Killer artificial antigen-presenting cells: a novel strategy to delete specific T cells

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Several cell-based immunotherapy strategies have been developed to specifically modulate T-cell–mediated immune responses. These methods frequently rely on the utilization of tolerogenic cell-based antigen-presenting cells (APCs). However, APCs are highly sensitive to cytotoxic T-cell responses, thus limiting their therapeutic capacity. Here, we describe a novel bead-based approach to modulate T-cell responses in an antigen-specific fashion. We have generated killer artificial APCs (κaAPCs) by coupling an apoptosis-inducing α-Fas (CD95) IgM mAb together with HLA-A2 Ig molecules onto beads. These κaAPCs deplete targeted antigen-specific T cells in a Fas/Fas ligand (FasL)–dependent fashion. T-cell depletion in cocultures is rapidly initiated (30 minutes), dependent on the amount of κaAPCs and independent of activation-induced cell death (AICD). κaAPCs represent a novel technology that can control T cell–mediated immune responses, and therefore has potential for use in treatment of autoimmune diseases and allograft rejection. (Blood. 2008;111:3546-3552)

Introduction

The major goal in the treatment of autoimmune and allograft rejection is controlling autoreactive cytotoxic T lymphocytes (CTLs) directed at self-antigens. Current therapeutic approaches are mostly based on immunosuppressive drugs that globally suppress the immune system.1,2 While successful, these treatments make the patients vulnerable to infections and possibly even the development of cancer. In order to selectively target autoreactive CTLs without compromising normal immune functions, new strategies need to be developed. The steadily increasing knowledge of antigen-presenting cell (APC)/T-cell interactions,3-6 newly identified APC and T-cell subsets,7,8 and the observation that some tumor cells express Fas ligand (FasL; CD95L) on their surface to evade T cell–mediated antitumor responses9-12 has led researchers to design new approaches to induce T-cell inhibition or deletion.

One approach to inhibiting or deleting autoreactive CTLs in the setting of autoimmune disease and allograft rejection is based on genetically modified dendritic cells (DCs) that express the apoptosis-inducing ligand FasL.13-20 We and others have previously demonstrated in various murine models and cell lines that FasL-expressing DCs can be used for antigen-specific deletion of unwanted CTLs.16,21 While these results are promising, there are several problems related to the use of autologous DCs. DC preparation is time, cost, and labor intensive, and the resulting cell product is highly variable in quality and quantity from donor to donor and from preparation to preparation. In addition, mature killer DCs express high levels of costimulatory molecules such as B7.1 and B7.2, which could be counterproductive by stimulating antiapoptotic processes.22 Furthermore, while killer DCs can be pulsed with peptides of interest, they will also present other previously acquired antigens, potentially leading to deletion of unwanted or unidentified T cells. For the same reason, gene transfer strategies using viral vectors are limited to in vitro studies, as virally transduced killer DCs will present viral antigens, thus causing a suppressed immune response if the patient becomes infected with the native virus. Moreover, all cellular-based DCs or APCs approaches are sensitive to their in vivo and in vitro environment, as the cytolytic effector activity of T cells23 may lead to DC depletion or unwanted changes in cell-cell signaling.

To overcome issues related to the use of cell-based killer APCs, we have developed a new bead-based approach to inhibit or delete self-reactive CTLs in an antigen-specific fashion for the treatment of autoimmune disease and allograft rejection. Based on our previous work using artificial APCs (aAPCs)24 and our experiences with FasL-expressing killer DCs,25,26 we have designed killer aAPCs (κaAPCs), made by coupling HLA-A2 Ig and α-Fas IgM mAb covalently to the surface of beads. In this study, we have successfully used κaAPCs to delete antigen-specific CTLs in vitro from a mixture of T cells with various specificities.

Methods

This study was approved by the ethical committee of the University of Regensburg.

Peptides

The following HLA-A2–restricted peptides have been synthesized by the Johns Hopkins University core facility and used throughout the experiments: human cytomegalovirus pp65–derived peptide (CMVpp65; NLVPMVATV) and modified melanoma-associated antigen peptide (Mart-1: ELAGIGILTV). The purity


An Inside Blood analysis of this article appears at the front of this issue.
Generation of human antigen-specific CTLs using aAPCs

aAPCs have been generated as previously described and used to expand Mart-1- and CMVpp65-specific CTLs.24 Briefly, purified CD8+APCs have been generated as previously described and used to expand CTLs. Medium and TCGF were replenished once a week. Specificity of CTLs was monitored each week and directly before use by fluorescence-activated cell sorting (FACS) analysis.

Generation of αAPCs and control beads

αAPCs were generated by coupling HLA-A2 IgG1 (0.018 μg/mL) and α-Fas IgM mAb, (clone CH11; Upstate, Chicago, IL; 3.64 μg/mL) onto 108 epoxy beads (Dynabeads, Oslo, Norway). Briefly, beads were washed twice in sterile 0.1 M borate buffer and then loaded in borate buffer overnight at 4°C on a rotator. The next day, the beads were washed twice with 1 mL bead wash (phosphate-buffered saline [PBS] with 0.001% NaN3, 2 mM EDTA, and 0.1% bovine serum albumin [BSA]) and incubated overnight at 4°C on a rotator. Finally, the beads were placed into 1 mL fresh PBS (GIBCO/Invitrogen) and loaded with either CMVpp65 or Mart-1 peptide (10 μg/mL) at 4°C overnight. Before use, αAPCs were washed several times. Control beads were prepared in identical fashion but lacking the α-Fas IgM mAb (clone CH11).

Cocultures

Cocultures were established by placing expanded antigen-specific CTLs in 96-well round-bottom plates at a density of 1.5 × 105 cells/well together with control beads (CBs) or αAPCs, which have been unloaded or loaded with indicated peptides. A bead-to-T-cell ratio of 1:1 was used throughout the experiments if not stated differently. Soluble α-Fas IgM (clone CH11) at a concentration of 1 μg/mL served as positive control. Cocultures established with complete RPMI-1640 medium supplemented with 3% autologous plasma or human AB serum (Cambrex, Walkersville, MD) and 3% TCGF were replenished once a week. Additionally, peptides (purity greater than 95%) were obtained from Merck (Darmstadt, Germany). Peptides were dissolved in 10% dimethyl sulfoxide (DMSO) at 1 mg/mL and sterile-filtered through a 0.22-μm SpinX (Corning, Corning, NY).

Figure 1. Composition and phenotypical characterization of αAPC and control beads (CB). (A) Composition of a HLA-A2 IgG1–based αAPC and CB. (B) FACS analysis of IgM (α-Fas mAb; left panels) and IgG1 (HLA-A2 IgG1; right panels) immobilized on CBs and αAPCs. ■ represents the isotype control, whereas □ indicates α-Fas mAb (clone CH11) and HLA-A2 IgG1.

15 minutes at room temperature in the dark. Samples were immediately analyzed.

Flow cytometry

Cells or beads were stained with α-mouse IgM mAb–FITC (clone R6-60.2; BD Pharmingen), α-mouse IgG1 mAb–PE (BD Pharmingen), and α-human CD8 mAb–FITC (clone UCHT-4; Sigma-Aldrich, St Louis, MO) at 4°C for 10 to 15 minutes. Prior to antibody stain, HLA-A2 tetramer (Immunomics, Beckmann Coulter, Marseilles, France) stain was performed for 30 minutes at room temperature in the dark. Apoptosis assays were conducted according to the manufacturer’s protocol (ApoAlert annexin V Apoptosis Kit; BD Pharmingen). FACS analysis was carried out on a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA). All flow cytometry data were analyzed with either FCSExpress 3 (De Novo Software, Thornhill, ON) and/or WinMDI 2.8 (http://facs.scripps.edu/software.html). Numbers of viable cells were determined by the amount of Annexin V and propidium iodide (PI) or 7-AAD double-negative cells. Untreated control cultures were set to 100%, whereas the percentage of viable cells in specifically treated cultures was calculated as % viable cells = (% treated cells / % untreated cells).

Results

Generation of αAPCs

Previously, we developed an HLA-A2 Ig–based aAPC for ex vivo expansion of antigen-specific CTLs.24 Based on this technology, we designed a αAPC that would induce the depletion of CD8+ T cells in an antigen- and HLA-dependent manner (Figure 1A). In
this system, peptide-loaded HLA-A2 Ig was used to provide an antigen-specific signal, and α-Fas IgM (clone CH11) provided an apoptosis-inducing signal. Together, these signals should initiate apoptosis in T cells specific for the peptide-HLA of interest via the Fas/FasL signaling pathway. CBs were made by coating with HLA-A2 Ig in the absence of any apoptosis signal.

To confirm the phenotype of αAPCs and CBs, FACS analysis was performed (Figure 1B). Staining with an α–mouse IgM mAb and an α–mouse IgG1 mAb demonstrated that αAPCs displayed both α-Fas IgM (clone CH11) and HLA-A2 Ig immobilized to the bead surface, whereas CBs lacked the apoptosis-inducing signal but had equal amounts of HLA-A2 Ig (Figure 1B left and right panels). Phenotypes of all αAPCs and the corresponding controls (CBs) used throughout the experiments have been routinely analyzed in this manner.

Antigen-specific apoptosis induction by αAPCs

To investigate whether αAPCs delete primary human CD8+ T cells in an antigen-specific manner, CD8+ T cells were purified from peripheral blood mononuclear cells (PBMCs) of healthy donors, expanded as described, and used as targets for αAPCs. Figure 2A shows a representative tetramer analysis of primary CMVpp65-specific CTLs that were used throughout the experiments.

To distinguish viable cells from apoptotic cells, we simultaneously stained with annexin V and PI. Annexin V is known to bind phosphorlidyserine (PS) under high Ca2+ conditions and therefore detects early apoptotic events. Annexin V(high) T-cell populations are almost exclusively apoptotic and also stain positive for PI. PI has been demonstrated to intercalate into fragmented DNA and therefore detects late apoptotic events. The combination of both annexin V(high) and PI(high) staining ensures a reliable exclusion of dead cells and therefore an accurate determination of viable, nonapoptotic cells (annexin V/PI double-negative).

CMVpp65-specific CTLs generated from different donors were cocultured with CMVpp65(αAPCs), αAPCs loaded with noncognate Mart-1 peptide (Mart-1(αAPCs)), unloaded αAPCs (unloaded(αAPCs)), or with corresponding CBs lacking the apoptosis signal (CMVpp65CBs, Mart-1CBs, and unloadedCBs; Figure 2B). The amount of viable cells (VCs; annexin V−/PI− population) after 48 hours of coculture was compared. There was a strong induction of apoptosis in CMVpp65-specific CTLs cocultured with CMVpp65(αAPCs); only 13% of the CTLs remained viable, whereas unloaded and noncognate peptide-loaded αAPCs and CBs induced apoptosis only slightly above background levels (VCs, 88%-97%).

CMVpp65 (NLVPVMATV) is known to be a high-affinity peptide. Additional experiments were performed using CD8+
Specific CTLs with CMVpp65 CD107a up-regulation. In contrast, cocultures of CMVpp65-stimulated with soluble CMVpp65 peptide, which induced strong CBs (Figure 3). As a positive control, CMV-specific CTLs were cocultured with Mart-1 CBs, CMVpp65 CBs, Mart-1 aAPCs, or unloaded aAPCs. CTL cultures stimulated with 7.5 ng/mL soluble CMVpp65 peptide served as positive control. Numbers of CD107a+ cells were determined 5 hours following initiation of cocultures. One representative experiment of 3 is shown.

T cells directed toward the low-affinity modified Mart-1 peptide (ELAGIGILTV), which is derived from a melanocyte self-antigen. Mart-1-specific CTLs generated from different donors were cocultured with Mart-1 aAPCs, CMVpp65 aAPCs, and unloaded aAPCs (Figure 2B). As observed with CMVpp65 aAPCs, Mart-1 aAPCs induced strong apoptosis during coculture with their cognate targets; thus, only 15% of the cells remained viable, whereas unloaded aAPCs did not induce apoptosis (VCs, 95%). In addition, apoptosis at background levels was observed in all control cultures with CMVpp65 aAPCs (VCs, 84%) and Mart-1 CBs, CMVpp65 CBs, and unloaded CBs (VCs, 87%-95%; data not shown). Apoptosis of CTLs was only slightly elevated in cocultures with unloaded and non-cognate-loaded aAPCs as compared with cocultures with CBs. These results demonstrate that aAPCs can be used to efficiently delete CTLs in an antigen-specific fashion.

To analyze the mechanism of aAPCs, we investigated whether cell/bead contact led to an activation of antigen-specific CTLs and subsequent activation-induced cell death (AICD). It has been demonstrated that CD107a is expressed on the cell surface of CD8+ T cells shortly after activation with cognate peptide, and that these cells mediate cytolytic activity in an antigen-specific manner. Therefore, we examined the amount of CD107a expressed on the cell surface of CD8+ T cells after activation with cognate peptide, and that these cells mediate cytolytic activity in an antigen-specific manner. Activation in CMVpp65-specific CTLs of a single donor cocultured with Mart-1 CBs, CMVpp65 CBs, Mart-1 aAPCs, or CMVpp65 aAPCs. CTL cultures stimulated with 7.5 ng/mL soluble CMVpp65 peptide served as positive control. Numbers of CD107a+ cells were determined 5 hours following initiation of cocultures. One representative experiment of 3 is shown.

AICD. Activation in CMVpp65-specific CTLs of a single donor cocultured with Mart-1 CBs, CMVpp65 CBs, Mart-1 aAPCs, or CMVpp65 aAPCs. CTL cultures stimulated with 7.5 ng/mL soluble CMVpp65 peptide served as positive control. Numbers of CD107a+ cells were determined 5 hours following initiation of cocultures. One representative experiment of 3 is shown.

To analyze if a short interaction of aAPCs with CTLs is sufficient, or if long-term interaction is needed for maximal apoptosis induction, we also characterized the kinetics of the aAPC-dependent CTL depletion. Antigen-specific CTLs were stimulated with aAPCs for different coculture periods (Figure 5). Cocultures established for 48 hours served as positive control for maximum depletion. To separate beads from CTLs, beads were magnetically removed from the cocultures at indicated time points. More than 95% of the aAPCs were removed during this purification step, as confirmed by light microscopy. Following purification,
CTLs were cultured for 48 hours, and apoptotic CTLs were determined by FACS analysis. High numbers of apoptotic CTLs could already be observed after a coculture period with CMVpp65-kaAPCs for just 30 minutes (VCs, 31%), compared with 15% viable CMVpp65-specific CTLs cocultured with CMVpp65-kaAPCs for 48 hours. In contrast, CTL apoptosis was within background levels (VCs, 85%-100%) in all other established cocultures with CMVpp65 peptide–loaded CBs (data not shown) as well as unloaded or non cognate peptide–loaded kaAPCs. These results indicate that even after only a short period of interaction, kaAPCs were able to delete CTLs.

**kaAPCs eliminate CTLs in an antigen-specific manner from T-cell populations with diverse antigen specificities**

To demonstrate that kaAPCs deplete CTLs in an antigen-specific fashion, additional coculture experiments were performed using a mixture of CTLs with different antigen specificities. For these experiments, an autologous T-cell mixture (T mix) was established containing PKH67-stained, CMVpp65-specific CTLs and PKH26-labeled, activated Fas+ effector-memory CTLs at a ratio of 1:1. Cells from this T mix were then cocultured with unloaded or non cognate peptide–loaded kaAPCs or CBs. This experimental set-up allowed us to determine apoptosis in each T-cell population by differential gating on PKH67-labeled, activated Fas+ containing PKH67-stained, CMVpp65-specific CTLs and PKH26-labeled, activated Fas+ effector-memory CTLs at a ratio of 1:1. Cells from this T mix were then cocultured with unloaded or CMVpp65 peptide–loaded kaAPCs or CBs. This experimental set-up allowed us to determine apoptosis in each T-cell population simultaneously. We determined the kaAPC-mediated CTL depletion in both populations by differential gating on PKH67+ CTLs and PKH26+ CTLs. T mix cocultures with CMVpp65-kaAPCs revealed clear differences between both populations (Figure 6). CMVpp65-kaAPCs specifically killed cognate CMVpp65–specific CTLs in the T mix; only 14% of the original CMVpp65 population remained viable. In contrast, non–CMV-specific activated Fas+ effector-memory CTLs were not depleted, and similar to non-treated CTLs, more than 70% of these CTLs remained viable. Considering that both populations were highly apoptosis sensitive when treated with soluble α-Fas mAb, our findings demonstrate that the detected differences were due to specific interactions of CMVpp65-kaAPCs with CMVpp65–specific CTLs. In addition, cocultures with unloaded kaAPCs or other CBs (data not shown) did not result in significant CTL depletion in any of the populations (VCs, 85%-97%) which further emphasizes that cognate-loaded kaAPCs are capable of depleting activated CTLs in an antigen-specific manner from a mixture of activated T cells with different antigen specificities.

**Discussion**

Antigen-specific elimination of T cells by FasL-expressing APCs has been demonstrated by various investigators in different in vitro and in vivo experimental models using both murine and human cells. Despite promising results indicating the therapeutic potential of FasL-expressing killer APCs as immunoregulatory cells for the treatment of allograft rejection, autoimmune disease, and chronic infections, there are several significant limitations associated with these approaches related to the cellular nature of the modified APCs. Ultimately, this has limited clinical utilization. There are several limitations. First, transduction with the FasL gene is required to generate killer APCs. Several virus- and nonvirus-based transduction methods have been used; however, all strategies result in different levels of FasL expression depending on the transduction efficiency. Therefore, purification of FasL-expressing APCs is needed to avoid activation of T cells by nontransduced APCs. In addition, production of soluble FasL has been observed in killer APCs, compromising its efficiency due to blockade of the Fas/FasL signaling pathway. Second, use of primary cells, including DCs, as APCs is time, cost, and labor intensive, and the resulting cell product is highly variable in quality and quantity. In particular, the phenotype of the killer APCs cannot be maintained from donor to donor, resulting in variable functional activity. Third, since killer APCs are also targets of CTLs, the therapeutic potential could be substantially reduced. Finally, presentation of new antigens derived from bacteria or viruses as well as viral vectors on the surface of FasL-expressing killer APCs could result in elimination of activated T cells directed against bacterial or viral antigens, resulting in a critical impairment of the protective immune response.

In light of these limitations and based on our previous work and the work of others, we generated an artificial killer APC by conjugation of HLA-A2 Ig dimer molecules and an apoptosis-inducing α-Fas IgM mAb onto epoxy-beads, which could then be loaded with different peptides. In contrast to our aAPCs, which express an optimal amount of HLA-A2 Ig and α-CD28 mAb required for efficient T-cell activation, the goal for the kaAPCs was to achieve maximal antigen-specific killing combined with minimal CTL activation. Therefore, it was necessary to carefully titer the amount of HLA-A2 Ig and α-Fas mAb during kaAPC preparation. To evaluate the optimal amount and ratio of HLA-A2 Ig and α-Fas mAb, we found that if more HLA-A2 Ig was coupled to the beads, they would induce antigen-specific activation and consequent expansion of the target cells, while if more α-Fas mAb was coupled to the kaAPCs, they would induce nonspecific killing of all Fas-expressing CTLs. Phenotypic and functional characterization of our current kaAPCs demonstrates deletion of CD8+ T cells in an antigen-specific fashion, which was directly related to the number of kaAPCs present in the cocultures. In addition, kaAPCs selectively eliminated CMVpp65–specific CTLs from mixtures of effector-memory T cells with unknown specificity, specifically reducing the targeted CMV-specific CTL population, while not significantly affecting the viability of the remaining non–CMV-specific CTLs. To our knowledge, this is the first report of the generation and functional characterization of bead-based “off the shelf” kaAPCs that might be of use in modulating T-cell responses in autoimmune and transplantation-related diseases.
In contrast to cell-based killer APCs, καAPCs do not promote the risk of activation of a T-cell response, or induction of tolerance toward other antigens. Furthermore, καAPCs are not eliminated by self or paracrine killing due to Fas/FasL signaling, and καAPCs are not targets of the cytokine effector functions of CTL. In addition, the ease of the καAPC system allows one to modify the phenotype through simple peptide exchange, and large numbers of καAPC with proven activity can be rapidly and reproducibly manufactured. The apoptosis-inducing signal could be modified as well by using other ligands or α receptor mAb of the TNFR superfamily.42,43 Also, καAPCs do not produce FasL. Cell-based killer APCs that express only minimal or even no FasL might initiate intracellular signaling cascades that ultimately lead to priming and expansion of antigen-specific T cells. The fact that this cannot happen using our system highlights the great advantage of a bead-based καAPC.

Stimulation with either καAPCs or CBs did not result in CD107a expression, indicating that the interaction with CTLs did not lead to complete activation.29 Furthermore, we found that only 30 minutes or less of interaction between CTLs and καAPCs were required to induce Fas-mediated apoptosis leading to CTL deletion. For technical reasons, we were not able to test shorter intervals of stimulation. Nevertheless, these experiments clearly demonstrate that apoptosis in CTLs was dependent on a combined signal, delivered via the T-cell receptor (TCR) and Fas, which was only provided by καAPCs loaded with the cognate peptide, and not καAPCs loaded with an irrelevant peptide or CBs. This could reflect the fact that the beads were made with low amounts of HLA-A2. Ig dimer and/or also did not have any costimulatory complexes. In either event, καAPCs that delivered simultaneous signals through both the TCR and Fas-receptor rosette into big bead-cell clusters so that one καAPC may simultaneously induce apoptosis in multiple CTLs. Due to the fact that all experiments are performed in a static 96-well system, it is not clear if καAPCs can also induce apoptosis to multiple CTLs through multiple contacts during migration.

Other novel approaches that could complement bead-based καAPCs have been developed. For example, Tykocinski et al have developed a CTLA-4–FasL fusion molecule that physically and functionally bridges APCs and T cells. Binding of CTLA-4 to B7.1 and B7.2 on the APCs blocks the important costimulatory interaction of CD28 on T cells and B7 on the APCs. At the same time, the FasL molecule binds to the Fas receptor on activated T cells and induces Fas/FasL-mediated T-cell apoptosis. It has been shown that this newly designed molecule, which combines the abilities to block an important costimulatory pathway and to induce apoptosis, can be used to delete alloantigen-specific CTLs in vitro. In addition, it was demonstrated that the CTLA-4–FasL fusion protein is more efficient at T-cell deletion than the separated or combined administration of soluble CTLA-4–Ig and FasL–Ig, again suggesting that the combination of signals is crucial for the observed effect.44

With regard to potential clinical applications, bead-based καAPCs in contrast to cell-based killer APCs cannot process antigens. This is advantageous in that it avoids the depletion of nonpathogenic T cells and the presentation of viral-vector antigens. However, it could be argued that the inability to process antigen is a limitation of καAPC functionality. καAPCs require the identification of relevant antigens for each disease. Currently, there are already several HLA-A2–restricted target antigens relevant for type 1 diabetes45,46 as well as for graft-versus-host disease (GVHD)50,51 and multiple sclerosis.52,53 Furthermore, the decoding of the human genome and the application of novel molecular technologies will permit the rapid identification of new auto-antigens in the near future.54 In addition, it has been demonstrated that during the onset phase of an autoimmune disease, only a few autoreactive T-cell clones are activated, and that antigen spreading occurs later during the disease course.55 More importantly, καAPCs provide the advantage of customizing signals and their strength to most efficiently target pathogenic T cells for immunotherapy. Cell-based approaches do not have this advantage, and furthermore, they may harbor other naturally occurring undesirable signals dependent on the cell scaffold used, which can be excluded when using καAPCs. In cell-based approaches, gene regulation issues affect signal strength and composition; this is not a concern with καAPCs. In addition, initial experiments in mice have shown that καAPCs are capable of activating CTLs in vivo (data not shown). Furthermore, when injected intravenously, the beads distribute into all organs analyzed, including spleen, lymph nodes, liver, pancreas, kidney, and lungs (data not shown). Therefore, in vivo activation of καAPCs appears to be a promising treatment strategy during the early phase of an autoimmune disease. The beads currently in use are available in Good Manufacturing Practice grade, and one could envision using other scaffolds such as biodegradable particles or latex, which have already been used in vivo in other clinical settings.56

In summary, we provide proof of concept that CTLs can be depleted in an antigen-specific fashion by bead-based HLA-A2-Ig καAPCs. The flexibility and versatility of the καAPC platform enables us not only to modulate the signal strength but also to compose new signal combinations to target other T-cell populations for immunotherapy. Therefore, this study shows proof of principle of a bead-based καAPC that has the potential to treat T-cell–related autoimmune diseases and allograft rejection.

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Authorship

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