Allograft tolerance induced by donor apoptotic lymphocytes requires phagocytosis in the recipient

E Sun[1,2], Y Gao[1], J Chen[1], AI Roberts[2], X Wang[3], Z Chen[4] and Y Shi*[,2]

[1] Transplantation Department, Zhujiang Hospital, Guangzhou 510282, China
[2] Department of Molecular Genetics, Microbiology and Immunology, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ 08854, USA
[3] Institute of Molecular Immunology, Guangzhou 510515, China
[4] Institute of Transplantation, Tongji Medical College, Huazhong University of Science and Technology, 1095 Jiefang Avenue, Wuhan, Hubei, 430030, China
* Corresponding author: Y Shi, Department of Molecular Genetics, Microbiology and Immunology, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, 661 Hoes Lane, Piscataway, NJ 08854, USA. Tel: +732-235-4501; Fax: +732-235-4505; E-mail: shiyu@umdnj.edu

Abstract

Cell death through apoptosis plays a critical role in regulating cellular homeostasis. Whether the disposal of apoptotic cells through phagocytosis can actively induce immune tolerance *in vivo*, however, remains controversial. Here, we report in a rat model that without using immunosuppressants, transfusion of apoptotic splenocytes from the donor strain prior to transplant dramatically prolonged survival of heart allografts. Histological analysis verified that rejection signs were significantly ameliorated. Splenocytes from rats transfused with donor apoptotic cells showed a dramatically decreased response to donor lymphocyte stimulation. Most importantly, blockade of phagocytosis *in vivo*, either with gadolinium chloride to disrupt phagocyte function or with annexin V to block binding of exposed phosphotidylserine to its receptor on phagocytes, abolished the beneficial effect of transfused apoptotic cells on heart allograft survival. Our results demonstrate that donor apoptotic cells promote specific allograft acceptance and that phagocytosis of apoptotic cells *in vivo* plays a crucial role in maintaining immune tolerance.
Cell Death and Differentiation (2004) **11,** 1258–1264.
doi:10.1038/sj.cdd.4401500
Published online 17 September 2004

Introduction

Cell death by apoptosis is an evolutionarily conserved biological process that occurs both physiologically and pathologically.[1] *In vivo*, apoptotic cells are hardly detectable as they are rapidly removed by phagocytic cells, thus preventing the release of intracellular contents that would cause local inflammation.[2] This phagocytic process is also highly conserved; similar mechanisms operate in *Caenorhabditis elegans* as well as in mammalian species.[3–6] Several cell types have been shown capable of phagocytosing apoptotic cells. Among them, tissue macrophages and dendritic cells (DC) play a major role in apoptotic cell clearance.[7–10] They possess receptors for molecules specifically present on the surface of apoptotic cells including phosphatidylserine (PS), which is exposed on the outer leaflet of the cell membrane early in apoptosis.[2,10] Significantly, there is evidence that phagocytes secrete immunosuppressive cytokines upon phagocytosing apoptotic cells.[11,12] Since these same phagocytes are the main antigen-presenting cells, phagocytosis of apoptotic cells may provide immunosuppressive signals to T cells.

The concept that apoptotic cells have an immunoregulatory role comes from early clinical observations in blood transfusion. It was found that blood transfusion after surgical operation or trauma was often accompanied by immunosuppression and opportunistic infection.[13,14] In addition, blood transfusion was reported to reduce autoimmune disease severity and to prolong survival of allografts.[13,14] Apoptotic granulocytes and lymphocytes have been detected in blood stored for clinical transfusion[15,16] and it has been shown that leukocytes are important for the immunoregulatory property of transfused blood, since depletion of these cells abolishes immunosuppression.[13,14,17] Recent studies have shown that apoptotic cells do indeed transmit immunosuppressive signals. Cells undergoing apoptosis secrete immunosuppressive cytokines, such as IL-10 and TGF-β, and also inhibit the secretion of proinflammatory cytokines by macrophages.[18] In fact, apoptotic cells greatly increase the susceptibility and mortality of animals to infections.[19] Normal apoptosis has also been demonstrated to be crucial for the establishment of immune privilege in the anterior chamber of the eye, while disruption of apoptotic mechanisms in mice deficient in Fas or Fas ligand (FasL) breaks tolerance and leads to inflammation.[20]

Apoptotic cells must be cleared at an early stage to prevent them from undergoing secondary necrosis that would deliver 'danger signals' to activate the immune system.[21] Therefore, it has been proposed that prompt clearance of apoptotic cells is crucial for the maintenance of self-tolerance, while their delayed removal promotes autoimmune disease.[22–25] In fact, accumulating evidence shows that apoptotic cells play an important role in autoimmune disease pathogenesis. Recent studies revealed that complement C1q and C-reactive protein are important molecules in mediating the phagocytosis of apoptotic cells. C1q specifically binds to membrane blebs of apoptotic cells, promoting phagocytosis.[26–27] In systemic lupus erythematosus (SLE) patients, both these molecules are decreased. Also, C1q-deficient mice have impaired

monocyte/macrophage phagocytosis of apoptotic cells and display increased numbers of apoptotic cells in the kidneys, with nephritis-like changes.[27] Finally, in diabetic BB rats or NOD mice, increased numbers of apoptotic islet β-cells are found to be accompanied by macrophage abnormalities, including decreased cell number and a deficiency in the recognition or phagocytosis of apoptotic cells, which may play a role in the pathogenesis of autoimmune diabetes.[28,29] These data clearly indicate that apoptotic cells are actively immunoregulatory. However, the mechanisms by which apoptotic cells mediate immunosuppression, especially *in vivo*, remain elusive.

Attempts have been made with *in vitro* systems to examine the influence of apoptotic cells on immune responses.[11,12] We have reported that apoptotic cells inhibit Con A-stimulated expression of CD69, an early marker of T-cell activation, demonstrating that apoptotic cells do actively suppress T-cell activation.[30] As most such investigations have used *in vitro* systems, the physiologic role of this apoptotic cell-mediated immune regulation (AMIR) is yet to be established *in vivo*. To determine whether apoptotic cells regulate immune responses *in vivo*, we employed a rat allogeneic whole-heart transplant model, in which donor heart blood vessels are anastomatized to those of the recipient. We found that administration of apoptotic donor cells 7 days before transplantation specifically prolonged cardiac graft survival. Furthermore, we also demonstrated that, both *in vitro* and *in vivo*, phagocytosis of apoptotic cells plays a crucial role in their ability to induce transplant tolerance induction. These findings indicate that transfused allogeneic apoptotic cells transmit immunoregulatory signals to phagocytes, an effect which plays a crucial role in the induction of allograft tolerance.

Results

Transfusion of donor apoptotic cells prolongs allograft survival

To test whether apoptotic cells from a transplant donor can prolong allograft survival, we employed ultraviolet (UV)- or γ-irradiation to induce apoptosis in freshly isolated splenocytes from a donor strain animal before transfusion into the recipient. Such physical methods are more advantageous than either chemical or biological means, as cells so treated do not have to be washed and thus there is no loss of apoptotic cells or apoptotic bodies. We also found that UV- or γ-irradiation are more effective in apoptosis induction and minimize the occurrence of necrotic cells in the resultant cell preparation, which otherwise would provide danger signals and generate an immune response upon transfusion.[31] Our UV-based protocol typically generates around 50% apoptotic cells with less than 3% necrotic cells, as detected by staining with annexin V and propidium iodide (PI).

Splenocytes from Wistar rats were thus induced by UV to undergo apoptosis and 5×10^7 cells were injected intravenously (i.v.) into each intended Sprague–Dawley (SD) recipient. Heterotopic donor heart transplantation was performed 1 week later. As shown in Figure 1a, the median graft survival time (MST) of rat cardiac allografts was 7 days in control recipients injected with saline alone. Strikingly,

transfusion with apoptotic donor splenocytes 7 days before transplantation resulted in significant prolongation of graft survival (MST = 53 days, $P < 0.01$ *versus* control group), while transfusion of live or necrotic donor splenocytes resulted in survival patterns similar to those of untreated animals (MST = 8 and 10 days, respectively). Therefore, transfusion of apoptotic cells prolongs the survival of allografts.

For determining the optimal preoperative induction period for graft acceptance, apoptotic cell transfusion was carried out 14, 7 or 3 days before transplantation and the effect on heart allograft survival time was measured. As shown in Figure 1b, an induction period of 7 days was the most effective: apoptotic cell transfusion on day −7 resulted in an MST of more than 50 days, which was significantly longer ($P < 0.01$) than that induced by transfusion on day −3 (MST = 8 days) or day −14 (MST = 10.5 days). Histological analysis of grafts 1 week after transplantation further revealed obviously attenuated rejection signs in grafts from apoptotic cell-transfused recipients, as evidenced by greatly reduced lymphocyte infiltration and less destruction of cardiac cells (Figure 1c).

In order to know whether the effect of apoptotic cells on graft survival depends on the method of apoptosis induction, we next employed γ-irradiation to induce apoptosis. A similar prolongation of graft survival occured (data not shown). Furthermore, graft acceptance was also achieved using another pair of inbred rat strains as donor and recipient: Dark Agouti (DA, RTI[a]) as donors and Lewis (RTI[l]) as recipients (Figure 2). Again, graft survival time in Lewis recipients administrated with apoptotic DA donor cells was significantly longer than that of a saline control group (MST = 35 *versus* 6 days, $P < 0.01$). Thus, prolongation of heart allograft survival by injection of apoptotic cells is neither dependent on the method of apoptosis induction nor is it limited to only a particular rat strain.

Prolongation of allograft survival by apoptotic cell transfusion is donor specific

If the immunoregulation induced by apoptotic cells is antigen specific, it should not prolong the survival of an allograft from a third-party strain. To test this prediction, DA (RTI[a]) apoptotic splenocytes were injected into Lewis (RTI[l]) recipients, as above, but the latter then received a heart allograft from Brown Norway (BN, RTI[n]) 1 week later. This scenario resulted in much shorter allograft survival as compared to that of the DA grafts (MST = 11 *versus* MST = 35 days, $P < 0.01$) (Figure 2). Similarly, injection of Lewis apoptotic splenocytes did not prolong survival of DA heart transplanted into Lewis recipients (MST = 6 days) (Figure 2). These results clearly demonstrate that the allograft-protective effect of transfused apoptotic cells is donor specific.

In order to investigate whether apoptotic cells modulate the response of lymphocytes in the recipient, we performed an *ex vivo* lymphocyte proliferation assay. At 1 week after transfusion of 5×10^7 DA apoptotic cells (induced by 1.5 Gy γ-radiation), splenocytes were isolated from Lewis rats and challenged with DA splenocytes or Con A *in vitro*, as described in Materials and Methods. Compared to cells from untreated controls, splenocytes from recipients transfused
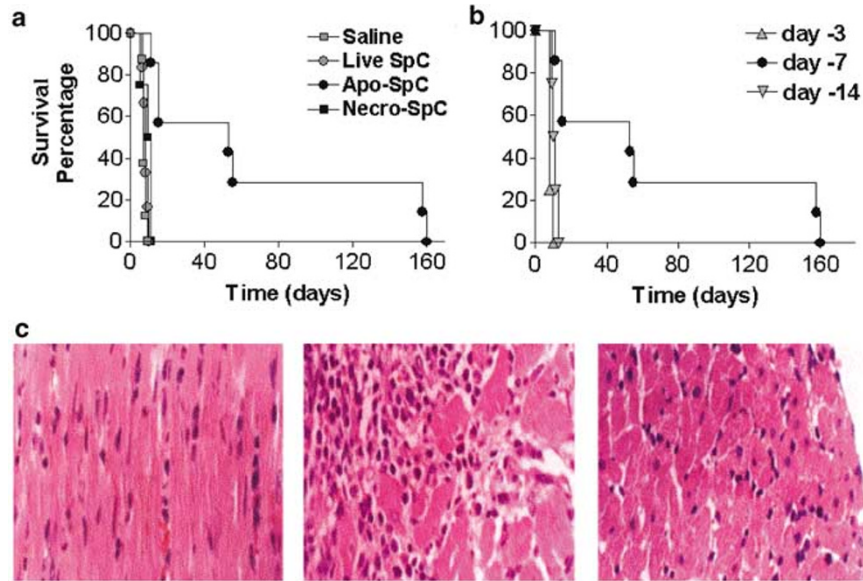
Figure 1 Donor apoptotic splenocytes prolong heart allograft survival. (**a**) Isolated splenocytes (SpC) from Wistar donor rats were resuspended in RPMI-1640 medium. They were exposed to either a 40 W UV source (312 nm, 10 min) at a distance of 40 cm to induce apoptosis (Apo SpC) or a hot water bath (56°C, for 60 min) to induce necrosis (Necro SpC). Control splenocytes were without any treatment (Live SpC). After incubation at 37°C in 5% CO_2 for 8 h, 5×10^7 donor splenocytes were injected i.v. into SD recipients. Saline injection served as a negative control. After 7 days, donor hearts were transplanted into the SD recipients and allograft survival scored as the day when palpable allograft heartbeat ceased. As shown, transfusion of UV-induced apoptotic cells significantly extended heart allograft median survival time (MST = 53 days) compared to all other treatments (MST for saline, Live SpC and Necro SpC were 7, 8 and 7 days, respectively) ($P < 0.01$). (**b**) In a similar experiment, intervals between transfusion of apoptotic cells and heart transplantation were 14, 7 and 3 days, and the effect on allograft survival was determined. Graft survival for the 7-day interval (MST = 53 days) was much higher than that for 14- or 3-day intervals (MST = 10.5 and 8 days, respectively) ($P < 0.01$). (**c**) Histological analysis 1 week after transplantation. Heart isograft (left), allograft (center) or allograft from recipient transfused with donor apoptotic cells (right) were procured. Hematoxylin and eosin staining of paraffin-embedded sections revealed massive lymphocyte infiltration and myocyte destruction in control allografts, while apoptotic donor splenocyte transfusion caused significantly reduced lymphocyte infiltration and preserved cardiomyocyte morphology

with donor apoptotic cells were much less responsive to stimulation by donor cells, although the same splenocytes responded briskly to Con A (Figure 3). These results further demonstrate that apoptotic cell-induced downregulation of lymphocyte responsiveness is donor antigen specific and is not due to a general immunosuppression.

Blocking phagocytosis *in vivo* abolishes the tolerogenic effect of apoptotic cell transfusion

Apoptotic cells are known to express specific molecules on their surface that allow their recognition by phagocytes.[2,7,10] Based on previous results, we propose that phagocytes in the recipient animal are pivotal for engulfing and degrading transfused donor apoptotic cells. Then, immunosuppressive cytokines secreted by the phagocytes may tolerize T cells to donor antigens derived from apoptotic cells. If so, blockade of phagocytosis should abrogate the effect of apoptotic cells in prolonging graft survival. To test this hypothesis, first we used gadolinium, a rare metal that is able to block completely the phagocytic activity of macrophages *in vivo*.[32,33] Gadolinium chloride ($GdCl_3$) was administered for 2 days to the intended transplant recipients before injection of donor apoptotic cells and heart transplantation was performed 1 week later. As shown in Figure 4, treatment with $GdCl_3$ completely abolished apoptotic cell-induced graft acceptance (MST = 6.5 days). The effect of $GdCl_3$ cannot be ascribed to toxicity for the transplants, since grafts survived well in recipients treated with $GdCl_3$ plus cyclosporine (CsA) (Figure 5), a widely used
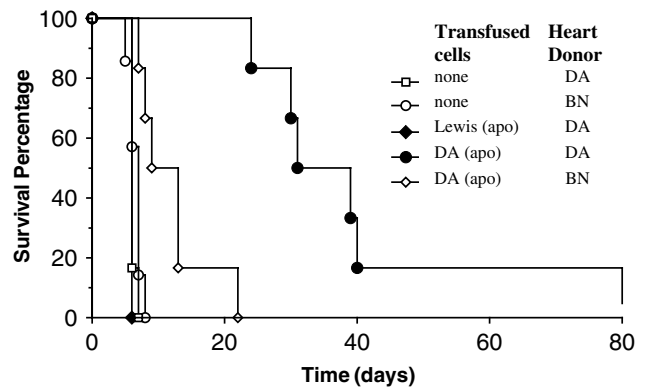

Figure 2 Prolongation of allograft survival by apoptotic cell transfusion is donor specific. DA (RT^a), Lewis (RT^l) and BN (RT^n) rats were used as donor, recipient and third-party control, respectively. Apoptosis was induced in donor splenocytes by γ-radiation (1.5 Gy) followed by incubation in RPMI-1640 medium at 37°C for 16 h, at which time approximately 54% of cells were apoptotic as detected by PI/ annexin V staining. Apoptotic splenocytes from DA (Apo DA SpC) or Lewis (Apo Lewis SpC) rats were transfused into Lewis recipients (as described in Figure 1), which then received DA or BN heart allografts. As shown, transfusion of live DA splenocytes (Live DA SpC) promoted accelerated acute rejection (MST = 4.5 days), as compared with saline controls (MST = 6 days, $P < 0.05$). Transfusion of apoptotic DA splenocytes dramatically prolonged allograft survival (Apo DA SpC + DA heart, MST = 35 days, $P < 0.01$ *versus* saline control group), while only slightly extending the survival of grafts from an irrelevant strain, BN (Apo DA SpC + BN heart) (MST = 11 days, $P < 0.01$ *versus* saline control group or Apo DA SpC group). Apoptotic cells from the same strain as the recipient (Apo Lewis SpC + DA heart) did not prolong the survival of grafts from DA donors (MST = 6 days)
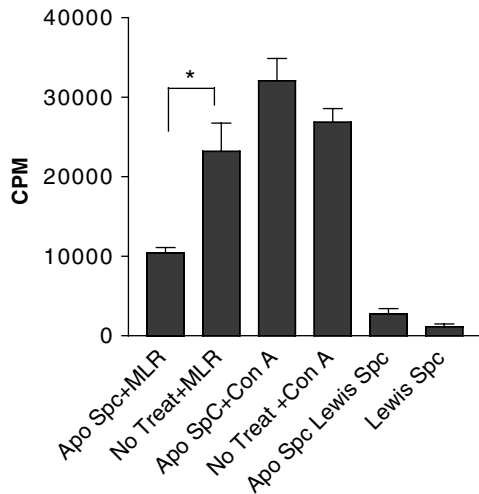
Figure 3 Transfusion of donor apoptotic splenocytes inhibits *ex vivo* MLR. Lewis rats received transfusion of apoptotic splenocytes from DA rats as in Figure 2. After 7 days, splenocytes (3×10^6/ml) from treated Lewis rats were isolated and cultured *in vitro* for 96 h with irradiated (16 Gy) DA splenocytes (3×10^6/ml) to induce MLR or with Con A (1 mg/ml) to induce nonspecific T-cell activation. Cultures were pulsed with [³H]thymidine for 4 h, harvested and [3H]thymidine incorporation assessed by liquid scintillation counting. MLR was inhibited by transfusion of apoptotic cells (*$P < 0.05$), while the Con A-induced response was not

clinical immunosuppressant (MST > 40 days, $P < 0.01$ *versus* GdCl$_3$-only group). These findings demonstrate that normal phagocytic function in the recipient is essential for the immunoregulatory property of transfused donor apoptotic cells *in vivo*, suggesting that phagocytosis of these cells is required to induce donor antigen tolerance. Furthermore, unlike studies carried out *in vitro*, these results demonstrate that any factors produced by apoptotic cells themselves are not important in their immunosuppressive effect.

Since GdCl$_3$ influences several functions of phagocytic cells, experiments with GdCl$_3$ alone cannot rule out the possibility that other functions of these cells besides phago-cytosis are critical for AMIR.[34,35] In order to further elucidate the role of phagocytosis, we employed annexin V to block phagocytosis of apoptotic cells specifically. Annexin V is well known for its high binding affinity for PS, a phospholipid normally situated on the inner leaflet of the cytoplasmic membrane. Early in apoptosis, PS flips to the outer leaflet where it serves as a crucial mediator of phagocytosis. Blocking PS with annexin V prevents binding to its receptor, thereby blocking phagocytosis of apoptotic cells.[4,36] Thus, the advantage of employing annexin V is that there is no influence on other cellular activities of phagocytes. To determine if blocking PS-mediated phagocytosis causes diminished apop-totic cell-induced graft tolerance, apoptotic donor splenocytes were coated with annexin V *in vitro*, transfused and allogeneic heart transplantation was performed 7 days later. As expected, annexin V pretreatment significantly attenuated allograft survival as compared to non-annexin V-treated controls (MST = 10 days, $P < 0.05$ *versus* apoptotic cell transfusion-only group) (Figure 5). These results further demonstrate that phagocytosis of transfused donor apoptotic cells is pivotal in the induction of allograft acceptance. Interestingly, unlike GdCl$_3$, which completely blocked the
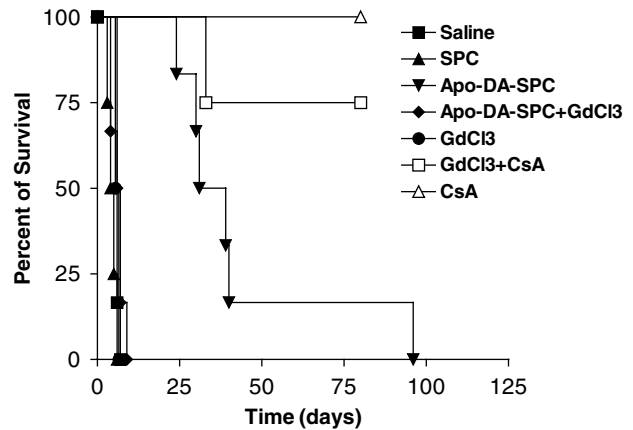

Figure 4 GdCl$_3$ abrogates prolongation of allograft survival induced by donor apoptotic splenocytes. Lewis rats were injected with GdCl$_3$ (10 mg/kg/day i.v.) for 2 days and DA apoptotic splenocytes transfused on the 3rd day. After another 7 days, DA hearts were transplanted and graft survival monitored as in Figure 1. MSTs are as shown, while GdCl$_3$ alone had no effect on allograft survival (MST = 6.5 *versus* 6 days for saline control group, $P > 0.05$), it completely blocked the graft prolonging effect of donor apoptotic splenocytes (Apo DA SpC + GdCl$_3$, MST = 6.5 *versus* 35 days for Apo DA SpC, $P < 0.01$). To rule out the possible toxicity of GdCl$_3$ to allografts, its effect on CsA-induced graft prolongation was tested, where Lewis rats were treated with GdCl$_3$ for two days, 8 days later DA heart was transplanted and CsA administrated (10 mg/kg/day) by gavages starting 1 day before transplant and stopping at 40 days after operation. GdCl$_3$ did not affect CsA-induced allograft survival (Gd + CsA, MST > 40 *versus* 6 days for saline control, or 6.5 days for GdCl$_3$ alone, $P < 0.01$)
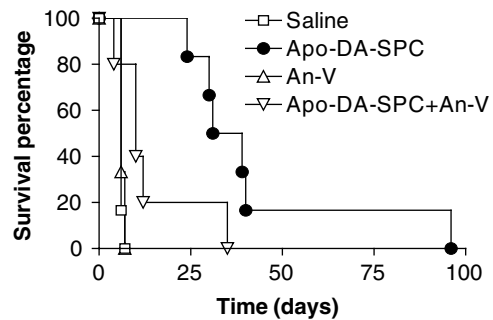

Figure 5 Annexin V abrogates the prolongation of allograft survival induced by donor apoptotic splenocytes. To determine whether specifically blocking PS-mediated phagocytosis prevents apoptotic cell-generated allograft protection, an experiment like that described in Figure 4 was carried out, but apoptotic cells were coated with purified recombinant annexin V (An-V, 100 μg in 600 μl RPMI-1640 medium) before transfusion. Precoating donor apoptotic splenocytes with annexin V significantly reduced their allograft prolonging effect (Apo SpC + An-V, MST = 10 days) compared to controls receiving non An-V-treated cells (Apo SpC, MST = 35 days for, $P < 0.05$). An-V without apoptotic splenocytes had no effect on graft survival (MST = 6 days)

effect of apoptotic cells, the effect of annexin V was incomplete as graft survival was significantly longer than that of the saline-treated group ($P < 0.05$).

Discussion

Recent progress in developing effective immunosuppres-sants has made transplantation the ultimate treatment for organ failure. However, the incidence of opportunistic infec-tions, toxic side effects and high cost associated with long-term application of these drugs remain the main obstacles for general adaptation of organ transplantation in clinical

practice.[37,38] Furthermore, when genetically modified xeno-grafts become available, the challenge of having the immune system properly regulated will be a major hurdle. Therefore, various attempts have been made to find more specific ways to induce transplant tolerance. Thus, administration of donor antigens and concurrent short-term administration of various immunosuppressants either to inhibit specifically T-cell activation or block costimulation signals are being tested to induce tolerance. The finding that apoptotic cells are not only rapidly scavenged by phagocytes but also actively transmit immunoregulatory signals to phagocytes suggests the uniqueness of apoptotic cells in transplantation tolerance induction. Since phagocytes devouring apoptotic cells secrete immunoregulatory cytokines, there is a strong possibility that antigens derived from apoptotic cells are presented to cognate T cells within the context of local immune regulatory factors. In this investigation, we employed the rat whole-heart transplantation model and found that a single injection of donor apoptotic cells into the recipient significantly prolonged the survival of alloheart transplants. In addition, the recipient's phagocytes play a critical role in inducing organ transplant tolerance. Therefore, our studies not only provide *in vivo* evidence that apoptotic cells can induce immune tolerance but also reveal a potentially novel strategy to maintain organ transplant tolerance in clinical practice.

In the present study, we demonstrated that i.v. administration of apoptotic cells of donor origin significantly prolongs the survival of allograft, while apoptotic cells from the recipient or unrelated strains have no effect. This seems to be inconsistent with previous studies showing that apoptotic cells inhibit xenogeneic T-cell activation *in vitro* and promote third-party or xenogeneic bone marrow engraftment.[39] However, this discrepancy may reflect temporal and spatial differences. In the studies performed by Bittencourt *et al.*,[39] graft cells are directly mixed with apoptotic cells. We believe that, in addition to the secretion of immunosuppressive cytokines, other mechanisms such as local antigen presentation are more prominent in such experimental setting. Since our experiments involve a solid organ, active antigen-specific immune tolerance is more important. Indeed, as shown in Figure 1, we have seen that a 7-day priming with apoptotic cells is required for generating immune tolerance. As heart transplantation between DA rats and Lewis rats represents a strong allograft rejection model, our results reflect a potent influence of apoptotic cells on immune regulation. The cells transfused in these experiments contain various types of cells. While it is possible that antigen-presenting cells such as DCs in the splenocyte mixture may undergo changes and acquire immune modulatory function, this is unlikely since we have shown that treatment with GdCl₃ 24 h prior to transfusion of apoptotic cells completely abolishes the tolerogenic effect. As the half-life of GdCl₃ in rats is only 15 min,[40] by 24 h postinjection, any remaining GdCl₃ is minimal, and would not be expected to affect the function of transfused antigen-presenting cells. In addition, we found that donor splenocytes treated with dexamethasone significantly prolong donor heart allograft survival and, most interestingly, indefinitely prolong liver allograft survival (data not shown). Although apoptotic cells have been shown previously to promote bone marrow engraftment in irradiated recipients, our experiments demon-

strate for the first time that transplantation tolerance can be induced in adult animals in the absence of any systemic immunosuppression.

Apoptotic cells are normally rapidly phagocytosed and cleared to prevent secondary necrosis that would usually cause local inflammation. Interestingly, apoptotic cells have also been demonstrated to play a crucial role in maintaining immune tolerance, while abnormal delay in their clearance often leads to autoimmune diseases. Apoptotic cells express specific molecules such as phosphotidylserine on their cell membrane, which play a critical role in the recognition by phagocytes. Phagocytes devouring apoptotic cells secrete large amounts of immunoregulatory cytokines, suggesting the importance of phagocytosis in apoptotic cell-induced tolerance. In our experiment, depletion of phagocytes by GdCl₃ completely blocked the effect of donor apoptotic cells in tolerance induction demonstrating the pivotal role of recipient phagocytes. CsA is a well-known immunosuppressant routinely used in clinical practice. It has been shown that CsA can effectively maintain a DA to Lewis heart transplant.[41] As shown in Figure 4, the attenuation of apoptotic cell-induced graft survival is not due to direct toxicity of GdCl₃ to the heart allograft, because GdCl₃ had no effect on CsA-induced heart engraftment. In fact, GdCl₃ has no toxicity for heart even at a dose as high as 92 mg/kg. Additionally, GdCl₃ is quickly eliminated with a half-life of about 15 min.[40,42] Therefore, we believe that GdCl₃ administered 8 days before heart transplantation should have minimal toxicity for the transplanted heart. The dependence on recipient phagocytes was also verified by coating donor apoptotic splenocytes with PS-specific annexin V. Therefore, the process of phagocytosis of apoptotic cells is a critical step in the induction of immune tolerance.

Although many studies have found that apoptotic cells and their phagocytosis can mediate inhibitory effects on the immune system, some investigations have paradoxically shown that apoptotic cells may also be stimulatory.[43–45] This controversy might reflect the complexity of the status of apoptotic cells while being administered. Cells in late stages of apoptosis may enter secondary necrosis and deliver stimulatory (danger) signals.[9] Similarly, different apoptotic inducers may also impact the outcome. For example, viral inducers may themselves transmit stimulatory danger signals.[43,45] We also found that inclusion of much higher numbers of apoptotic cells in the culture resulted in lymphocyte proliferation even greater than that without apoptotic cells, suggesting that excessive apoptotic cells in the culture are actually stimulatory for T cells (data not shown). Our findings that immune regulation by apoptotic cells depends on cell numbers *in vitro* and phagocytosis *in vivo* support the concept that efficient phagocytosis is critical for apoptotic cell-mediated immune regulation. Excessive apoptotic cells surpassing the phagocytotic capacity could have stimulatory effects.[44]

In conclusion, we believe that transfused apoptotic cells are scavenged by recipient phagocytes, and that donor antigens, including MHC-derived antigenic peptides, are then presented to recipient T cells. Concurrently, phagocytosis of apoptotic cells renders phagocytes to deliver immunoregulatory signals and prevent unwarranted T-cell activation. Thus,

T cells reactive to donor alloantigens are silenced and allograft survival prolonged. Although the role of apoptotic cells in the maintenance of tolerance has long been suspected and some immunomodulatory effects have been observed *in vitro*,[18,23,46] it is striking that AMIR in our models is so effective that only a single injection of apoptotic cells is sufficient to prolong allograft survival in non-immunosuppressed recipients. Our findings also explain the clinical observation that blood transfusion can prolong graft survival.[47,48] In fact, we have found that, like splenocytes, whole blood treated with γ-irradiation also prolongs allograft survival (data not shown). The powerful immunoregulatory effect of apoptotic cells on immune responses *in vivo* supports the notion that apoptotic cells play a paramount physiologic role in preserving self-tolerance. More studies are needed to further evaluate the mechanisms and significance of this new strategy of tolerance induction, to explore more reliable models and protocols to direct apoptotic cell initiated immune regulation, and to pave the way to clinical applications.

Materials and Methods

Animals and reagents

Male SD (RT1[b]) and Wistar (RT1[k]) rats (8–10 weeks old) were purchased from the Experimental Animal Center, Zhongshan Medical University, and maintained in the animal facilities of Zhujiang Hospital. DA (RTI[a]) and Lewis (RTI[l]) rats were purchased from Harlan Corp. (Indianapolis, IN, USA) and maintained in the Vivarium of Robert Wood Johnson Medical School. In transplantation studies, Wistar or DA rats served as donors, while SD or Lewis rats were recipients. AnnexinV–PI apoptosis detection kits and purified recombinant annexin V were purchased from BD Biosciences (San Diego, CA, USA). GdCl$_3$ was from Aldrich Chemical Company, Inc. (Milwaukee, WI, USA). CsA Neoral solution was from Norvartis Corp.

Apoptosis induction and detection

Single-cell suspensions of rat splenocytes were prepared by mincing the spleen and filtering the cells through a nylon mesh. To induce apoptosis, 3×10^6 splenocytes were resuspended in 6 ml phenol red-free Hank's solution, transferred to a six-well plate and exposed to a 40 W UV source (320 nm) at a distance of 40 cm for 10 min. Alternatively, cells were treated with 1.50 Gy γ-irradiation from a Cobalt-60 source. After irradiation, cells were resuspended in RPMI-1640 medium and cultured at 37°C in 5% CO$_2$ for 8 or 16 h, for UV- or γ-irradiated cells, respectively. Necrotic cells were generated by incubation in a hot water bath (56°C) for 60 min. Cell death resulting from these treatments was analyzed by flow cytometry after staining with annexin V and PI; annexin V$^-$/PI$^-$, annexin V$^+$/PI$^-$ and annexin V$^+$/PI$^+$ cells were identified as live, apoptotic and necrotic cells, respectively. To block surface PS, 3×10^8 DA apoptotic splenocytes were incubated with 100 μg purified recombinant annexin V in 600 μl RPMI-1640 medium at room temperature for 20 min.

Animal treatment and heart transplantation

Apoptotic, necrotic or untreated rat splenocytes (50×10^6) were injected via the penile vein on days -14, -7 and -3 (the day of heart transplant was designated as day 0). In some instances, GdCl$_3$ (10 mg/kg) was injected via the penile vein on days -9 and -8, and apoptotic splenocytes were administered on day -7. CsA was administrated by gavage from day -1 to day $+40$. Heterotopic heart transplantation was performed using a modified technique of Ono and Lindsey,[49] with donor aorta and pulmonary artery anastomosed end-to-side to the recipient's abdominal aorta and inferior vena cava, respectively. Graft heartbeat was checked daily by palpation and rejection scored when the graft stopped beating. All animal studies were approved by the Institutional Animal Care and Use Committee of Robert Wood Johnson Medical School.

Lymphocyte proliferation

For the mixed lymphocyte reaction (MLR), equal numbers (3×10^5) of responder splenocytes and irradiated stimulator splenocytes (16 Gy) were cocultured in 200 μl in a 96-well plate. For the Con A response, splenocytes were cultured with 1 μg/ml Con A. In some experiments, apoptotic DA splenocytes were also added to the culture. After incubation at 37°C in 5% CO$_2$ for 96 h, cells were pulsed with 1 μCi [³H]thymidine per well for 4 h, harvested and [³H]thymidine incorporation was measured by liquid scintillation counting in a Microbeta (Perkin-Elmer).

Histology

Heart allografts were removed 1 week after transplantation, fixed in 10% phosphate-buffered formalin and embedded in paraffin. Thin sections (4 μM) were stained with hematoxylin and eosin, and representative areas photographed by light microscopy.

Statistics

All data were analyzed with Prism Software (Graphpad Software Inc., San Diego, CA, USA). Comparisons of lymphocyte proliferation were made by unpaired Student's *t*-test. Graft survival was analyzed using the log-rank test and survival curves plotted by the Kaplan–Meier method.

Acknowledgements

References

1. Savill J, Fadok V, Henson P and Haslett C (1993) Phagocyte recognition of cells undergoing apoptosis. Immunol. Today 14: 131–136
2. Savill J (1998) Apoptosis. Phagocytic docking without shocking. Nature 392: 442–443
3. Henson PM, Bratton DL and Fadok VA (2001) Apoptotic cell removal. Curr.Biol. 11: R795–R805
4. Wang X, Wu Y-C, Fadok VA, Lee M-C, Gengyo-Ando K, Cheng L-C, Ledwich D, Hsu P-K, Chen J-Y, Chou B-K, Henson P, Mitani S and Xue D (2003) Cell corpse engulfment mediated by *C. elegans* phosphatidylserine receptor through CED-5 and CED-12. Science 302: 1563–1566
5. Wu YC and Horvitz HR (1998) The *C. elegans* cell corpse engulfment gene ced-7 encodes a protein similar to ABC transporters. Cell 93: 951–960
6. Wu YC and Horvitz HR (1998) *C. elegans* phagocytosis and cell-migration protein CED-5 is similar to human DOCK180. Nature 392: 501–504

7. Devitt A, Moffatt OD, Raykundalia C, Capra JD, Simmons DL and Gregory CD (1998) Human CD14 mediated recognition and phagocytosis of apoptotic cells. Nature 392: 505–509

8. Fadok VA, Bratton DL, Rose DM, Pearson A, Ezekewitz RA and Henson PM (2000) A receptor for phosphatidylserine-specific clearance of apoptotic cells. Nature 405: 85–90

9. Sauter B, Albert ML, Francisco L, Larsson M, Somersan S and Bhardwaj N (2000) Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. J. Exp. Med. 191: 423–434

10. Savill J and Fadok V (2000) Corpse clearance defines the meaning of cell death. Nature 407: 784–788

11. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY and Henson PM (1998) Macrophages that have ingested apoptotic cells *in vitro* inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2, and PAF. J. Clin. Invest. 101: 890–898

12. Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR and Girkontaite I (1997) Immunosuppressive effects of apoptotic cells. Nature 390: 350–351

13. Opelz G and Terasaki PI (1978) Improvement of kidney-graft survival with increased numbers of blood transfusions. N. Engl. J. Med. 299: 799–803

14. Opelz G and Terasaki PI (1974) Poor kidney-transplant survival in recipients with frozen-blood transfusions or no transfusions. Lancet 2: 696–698

15. Smit Sibinga CT (1999) Immune effects of blood transfusion. Curr. Opin. Hematol. 6: 442–445

16. van Prooijen HC, Visser JJ, van Oostendorp WR, de Gast GC and Verdonck LF (1994) Prevention of primary transfusion-associated cytomegalovirus infection in bone marrow transplant recipients by the removal of white cells from blood components with high-affinity filters. Br. J. Haematol. 87: 144–147

17. Frabetti F, Musiani D, Marini M, Fanelli C, Coppola S, Ghibelli L, Tazzari PL, Bontadini A, Tassi C and Conte R (1998) White cell apoptosis in packed red cells. Transfusion 38: 1082–1089

18. Fadok VA, Bratton DL, Freed PW, Westcott JY and PM H (1998) Macrophages that have ingested apoptotic cells *in vitro* inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF-beta, PGE2 and PAF. J. Clin. Invest. 101: 890–898

19. Freire-de-Lima CG, Nascimento DO, Soares MB, Bozza PT, Castro-Faria-Neto HC, de Mello FG, DosReis GA and Lopes MF (2000) Uptake of apoptotic cells drives the growth of a pathogenic trypanosome in macrophages. Nature 403: 199–203

20. Griffith TS, Yu X, Herndon JM, Green DR and TA F (1996) CD95-induced apoptosis of lymphocytes in an immune privileged site induces immunological tolerance. Immunity 5: 7–16

21. Matzinger P (1994) Tolerance, danger, and the extended family. Ann. Rev. Immunol. 12: 991–1045

22. Barr ML, Meiser BM, Eisen HJ, Roberts RF, Livi U, Dall'Amico R, Dorent R, Rogers JG, Radovancevic B, Taylor DO, Jeevanandam V and Marboe CC (1998) Photopheresis for the prevention of rejection in cardiac transplantation. Photopheresis Transplantation Study Group. N. Engl. J. Med. 339: 1744–1751

23. Sun EW and Shi YF (2001) Apoptosis: the quiet death silences the immune system. Pharmacol. Ther. 92: 135–145

24. Andrade F, Casciola-Rosen L and Rosen A (2000) Apoptosis in systemic lupus erythematosus. Clinical implications. Rheum. Dis. Clin. North Am. 26: 215–227 v

25. Lorenz HM, Herrmann M, Winkler T, Gaipl U and Kalden JR (2000) Role of apoptosis in autoimmunity. Apoptosis 5: 443–449

26. Korb LC and Ahearn JM (1997) C1q binds directly and specifically to surface blebs of apoptotic human keratinocytes: complement deficiency and systemic lupus erythematosus revisited. J. Immunol. 158: 4525–4528

27. Botto M, Dell'Agnola C, Bygrave AE, Thompson EM, Cook HT, Petry F, Loos M, Pandolfi PP and Walport MJ (1998) Homozygous C1q deficiency causes glomerulonephritis associated with multiple apoptotic bodies [see comments]. Nat. Genet. 19: 56–59

28. Luan JJ, Monteiro RC, Sautes C, Fluteau G, Eloy L, Fridman WH, Bach JF and Garchon HJ (1996) Defective Fc gamma RII gene expression in macrophages of NOD mice: genetic linkage with up-regulation of IgG1 and IgG2b in serum. J. Immunol. 157: 4707–4716

29. Trudeau JD, Dutz JP, Arany E, Hill DJ, Fieldus WE and Finegood DT (2000) Neonatal beta-cell apoptosis: a trigger for autoimmune diabetes? Diabetes 49: 1–7

30. Sun E, Zhang L, Zeng Y, Ge Q, Zhao M and Gao W (2000) Apoptotic cells actively inhibit the expression of CD69 on con A activated T lymphocytes. Scand. J. Immunol. 51: 231–236

31. Gallucci S, Lolkema M and Matzinger P (1999) Natural adjuvants: endogenous activators of dendritic cells. Nat. Med. 5: 1249–1255

32. Sato K, Yabuki K, Haba T and Maekawa T (1996) Role of Kupffer cells in the induction of tolerance after liver transplantation. J. Surg. Res. 63: 433–438

33. Callery MP, Kamei T and Flye MW (1989) Kupffer cell blockade inhibits induction of tolerance by the portal venous route. Transplantation 47: 1092–1094

34. Roland CR, Naziruddin B, Mohanakumar T and Flye MW (1996) Gadolinium chloride inhibits Kupffer cell nitric oxide synthase (iNOS) induction. J. Leukocyte Biol. 60: 487–492

35. Roland CR, Naziruddin B, Mohanakumar T and Flye MW (1999) Gadolinium blocks rat Kupffer cell calcium channels: relevance to calcium-dependent prostaglandin E2 synthesis and septic mortality. Hepatology 29: 756–765

36. Li MO, Sarkisian MR, Mehal WZ, Rakic P and Flavell RA (2003) Phosphatidylserine receptor is required for clearance of apoptotic cells. Science 302: 1560–1563

37. Rabkin JM, Oroloff SL, Corless CL, Benner KG, Flora KD, Rosen HR and Olyaei AJ (2000) Association of fungal infection and increased mortality in liver transplant recipients. Am. J. Surg. 179: 426–430

38. Serkova N and Christians U (2003) Transplantation: toxicokinetics and mechanisms of toxicity of cyclosporine and macrolides. Curr. Opin. Invest. Drugs 4: 1287–1296

39. Bittencourt MdC, Perruche S, Contassot E, Fresnay S, Baron M-H, Angonin R, Aubin F, Herve P, Tiberghien P and Saas P (2001) Intravenous injection of apoptotic leukocytes enhances bone marrow engraftment across major histocompatibility barriers. Blood 98: 224–230

40. Dean PB, Niemi P, Kivisaari L and Kormano M (1988) Comparative pharmacokinetics of gadolinium DTPA and gadolinium chloride. Invest. Radiol. 23 (Suppl 1): S258–S260

41. Schuurman HJ, Pally C, Fringeli-Tanner M and Papageorgiou C (2001) Comparative efficacy of mycophenolate sodium (MPS) and mycophenolate mofetil (MMF) with and without cyclosporine in rat transplantation models. Transplantation 72: 1776–1783

42. Spencer AJ, Wilson SA, Batchelor J, Reid A, Rees J and Harpur E (1997) Gadolinium chloride toxicity in the rat. Toxicol. Pathol. 25: 245–255

43. Albert ML, Sauter B and Bhardwaj N (1998) Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. Nature 392: 86–89

44. Rovere P, Sabbadini MG, Vallinoto C, Fascio U, Zimmermann VS, Bondanza A, Ricciardi-Castagnoli P and Manfredi AA (1999) Delayed clearance of apoptotic lymphoma cells allows cross-presentation of intracellular antigens by mature dendritic cells. J. Leukocyte Biol. 66: 345–349

45. Yrlid U and Wick MJ (2000) *Salmonella*-induced apoptosis of infected macrophages results in presentation of a bacteria-encoded antigen after uptake by bystander dendritic cells. J. Exp. Med. 191: 613–624

46. Voll RE, Herrmann M, Roth EA, Stach C and Kalden J (1997) Immunosuppressive effects of apoptotic cells. Nature 390: 350–351

47. Frabetti F, Tazzari PL, Musiani D, Bontadini A, Matteini C, Roseti L, Tassi C, Viggiani M, Marini M and Conte R (2000) White cell apoptosis in platelet concentrates. Transfusion 40: 160–168

48. Snyder EL and Kuter DJ (2000) Apoptosis in transfusion medicine: of death and dying—is that all there is? Transfusion 40: 135–138

49. Ono K and Lindsey ES (1969) Improved technique of heart transplantation in rats. J. Thorac. Cardiovasc. Surg. 57: 225–229