leukemia cells. We found ATG to have a broad complement-independent apoptosis-inducing activity to the cell compartments of human PBMNC. Obviously, a wide spectrum of different antibody specificities is present in the ATG preparation generated against antigens shared between Jurkat cells and the different PBMNC cell subsets. At present, a few of these antigens have been specified, such as CD11a, CD18, CD50, CD54, CD58, CD102, CD40, CD45, HLA-DR, or chemokine receptors CXCR4, CC5, CCR7 [14,15]. Our study shows that crosslinking of cell surface antigens on all PBMNC compartments induces signal transduction dependent apoptosis. According to the current model of GVHD, the non-T-lymphocyte PBMNC compartments are also involved in the pathopysiology by maintaining inflammation and tissue damage. Interestingly, we found the strongest activity of ATG to be against B-cells and monocytes. The crucial role of antigen presenting cells (APC) in the initiation of acute GVHD has long been recognized; hence, monocyte depletion by ATG would be contributing to the prevention of GVHD [3,16-18]. B-cells in contrast have only recently been implicated in the pathophysiology of chronic GVHD [19,20]. The weakest activity of ATG in our study was shown to be against NK-cells. Recent data show that NK-cells appear to have a rather protective role against GVHD and a crucial role in the development of a graft-versus-leukemia reaction (GVL) [21-23]. Hence, the relative resistance of NK-cells to apoptosis by ATG would be a rather beneficial toward HSC outcome.

The activity of horse ATGs on HSC of patients with aplastic anemia (AA) has been investigated in a couple of studies [10,24,25]. Killick et al. [24] found a reduction of spontaneous apoptosis in HSC from AA patients treated with ATG. We found healthy HSC to be highly resistant to rabbit ATG-mediated apoptosis. One study also reported the stimulation of HSC colony growth by low-dose horse ATG in healthy donors and some patients with AA and myelodysplasia [26]. However, that study used $CD34^+$ selected cells. We, in contrast, detected colony growth stimulation by low doses of rabbit ATG only in the presence of bystander cells, indicating that rabbit ATG presumably acts on HSC by a different mechanism requiring the secretion of stimulatory cytokines [27]. Further, the presence of high ATG concentrations abrogated HSC colony growth. One possible explanation for this might be ATG induced apoptosis of bystander cells. However, the colony growth-inhibitory effect of high ATG concentrations also observed in $CD34^+$ selected cells indicates at a direct antiproliferative action of ATG at high doses during longer term culture. A hypothetic mechanism could be the blockage of growth factors and their receptors. In vivo, the concentrations of ATG have been found to be above 100 μg/

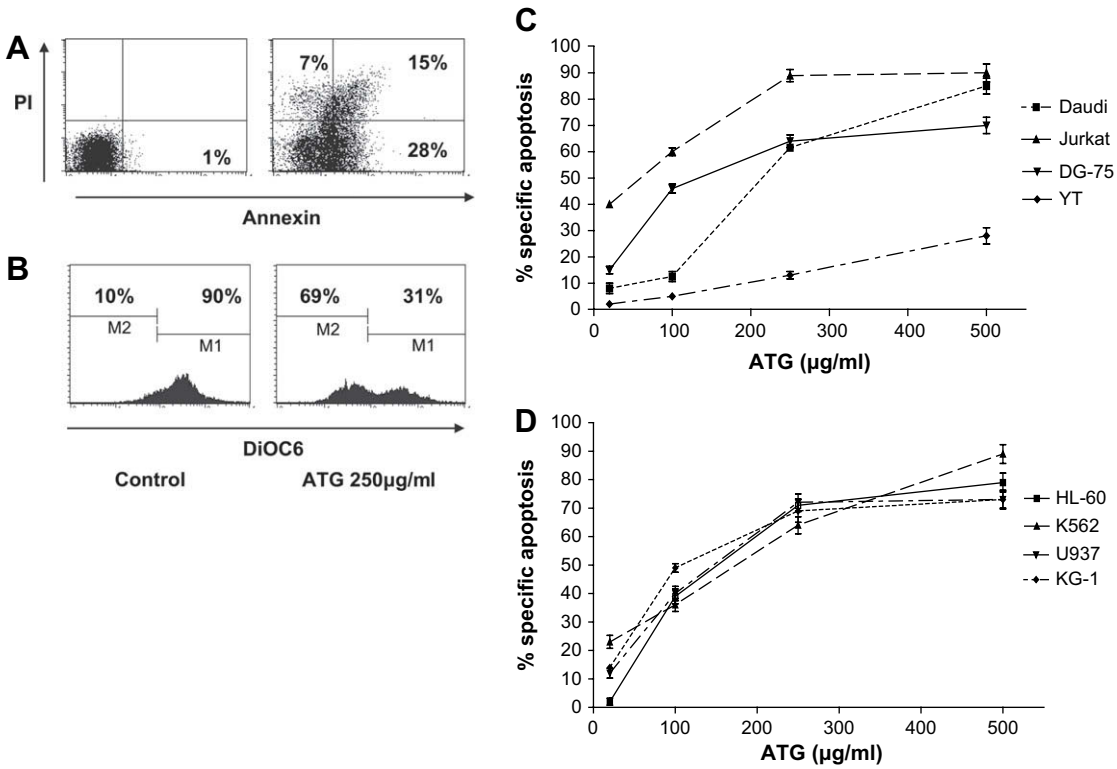

Figure 6. ATG induces apoptosis in leukemia cell lines. HL-60 cells incubated with ATG 250 µg/mL for 6 hours show high rates for Annexin V/PI positivity (A) and comparable MMP as measured by loss of $\Delta\Psi$ (reduced DIOC6 uptake) (B). Concentration-dependent induction of apoptosis by ATG in myeloblastic cell lines KG1, K562, HL-60, and U937 (C) and lymphoblastic cell lines Jurkat, Daudi, DG 75, and YT (D) after incubation with the indicated concentrations of ATG for 6 hours by Annexin V/PI staining (mean ± SEM from 2 independent experiments).

mL for about 24 hours following the final infusion and than drop into a range between 10 to 50 µg/mL, at which they are maintained for the next 2 weeks [12], indicating that HSC will be exposed to low-dose ATG during most of the time preengraftment.

Because many of the known epitopes targeted by ATG are also expressed on malignant cells, we postulated activity against leukemia cells. Ayuk et al. [28] demonstrated activity of horse ATG against myeloma cells, albeit at concentrations twice as high as used in our study. We found that anti-Jurkat rabbit ATG has pro-apoptotic activity on leukemia cells regardless of the originating lineage. We found a strong apoptotic activity against cell lines of the myeloid and lymphatic lineage. Only the human NK-cell leukemia derived line YT was shown to be resistant to ATG. Interestingly, this correlates to the relative resistance of healthy NK-cells. In primary leukemia cells the response rate to apoptosis by ATG varied individually. Generally, the strongest pro-apoptotic activity could be demonstrated against CLL cells. However, in 1 of 10 patients with CLL the leukemia cells were completely resistant. Leukemia cells from all 6 ALL patients were responsive to ATG at different rates, whereas leukemia cells from 4 of 8 patients with AML were also resistant to ATG induced apoptosis. It is of note that this activity was observed at concentrations of ATG that are pharmacologically reached during ATG infusion [12]. Although these differential response rates were observed for signal transduction-induced apoptosis, there is no evidence for similar effects in complement-dependent lysis induced by ATG, which proceeds in all cell types at very rapid kinetics provided the presence of high concentrations of ATG and complement [7,28].

In conclusion, we demonstrate that ATG acts on a broad range of malignant and nonmalignant hematopoetic cells. Its activity is not limited to T cell depletion, but apoptosis induction in monocytes and B cells could contribute to the prevention of acute GVHD (aGVHD) and cGVHD. HSC are resistant to the pro-apoptotic activity of ATG, and engraftment might even be supported by growth stimulation with low-dose ATG. Furthermore, antileukemic activity of ATG could be an additive factor to conditioning chemotherapy for the prevention of recurrences.

ACKNOWLEDGMENTS

The authors thank Eva Samek for her excellent technical assistance in HSC colony assays.

Financial disclosure: The work was in part supported by funds of the Albert Ludwigs University, Freiburg.

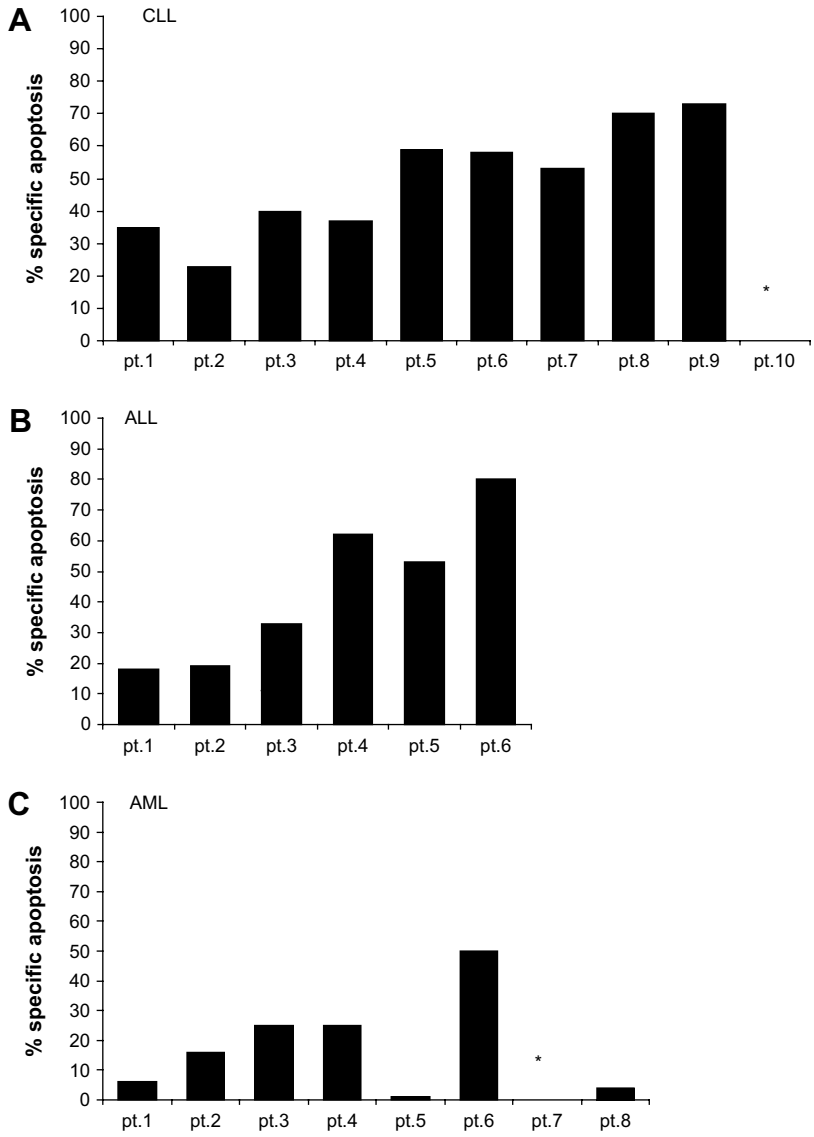

Figure 7. ATG induces apoptosis in primary leukemia cells. Specific Apoptosis rates for primary leukemia cells from 10 patients with CLL (A), 6 patients with ALL (B), and 8 patients with AML (C) incubated with ATG 250 μg/mL for 24 hours measured by triple fluorescence for leukemia lineage marker and Annexin V/PI (mean from 2 experiments) (*apoptosis rate slightly below control).

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