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Anti-thymocyte globulin facilitates alloreactive T-cell apoptosis via caspase-3: potential implications for monitoring rejection-free outcomes

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Abstract

Background—Alloreactive T-cell apoptosis may explain reduced immunosuppression requirements with pro-apoptotic immunosuppression and among rejection-free recipients. This possibility remains unproven.

Methods—Apoptotic (caspase-3+, cathepsin-B+) and inflammatory (CD154+) T-cell subsets were evaluated before and after adding rabbit anti-thymocyte globulin (rATG) to mixed lymphocyte co-cultures (MLC) between HLA-mismatched peripheral blood lymphocytes (PBL) from healthy adults. In random samples from children with liver (LTx-20) and intestine (ITx-13) transplantation, apoptotic T-cells were evaluated for association with rejection-free outcomes using the caspase-3 substrate, phipphilux.

Results—In MLC between normal human PBL, 1) frequencies of memory (M) and naïve (N) Th and Tc, which expressed activated caspase-3, were enhanced most by the combination of allostimulation and rATG, than either stimulus alone. These findings were confirmed with

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antibody to activated caspase-3, phiphilux, and TUNEL assay, 2) frequencies of Th subsets, which expressed activated cathepsin-B, were similarly increased with combined stimulation. Tc appeared resistant to cathepsin-B activation. 3) with increasing rATG concentrations, proportionately more allospecific CD154+TcM survived than TcM, resulting in relative enrichment of allospecific CD154+TcM. In random blood samples, phiphilux+T-cell subset frequencies were higher among 14 rejection-free LTx and ITx recipients, and demonstrated a greater increase with ex-vivo rATG pre-treatment, than 19 rejectors. In logistic regression analysis, phiphilux+TcM associated best with rejection-free outcomes with sensitivity/specificity of 57%/89%, respectively.

Conclusions—rATG facilitates apoptosis of alloreactive T-cells via caspase-3 activation, which may explain its steroid-sparing effect in pediatric liver and intestine recipients. Apoptotic susceptibility of T-cytotoxic memory cells, which resist cathepsin-B activation, may distinguish rejection-free and rejection-prone liver recipients.

Keywords

alloreactive T-cells; activation-induced apoptosis; anti-thymocyte globulin; caspase-3; T-cytotoxic memory cell

Introduction

Reducing immunosuppressant usage has the potential to improve survival and quality of life of transplant recipients, because side effects such as infections and malignancies account for most deaths after the first post-transplant year (1). This objective can be achieved by promoting alloreactive T-cell apoptosis with proapoptotic immunosuppressants. In animal models of transplantation, alloactivation-induced T-cell apoptosis is a potent mechanism by which rejection-free outcomes are achieved with reduced or no immunosuppression (2). Induction immunosuppression with antibodies, which induce lymphocyte apoptosis, is thought to lower dependence on immunosuppressants such as steroids, in part by facilitating this mechanism (3). Whether this facilitation actually occurs remains to be demonstrated. Minimization of drugs may also be facilitated by monitoring tolerogenic mechanisms, which are associated with rejection-free outcomes (4). Whether T-cell susceptibility to apoptosis is an attribute of rejection-free recipients is also not known. These knowledge gaps are largely technology-driven. The best-known traditional approach infers apoptotic depletion of alloreactive T-cells from failure to demonstrate donor-reactive inflammatory T-cells at limiting dilutions (5, 6). Increasingly, apoptosis mediators can be measured in single cells with specific reagents using flow cytometry. Examples include specific antibodies to activated apoptotic enzymes, and sensitive substrates, which become fluorescent upon cleavage by apoptotic enzymes and can be detected intracellularly by simple non-permeabilizing procedures. These advances can directly evaluate whether T-cell allostimulation is accompanied by apoptosis and not just an inflammatory alloresponse, whether a particular drug regimen promotes apoptosis, and whether measurements of apoptosis can expand the immune surveillance repertoire to predict graft acceptance.

In this report, we evaluate T-cell apoptosis for its association with a potentially tolerogenic immunosuppressant, and with rejection-free outcomes. *First*, we evaluate using peripheral blood lymphocytes (PBL) from HLA-mismatched normal human subjects, whether

allostimulation-induced T-cell apoptosis is enhanced by rabbit anti-thymocyte globulin (rATG, Genzyme, Cambridge, MA) via caspase and cathepsin pathways. The pro-apoptotic agent, rATG, causes T-cell apoptosis via cathepsin-B, and B-cell apoptosis via caspase-3 (7, 8). Considered an “executioner” caspase, caspase-3 is a key member of cytoplasmic proteases called the caspases (9). Caspase-3 initiates activation-induced cell death in response to initiator caspases, which are either activated by intrinsic mitochondrial or extrinsic cell-surface events. Ligation of the T-cell receptor is an example of an extrinsic event, which induces apoptosis via caspase-3 activation (10, 11). Exemplified by cathepsin-B, cathepsins are lysosomal proteases (12). Upon release into the cytoplasm, cathepsin-B induces apoptosis by several pathways including caspase activation and mitochondrial release of pro-apoptotic factors. All experiments have been conducted in culture medium containing heat-inactivated fetal bovine serum, to avoid the confounding effects of complement-mediated T-cell lysis by rATG (13).

Next, we evaluate in random blood samples, whether frequencies of circulating apoptotic T-cells which express activated caspase-3, are higher in rejection-free children compared with children who have experienced early rejection after liver or intestine transplantation (rejectors). Early rejection occurs when therapeutic immunosuppression targets are at their highest, is a risk factor for recurrent ACR during drug minimization, and is associated with pre-transplant T-cell sensitization (14-16). *As an extension of this hypothesis*, we evaluate whether rejection-free recipients also demonstrate greater T-cell susceptibility to the pro-apoptotic effects of rATG, compared with rejection-prone recipients. These experiments presuppose that circulating apoptotic T-cells in the post-transplant setting represent ongoing alloactivation by indwelling liver allografts. Measuring apoptotic response with several parallel mixed lymphocyte co-cultures (MLC) under a variety of conditions would have required amounts of PBL, not safely obtained from pediatric recipients averaging 5 years in age.

Results

Human Subjects

Six healthy adult human subjects and 20 pediatric LTx provided blood samples for studies approved by the University of Pittsburgh's Institutional Review Board (NCT#01163578). As in our previous work, rejectors are those children who experienced acute cellular rejection during the first 60 days after liver or intestine transplantation (14, 15). Non-rejectors did not experience rejection during this early time period after transplantation. Five of fourteen non-rejectors experienced late rejection (after the first 60 post-transplant days) at days 99, 1067, 1334, 1841, and 5146 days after transplantation. These events were separated from the date of sample collection for apoptosis studies by an average of 652 days, ranging from 244 days to 1470 days. Therefore, apoptotic susceptibility measured in these samples was not influenced by late rejection episodes among non-rejectors.

No differences were seen between rejectors and non-rejectors in mean age, tacrolimus whole blood concentrations (FKWBC), use of immunosuppressants such as tacrolimus, steroids, sirolimus and cellcept, HLA match between donor and recipient, time between transplant

and assay, and presence of EBV or CMV viremia within the 60-day post-sampling period (Table 1).

rATG augments caspase-dependent allostimulation-induced apoptosis of all T-cell subsets, and enhances cytokine release

Responder PBL were pre-treated with increasing rATG concentrations, from 0-200 µg/ml, and were incubated alone and with irradiated stimulator PBL from HLA-mismatched normal human subjects. Six different responder-stimulator pairs were used for these MLC for which results are summarized in Figures 1-3. These MLC 1) Evaluated T-cell apoptosis using fluorochrome-labeled antibodies to activated caspase-3 and activated cathepsin-B with permeabilizing intracellular staining (ICS), 2) Corroborated caspase-3-dependent T-cell apoptosis with non-permeabilizing ICS using the caspase-3 and -7 substrate, phipphilux (Oncoimmune, Gaithersburg, MD), 3) Corroborated T-cell apoptosis with DNA damage in flow-sorted CD3+T-cells using flow cytometric measurements of DNA fragments with the APO-BrdU™ TUNEL Assay Kit (Invitrogen, Carlsbad, CA, USA). Large numbers of cells were required for this component of the experiment. Therefore, DNA damage was measured in CD3+T-cells and not in additional subsets, 4) Measured the cytokines, IL-6, IL1β, and TNFα, which are potentially released by rATG-induced lympholysis, by ELISA in supernates using Multianalyte ELISArrays from SABiosciences- Qiagen (Valencia, CA).

All T-cell subsets expressing activated caspase-3 measured by ICS increased with either allostimulation, or increasing rATG concentrations (Figure 1, Supplementary Table 1). This effect was greatly increased when allostimulation and rATG were used together, than with either treatment alone, and achieved significance for Tc subsets (Supplementary Table 1). Interestingly, rATG and allostimulation together induced significantly more naive and memory Th, which expressed activated cathepsin-B than either intervention alone, but did so minimally among Tc subsets (Figure 1, Supplementary Table 1). In parallel MLC, changes in apoptotic cells measured with caspase-3 were corroborated by a corresponding increase in most phipphilux+Th and Tc subsets (Figure 2), and by DNA damage measured by flow cytometry in flow-sorted CD3 cells (Figures 3a and b). Supernates from MLR co-cultures showed an increased content of the inflammatory cytokines IL6, IL1β and TNFα with increasing rATG concentrations (Figure 3c).

Allospecific CD154+TcM are relatively spared among surviving PBL with high doses of rATG

Parallel MLC (n=6) with normal human PBL tested the inflammatory alloresponse of responder PBL pre-treated with increasing rATG concentrations, to stimulation with irradiated HLA-mismatched stimulators. The inflammatory alloresponse was measured with allospecific CD154+T-cytotoxic memory cells (TcM), which are highly sensitive and specific for rejection after liver, intestine or renal transplantation (14, 17, 18). Intracellular CD154 expression was measured with non-permeabilizing ICS, which incorporates monensin and anti-CD154-phycoerythrin in culture medium for the duration of MLC as described previously (14).

After rATG pre-treatment and allostimulation, absolute counts of all T-cell subsets decreased markedly. For e.g. absolute counts of TcM decreased by 97%, from mean 6065 ± 3653 cells without rATG pre-treatment to 142 ± 97 cells after pretreatment with 200 $\mu\text{g/ml}$ rATG (Figure 3d). The corresponding decrease in allospecific CD154+TcM was less pronounced, with counts decreasing by roughly 75%, from 113 ± 85 cells among untreated PBL, to 29 ± 24 cells after pretreatment with 200 $\mu\text{g/ml}$ rATG. This relative sparing results in an increased frequency of allospecific CD154+TcM among surviving TcM, from mean 2.4 ± 1.3 % among untreated PBL to mean 22 ± 11 % after high-dose rATG pretreatment (Figure 3e).

Rejection-free outcomes are associated with enhanced phipphilux+T-cytotoxic memory cells (TcM) and their increased susceptibility to rATG-induced apoptosis

The cell-permeable caspase-3 substrate phipphilux (Oncoimmune, Gaithersburg, MD), is cleaved by the caspases to a fluorogenic compound, which is detected by flow cytometry. The proportion of phipphilux+ memory (M) and naïve (N) T-helper (Th, CD4) and T-cytotoxic (Tc, CD8) subsets were measured in PBL from each transplant patient, which were pre-treated for 60 minutes in vitro with 0, 5, 20 and 200 $\mu\text{g/ml}$ rATG, and then incubated for 15 minutes with phipphilux. The non-rejector: rejector distribution was 8: 12. Apoptotic subset frequencies increased in a dose-dependent manner for all subsets within each outcome group ($p < 0.001$, repeated-measures 2-way ANOVA) (Figure 4). Further, apoptotic frequencies were higher among non-rejectors compared with rejectors, for each of four T-cell subsets, naïve Th ($p = 0.063$, NS), memory Th ($p = 0.021$), naïve Tc ($p = 0.012$) and memory Tc ($p = 0.017$, repeated measures, 2-way ANOVA) (Figure 4).

Stepwise logistic regression analysis was used to identify the cell type that best predicted rejection-free outcomes in LTx recipients, among Th, Tc, and their naïve and memory subsets, which expressed caspase-3 and -7 by phipphilux staining. Covariates entered into the model were age, gender, race, tacrolimus whole blood concentrations, rATG induction vs no induction, time between transplant and blood sampling, and rATG concentration used in ex-vivo experiments. The optimal model was built with phipphilux+TcM. A threshold phipphilux+TcM frequency 28.2% was reached in 5 of 8 non-rejectors for sensitivity of 62.5%. This threshold frequency was not reached in 10 of 12 rejectors for a specificity of 83.3%. The positive and negative predictive values (PPV, NPV) were 71.4% and 76.9%, respectively. Logistic regression analysis also identified phipphilux+TcM frequency 31.6% as the best predictors of rejection-free outcomes after ITx, with sensitivity, specificity, PPV and NPV of 66.7%, 85.7%, 80%, and 75% (supplementary Figure 1). In the combined population of 33 LTx and ITx recipients, phipphilux+TcM frequency 29.45% predicted rejection-free outcomes with sensitivity, specificity, PPV and NPV of 57%, 89.5%, 80% and 73.9% (Supplementary Table 2).

Discussion

We show for the first time that T-cell apoptosis mediated by activated caspases, which occurs with allostimulation, and also with rATG, is enhanced to the greatest extent by the combination of allostimulation and rATG, than with either stimulus alone. These findings

have been made using two different detectors for activated caspase-3, the antibody to activated antigenic caspase-3, and the caspase-3 substrate, phipphilux, which also serves as a substrate for caspase-7 (Figures 1 and 2). These findings have also been corroborated with the TUNEL assay, which assesses global apoptosis (Figures 3a and b). Therefore, facilitated allostimulation-induced apoptosis of T-cells is indeed another mechanism by which lymphocyte-depleting agents may facilitate graft acceptance and early reduction in immunosuppression requirements. Enhancement of caspase-3-mediated apoptosis with the combination of allostimulation and rATG is seen across all T-cell subsets, and achieves significance for Tc subsets in this small cohort of subjects, when compared with either treatment alone (Figure 1, Supplementary Table 1). In contrast, the enhancement of cathepsin-B-mediated apoptosis by combination treatment, when compared with either treatment alone is more consistently seen among Th subsets. Tc appear resistant to cathepsin-B activation in all conditions tested (Figure 1). Cathepsin-B is also found on the cell-surface of Tc, where it is known to protect against self-apoptosis by released perforin (19). Reduced susceptibility to apoptosis may also explain why Tc are among the first lymphocyte subsets to be reconstituted after rATG induction in children with either liver or intestine allografts (20, 21).

Another finding of interest is the effect of rATG on allo-antigen-specific CD154+TcM. This cell subset is highly sensitive and specific for association with rejection after several types of organ transplants, and has been used as a measure of the inflammatory alloresponse in our current study (14, 17, 18). Although both, parent TcM and allospecific CD154+TcM decrease upon exposure to rATG in a dose-dependent manner, allospecific CD154+TcM are not depleted to the same extent, leading to their relative enrichment among surviving TcM at increasing rATG concentrations (Figure 3d). To illustrate, allospecific CD154+TcM frequencies increased from (mean $\pm$ SD) 2.4 ± 1.3 % in untreated PBL, to mean 22 ± 11 % after high-dose rATG pretreatment (Figure 3e). Possible reasons include an “rATG-resistant” or apoptosis-resistant TcM clone, or cathepsin-B-mediated resistance to self-apoptosis. Another explanation may be antagonism of rATG-mediated apoptosis by the trophic effect of the cytokines IL6, IL1 β , or TNF α (Figure 3c). Released in increasing amounts with increasing rATG doses in our study, these cytokines are known to mediate dose-dependent systemic side effects of rATG (22). Therefore, it is likely that the optimal “tolerogenic” effect of anti-lymphocyte antibodies is also dose-dependent.

The abovementioned findings suggest that caspase-3, which is activated by allostimulation and a pro-apoptotic immunosuppressant in all T-cell subsets is more suited to evaluate apoptotic T-cells as correlates of clinical outcomes, compared with cathepsin-B. Using phipphilux as the caspase-3 target, we find that frequencies of circulating apoptotic T-cells are higher among rejection-free LTx recipients, compared with rejection-prone recipients (Figure 4). Apoptotic cell frequencies are enhanced further with ex-vivo addition of pro-apoptotic induction immunosuppressants such as rATG among all subsets, with non-rejectors showing a greater increase than rejectors. The similarity in apoptotic responses to an indwelling alloantigen and ex vivo rATG treatment within each outcome group suggests that relative susceptibility to apoptosis may be an attribute of the individual recipient, and may characterize the response of that individual to several stimuli (Figure 4). Of greater practical interest is the finding that circulating apoptotic TcM emerge as the subset best

associated with clinical outcomes in logistic regression analysis of all apoptotic subsets. This finding regarding TcM is biologically relevant. In non-human primate models, durable transplant tolerance is only achieved after depletion of TcM (23). In our previous studies, rejection and non-rejection outcomes were predicted more accurately with allospecific CD154+TcM than with other T-cell subsets (14, 17, 18).

Our findings have potential implications for immune monitoring during post-transplant drug minimization. The limiting dilution or trans-vivo delayed type hypersensitivity assays infer tolerance indirectly, from loss of donor-specific inflammatory cells. Apoptosis can be measured directly with a cell permeable indicator within an hour, in a variety of cells, making the assay clinically applicable. Further, this apoptotic susceptibility predicts rejection-free outcomes in two different types of organ transplant recipients, LTx and ITx suggesting a potentially broader utility for apoptosis monitoring in the clinical setting. Larger studies are needed to confirm whether rejection-free outcomes can be detected, and whether the sensitivity and specificity of 57% and 89%, respectively, seen in our preliminary study, can be replicated or improved upon. As with other tolerance tests, results will likely need to be combined with other tests to achieve robust clinical prediction. Another inference is that pro-apoptotic agents may enable early drug minimization in tolerogenic regimens, albeit at optimal doses. Testing pre-transplant susceptibility to apoptosis may facilitate selection of optimal doses toward maximizing successful outcomes.

Materials and Methods

The cell-permeable caspase-3 and -7 substrate, phipphilux (Oncoimmune, Gaithersburg, MD), and fluorochrome-labeled antibodies to activated caspase-3 and cathepsin-B, were used to detect apoptosis in subsets of unfractionated PBL using flow cytometry. The APO-BrdUTM TUNEL Assay Kit (Invitrogen, Carlsbad, CA, USA), was used to detect apoptosis by measuring DNA damage in flow-sorted CD3+T-cells using flow cytometry. Allospecific CD154+TcM were detected with non-permeabilizing ICS using previously described procedures (14-17). Responder PBL were pre-labeled with anti-CD45-APC (allophycocyanin, BD Biosciences, San Jose, CA.), and incubating these cells 1: 1 with irradiated HLA-mismatched stimulator cells. Previously described gating strategy was used to acquire events, which were expressed as percent positive cells. Cytokine released by rATG-induced lympholysis were measured in supernates with Multianalyte ELISArrays from SABiosciences-Qiagen (Valencia, CA).

All fluorochrome-labeled antibodies were obtained from BD Biosciences (San Jose, CA). The exception was anti-CD45RO-Texas Red from Beckman Coulter, (Brea, CA) and anti-cathepsin-B from Novus Biologicals LLC (Littleton, CO). T-cell subsets were identified with fluoro-chrome-labeled anti-CD3 (T-cells), anti-CD4 (Th), and anti-CD8 (Tc). Anti-CD45RO was used to identify the memory phenotype within each subset. Flow cytometry was performed using 8- or 9-color detection on a BD-LSRII system with 488, 635, 405, and 535 nm lasers, and the FACS-DIVA software.

Statistical procedures included t-tests for between-group comparisons, and 2-way repeated measured ANOVA for within and between-group comparisons. Using previously described

methods, logistic regression incorporating six potentially confounding variables described above, were used to identify the apoptotic cell type best associated with rejection-free outcomes in children with LTx.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

LTx	liver transplantation
M	memory
MLC	mixed lymphocyte co-cultures
N	naive
PBL	peripheral blood lymphocytes
rATG	rabbit anti-human thymocyte lobulin
Tc	T-cytotoxic cells (CD8)
Th	T-helper cells (CD4)

Figure 1.

Frequencies of naive (N) and memory (M) Th and Tc which express activated caspase-3 (panels on left) and activated cathepsin-B (panels on right) are shown after no treatment, rATG-pretreatment, allostimulation, and combined allostimulation and rATG pre-treatment of normal human responder PBL. Data are shown as representative histograms for ThM and TcM from one replicate (upper two panels), summary of data from four replicates for a single rATG concentration of 20 µg/ml (bar diagram), and summary of data for four replicates for each of four rATG concentrations. Activated caspase-3 and cathepsin-B are measured with specific fluorochrome-labeled antibodies and ICS.

Figure 2.

Frequencies of phiphilux+ThM and TcM subsets (upper and middle panels) are shown after no treatment, rATG-pretreatment, allostimulation, and combined allostimulation and rATG pre-treatment of normal human responder PBL. Phiphilux measures activated caspase-3 without ICS. Bar diagram in lower panel shows summary of data from six replicates for a single rATG concentration of 20 µg/ml.

Figure 3.

a. Representative histograms show DNA damage in flow-sorted CD3+T-cells by TUNEL assay after no treatment, rATG-pretreatment, allostimulation, and combined allostimulation and rATG pre-treatment of normal human responder PBL from one of the four replicate. **b.** Summary data (n=4) for frequencies of phiphilux+TcM, and CD3+T-cells manifesting DNA damage in normal human PBL pretreated with increasing rATG concentrations. **c.** Summary data (n=3) showing increased production of the cytokines IL6, IL1β and TNFα by allostimulated T-cell with increasing rATG concentrations. **d.** Summary data from MLC showing that (97%) decrease in allostimulated TcM with increasing rATG concentrations is accompanied by a numerically smaller (75%) decrease in allospecific CD154+TcM (n=6). **e.** Summary data from MLC (n=6) showing increased frequency of allospecific CD154+TcM among surviving TcM at increasing rATG concentrations.

Figure 4.

Summary data for apoptotic memory (M) (upper row) and naïve (N) (bottom row) Tc (column on left) and Th (column on right) among PBL from 20 children with LTx, after ex vivo pre-treatment with 0-200 µg/ml rATG. Apoptotic subsets are measured with the caspase-3 substrate, phipphilux. The population consists of 8 non-rejectors (NR, black squares) and 12 rejectors (R, grey triangles). P-values for between-group comparisons using 2-way repeated measures ANOVA are shown.

Table 1

Demographics and clinical information forLTx and ITx recipients.

	Non-rejector	Rejector	p-value
Total Patients	14	19	
LTx	8	12	
ITx	6	7	
Outcome	14	19	
Gender(M:F)	7:7	8:11	0.732 (NS)
Race(Caucasian:non-caucasian)	11:3	15:4	0.999 (NS)
Age at Transplant (Mean±SEM)	5.2±1.7	4.6±1.2	0.768 (NS)
FKWBLTx	4.5 ± 1.0	8.2 ± 1.4	0.055 (NS)
FKWBITx	6.1± 1.7	8.2 ±1.5	0.372 (NS)
Induction: no induction	12:2	13:6	0.698 (NS)
Time to Tx and sample (Mean±SEM)	1596 ±620	977 ± 270	0.372 (NS)
Tacrolimus alone	12 of 13	17 of 19	0.999 (NS)
Sirolimus alone	1of13	2 of 19	0.999 (NS)
Tacrolimus +Prednisone	5 of 13	4 of 19	0.427 (NS)
Tacrolimus +Prednisone+Cellcept	0of 13	1of19	0.999 (NS)
HLA match			
HLA-A (Mean±SEM)	0.38 ±0.14	0.37 ±0.11	0.929 (NS)
HLA-B (Mean±SEM)	0.15 ±0.15	0.47 ±0.12	0.111 (NS)
HLA-DR (Mean±SEM)	0.54 0.18	0.58 ±0.12	0.854 (NS)
All (Mean±SEM)	1.07 ±0.33	1.42 ±0.28	0.433 (NS)
Detectable Viral load			
EBV	1of14	3 of 19	0.619 (NS)
CMV	0 of 14	0 of 19	0.999 (NS)