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# MEDIATORS INVOLVED IN THE IMMUNOMODULATORY EFFECTS OF APOPTOTIC CELLS<sup>1</sup>

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## FOOTNOTES

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## ABBREVIATIONS

BM	Bone marrow
DC	Dendritic cell
Foxp3	Forkhead-box transcription factor p3
GVHD	Graft-versus-host disease
HC	Hematopoietic cell
i.v	Intravenous
TGF- $\beta$	Transforming growth factor- $\beta$
Treg	Regulatory T cells;

## **ABSTRACT**

Immunomodulatory properties are attributed to apoptotic cells. These properties have been used to modulate allogeneic immune responses in experimental transplantation settings. In independent studies, apoptotic cell infusion has been shown to favor hematopoietic cell engraftment, to increase heart graft survival and to delay the lethal onset of graft-versus-host disease (GVHD). The goal of this review was to discuss how apoptotic cell infusion interferes with graft rejection or host rejection (i.e., GVHD) and to focus on the potential mediators or "perpetuators" involved in apoptotic cell-induced immunomodulation. Particular emphasis on apoptotic cell phagocytosis, TGF- $\beta$  secretion and regulatory T cell induction was performed. Stimulating "naturally" immunosuppressive molecules (i.e., TGF- $\beta$ ) or immunomodulatory cells ("alternatively-activated" macrophages, certain DC subsets or regulatory T cells) in a physiological manner by using apoptotic cell infusion can be a promising way to induce tolerance.

Although the use of non specific immunosuppressive drugs has significantly reduced the incidence of acute graft rejection, the benefits of such therapies on chronic rejection and overall long-term graft survival are uncertain. Persistent excessive immunosuppression after therapeutic approaches of this type is associated with considerable long-term toxicity including increased incidence of cancers, infectious complications as well as metabolic diseases (e.g., diabetes, atherosclerosis, etc). Immunomodulatory approaches allowing for increased specificity are therefore needed. Cell-based therapy is one way of achieving tolerance to donor antigens. The use of donor apoptotic cell infusion has been proposed as an "alternative" cell-based therapy approach to induce tolerance. The mediators or "perpetuators" involved in the immunomodulatory properties of apoptotic cells are discussed in this review.

### **Immunomodulatory properties of apoptotic cells**

Immunomodulatory properties are attributed to apoptotic cells. This is due to apoptotic cells themselves (1, 2) or to cells phagocytosing apoptotic cells (3, 4). Professional phagocytes, such as macrophages (3) or certain dendritic cells (DC) subsets (4), quickly capture apoptotic bodies, thereby preventing both the release of harmful products from dying cells turning to necrosis and the associated inflammatory responses. Several mechanisms have been described to explain the immunomodulatory effects of apoptotic cells (5). Engulfment of apoptotic cells inhibits proinflammatory cytokine production by blocking NF- $\kappa$ B activation in phagocytosing cells (6). Apoptotic cell uptake by phagocytes blocks also their production of the type 1 cytokine IL-12 (7) as well as the Th17-related cytokines, IL-17 and IL-23 (8). Moreover, apoptotic neutrophils and T cells may prevent inflammatory cell recruitment by sequestering chemokines (2). In addition, apoptotic cell death is frequently associated with an immunomodulatory milieu that may neutralize immune cells present in the environment of dying cells. Such milieu consists mainly of anti-inflammatory factors like TGF- $\beta$ , IL-10, IL-1 receptor antagonist, prostaglandin-E2 and hepatocyte growth factor (3, 9-11). These factors can be released by phagocytes (3) and/or by apoptotic cells themselves (1). However, the

immunomodulatory effects induced by apoptotic cells can be modulated or influenced by several factors (5, 12) [Figure 1] such as cell death-inducing agents (13), soluble factors that bridge apoptotic cells to phagocytes (5, 14), or ligand receptor interactions involved in apoptotic cell uptake (5, 15). Rapidly after the initiation of the apoptotic process, changes including alterations in glycosylation, charge and surface lipids occur at the cell surface of dying cells. The best-characterized modification is the loss of phospholipid asymmetry and the exposure of phosphatidylserine on the outer surface. Recently, exposure of calreticulin after tumor cell death has been shown to trigger an efficient immune response against dying tumors (16). The route of administration and the site where apoptotic cells die may trigger inflammation and vigorous immune responses. This can lead to immune responses directed against apoptotic cell-derived antigens (Fig. 1), as mentioned above (13, 16). Thus, in order to use apoptotic cells as a cell therapy product, all of the latter factors, including the route of administration, have to be defined. Intended to induce immunomodulation, the tolerogenic intravenous (i.v.) route will be requisite.

### **Use of apoptotic cell infusion in transplantation settings**

Physiological mechanisms that maintain immune homeostasis and self-tolerance have been exploited for a long time to induce tolerance to allografts. This is why the inhibitory effects of apoptotic cell infusion have been used to modulate alloreactive responses in experimental transplantation models (17-21) [Table 1]. In hematopoietic cell (HC) transplantation, i.v. apoptotic cell infusion favors allogeneic HC engraftment (17), delays the onset of lethal acute GVHD (18) and prevents the anti-donor humoral response (19). In solid organ transplantation models, donor apoptotic cell infusion increases heart graft survival (20, 21) whatever the timing of infusion: 7 days before heart transplantation (19) (as previously done with donor specific blood transfusion) or 7 days after (20). The timing of apoptotic cell infusion does not seem to be a critical factor in the interference of alloreactive immune responses. The next questions to address are how i.v. apoptotic cell infusion may interfere with alloreactive

responses and by which mediators (and mechanisms) the immunomodulatory effects are propagated?

### **Mediators involved in the immunomodulatory effects of apoptotic cells**

All three studies performed in transplantation settings (18, 20, 21) shared the requirement for apoptotic cell phagocytosis in a limited area (the spleen), but the nature of phagocytes differed. Two studies (18, 20) identified recipient macrophages as critical for the apoptotic cell infusion-induced immunomodulatory effects. This was attested by macrophage depletion before apoptotic cell infusion using gadolinium chloride (20) or clodronate-loaded liposomes (18). Conversely, host CD8 $\alpha^+$  DC allowed prolonged graft survival when apoptotic cells were infused after heart transplantation, as shown by adoptive transfer experiments (21).

However, a direct role of DC was excluded in Ref. 18 where despite efficient depletion of CD11c<sup>high</sup> DC in a transgenic mouse model, apoptotic cell infusion still favored HC engraftment. The read out used (depletion (18, 20) versus adoptive transfer, Ref.21) may influence the result. Nevertheless, it makes sense to identify different phagocytes depending on the transplantation model used: heart (21) versus HC (18). Macrophages and DC diverge in their antigen presentation capacity due to their lysosomal protease content (22).

Macrophages rapidly degrade internalized antigens without presenting them, while DC exhibit limited lysosomal degradation capacity (22) favoring donor alloantigen presentation. A restricted donor-specific effect related to donor apoptotic cell phagocytosis by DC and subsequent donor alloantigen presentation may account for prolonged heart graft survival (21). Donor apoptotic cell-loaded DC may migrate to the thymus to perform alloantigen-specific T cell deletion. In HC transplantation, apoptotic cell phagocytosis by macrophages (18) may lead to a non-specific anti-inflammatory effect associated with TGF- $\beta$  production (19, see below), independent of donor alloantigen presentation. Indeed, HC engraftment is favored whatever the origin (donor/recipient/other) of apoptotic cells (17, 19). The subsequent anti-inflammatory effect related to TGF- $\beta$  secretion may neutralize host immune cells to allow HC engraftment and reduce the cytokine storm responsible for GVHD initiation.



To reconcile data obtained from different transplantation models (17, 19), an alternative explanation would be that both macrophages and DC are necessary for apoptotic cell-induced engraftment. In this scenario, anti-inflammatory cytokines produced by macrophages after phagocytosis (i.e., TGF- $\beta$ ) may induce DC to present alloantigens from apoptotic cells in an inefficient manner. This may explain why donor apoptotic cell-loaded DC infusion after heart transplantation induces an initial proliferation of alloreactive T cells followed by their deletion (21). This hypothesis is currently under investigation.

Another mediator involved in the immunomodulatory effects of apoptotic cell infusion in transplantation settings is TGF- $\beta$ . Increased *TGF- $\beta$*  transcripts were detected on day 3 post-transplantation in the spleen of mice that had received apoptotic spleen cells simultaneously with bone marrow (BM) grafts (Figure 2). Neutralization of this cytokine abrogates the effects of i.v. apoptotic cell infusion on BM engraftment (19) and the prevention of anti-donor alloimmunization (19). Indeed, i.v. apoptotic cell infusion given simultaneously with hematopoietic allografts prevents anti-BM donor immunization even in mice rejecting their graft (19). This alteration of allogeneic humoral responses was also observed when donor apoptotic cells were infused after heart transplantation (21). TGF- $\beta$  is implicated in the conversion of naive CD4<sup>+</sup> CD25<sup>-</sup> T cells into regulatory T cells (Treg) (23); analysis of Treg after apoptotic cell infusion was thus performed. Foxp3<sup>+</sup> Treg were identified as propagating the effect of i.v. apoptotic cell infusion both in heart (21) and BM (18) transplantation models. In the BM transplantation model, the involvement of TGF- $\beta$  secretion and splenic macrophages in the induction of Treg was demonstrated (18). Indeed, TGF- $\beta$  neutralization or clodronate-loaded liposome infusion blocked Treg expansion induced by i.v. apoptotic cell infusion (18). In this model, a direct link between macrophage and TGF- $\beta$  secretion has yet to be demonstrated. Interleukin-10 dependent-Treg induction after infusion of syngeneic splenocytes rendered apoptotic by photopheresis has also been reported (24). Interestingly, Treg induction after random blood product transfusion has been reported previously (25) and nonspecific immunomodulatory effects of preoperative blood product transfusion has been proposed to be related to the presence of apoptotic leukocytes in stored blood products (26).

Treg induction after blood cell apoptosis –a frequent phenomenon, mimicked by i.v. infusion of apoptotic cells– may constitute an additional mechanism to prevent autoimmunity that could be used in transplantation.

## **Conclusion**

In conclusion, we propose infusing apoptotic cells to interfere with graft rejection or host rejection (GVHD). This approach permits to stimulate "naturally" immunosuppressive molecule production (i.e., TGF- $\beta$ ) or to activate immunomodulatory cells (such as Treg and particular DC or macrophage subsets) in a physiological manner. Exploiting such mechanisms may facilitate the induction of immunological tolerance to transplants. However, before the possible application of such therapy to clinical transplantation, several obstacles need to be overcome, including the interactions of such an approach with immunosuppressive regimens. In the future, i.v. donor apoptotic cells may become a well-defined cell therapy product and a substitute for the blood products formerly used in transfusion practice to prolong graft survival. Indeed, similitude exists between donor-specific blood transfusion and i.v. apoptotic leukocyte infusion including induction of Treg (25) or of TGF- $\beta$  production (27). Some biological effects of transfusion may be related to the presence of apoptotic leukocytes in blood products (26). This idea of using the immunomodulatory properties of apoptotic cells in cell-based therapy is moving forward, since such an approach has also been proposed to increase cardiac muscle repair (28).

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**TABLE 1. Immunomodulatory effects of intravenous apoptotic cell infusion in transplantation settings: an overview.**

Experimental transplantation models:	Hematopoietic cell engraftment	Rat heart transplantation	Graft-versus-Host Disease	Mouse heart transplantation
Dependent on the origin of infused apoptotic cells:	no (17,19)	yes (only donor apoptotic cells prolong survival, [20])	yes <sup>a</sup>	yes (only donor apoptotic cells prolong survival, [21])
Timing of apoptotic cell infusion:	with the bone marrow graft (17-19)	7 days before heart transplant (20)	with the bone marrow graft (18)	7 days after transplant (21)
Required phagocytes:	splenic host macrophages (18)	host macrophages (20)	ND	splenic host CD8 $\alpha$ + DC (21)
Required cytokines:	TGF- $\beta$ (18, 19)	ND	ND	intra-graft TGF- $\beta$ and IL-10 (21)
Treg induction:	yes, but not critical in the graft facilitating effects (18)	ND	yes, Treg participate to delay the onset of lethal GVHD (18)	suspected, Foxp3 <sup>+</sup> Treg present in the graft (21)
Inhibition of anti-donor immunization:	yes (19) in a TGF- $\beta$ -dependent manner	ND	ND	yes (suppress systemic anti-donor response; IFN- $\gamma$ inhibition, [21])

<sup>a</sup>Kleinclauss F, de Carvalho Bittencourt M, Saas P, unpublished results

ND, not determined.

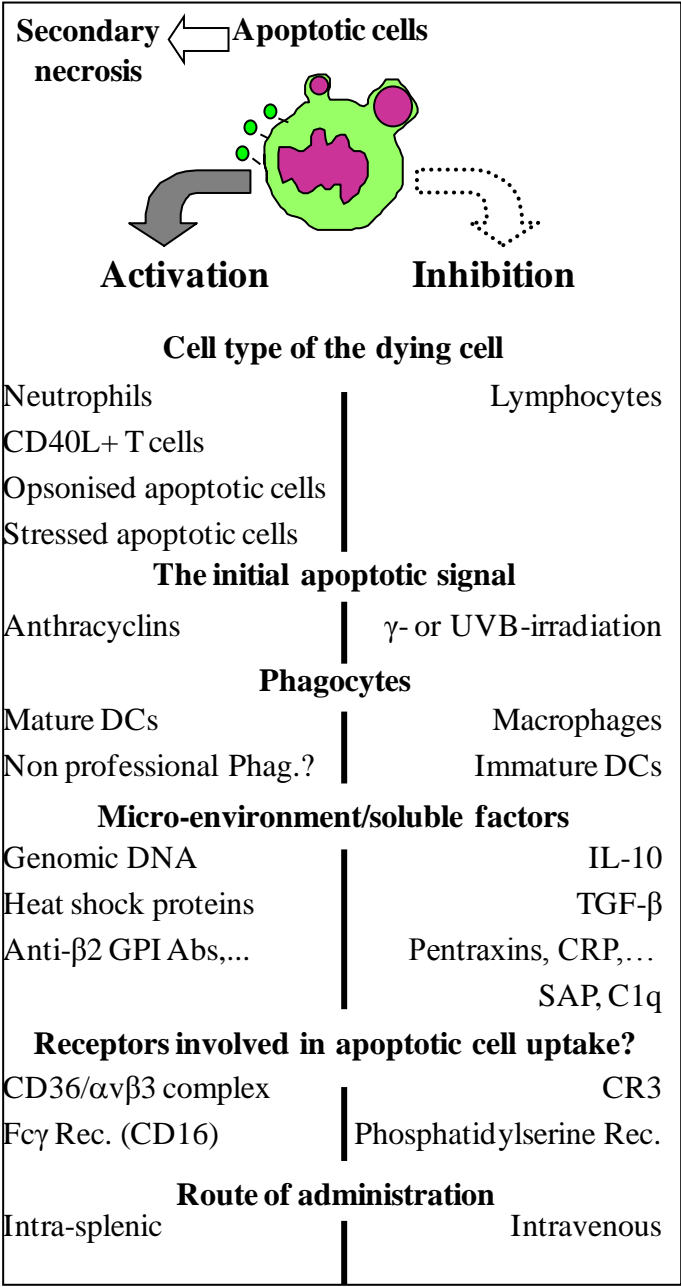
## FIGURE LEGENDS

**FIGURE 1. Factors influencing the immune response to apoptotic cells.** Several factors may influence the immune response to apoptotic cells. Factors involved in the activation of the immune response are listed on the left hand side, while those implicated in its down-regulation are mentioned on the right hand side. This figure is modified from Ref. 12 and updated with recent references (see text). Abbreviations: DC: dendritic cell; Phag: phagocytes; Rec: receptor; CR3: Complement receptor 3; CRP: C reactive protein; SAP: serum amyloid P component; C1q: one of the first fractions of the complement.

**FIGURE 2. Early increase of TGF- $\beta$  mRNA transcript after intravenous donor apoptotic cell infusion simultaneously with bone marrow graft.** Total RNA was extracted from spleen of BALB/c mice grafted with  $10^6$  FVB BM (black triangles) or with  $5 \times 10^6$  FVB apoptotic splenocytes (Apo C) plus  $10^6$  FVB BM (open square) using RNA extraction kit (Qiagen, Valencia, CA) and reverse transcribed using random hexamers and M-MLV reverse transcriptase (Life Technologies, Rockville, MD) to use as template for quantitative real time RT-PCR (QRT-PCR). QRT-PCR reactions were performed as described (18). Primers and dual labeled fluorescent probes were designed using Primer Express® software (Applied Biosystems, Forster City, CA) to be specific for RNA but not for genomic DNA. QRT-PCR primer pairs and related probes were as follow (sense, antisense and probe respectively). TGF- $\beta$  : 5'-GCTCTTGTGACAGCAAAGATAACAA-3', 5'-GGTCGCCCCGACGTTT-3' and 5'-FAM-CACGTGGAAATCAACGGGATCAGCC-TAMRA-3'; Primer pairs for GAPDH (used as endogenous reference) were already described (18). The relative quantity of each unknown sample was determined automatically with the iCycler iQ® software (Bio-Rad Laboratories, Marnes-la-Coquette, France), using the threshold cycle (Ct). Data were expressed as normalized TGF- $\beta$ 1 expression, which was obtained by dividing the relative quantity of TGF- $\beta$ 1 mRNA for each sample by the relative quantity of GAPDH mRNA of the same sample. \* $P < 0.05$ .



Figure 1



**Figure 2.**

