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## **Prospects of apoptotic cell-based therapies for transplantation and inflammatory diseases**

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**ABSTRACT/SUMMARY:**

Apoptotic cell removal or interactions of early stage apoptotic cells with immune cells are associated with an immunomodulatory microenvironment that can be harnessed to exert therapeutic effects. While the involved immune mechanisms are still being deciphered, apoptotic cell infusion has been tested in different experimental models where inflammation is deregulated. This includes: chronic and acute inflammatory disorders such as arthritis, contact hypersensitivity, or acute myocardial infarction. Apoptotic cell infusion has also been used in transplantation settings to prevent or treat acute and chronic rejection, as well as to limit acute graft-versus-host disease associated with allogeneic hematopoietic cell transplantation. Here, we review the mechanisms involved in apoptotic cell-induced immunomodulation and data obtained in preclinical models of transplantation and inflammatory diseases.

**KEYWORDS:** apoptotic cells; inflammation; transplantation; macrophages; dendritic cells; regulatory T cells; immunotherapy; cell therapy; arthritis; tolerance

Drugs and biotherapies are not devoid of side effects and improvements in patient treatment are needed. Indeed, biological agents, such as anti-TNF, have significantly improved the treatment of inflammatory chronic diseases, such as rheumatoid arthritis (RA). However, 30 to 40% of RA patients are considered to be refractory or contraindicated to these agents. In solid organ transplantation settings, acute graft rejection is now significantly controlled by immunosuppressive drugs. In contrast, the benefits of such immunosuppressive drugs on chronic rejection and overall long-term graft survival are still uncertain. Allogeneic hematopoietic cell transplantation is an efficient immunotherapy to cure leukemia. However, this approach is associated with major life-threatening complications, notably graft-versus-host disease (GvHD) which occurs in 30 to 80% of allografted patients. Cell-based therapies are new alternative therapeutic strategies that can be used to limit, or even, control all these pathologies with limited toxic effects. More than ten years ago, we propose to use intravenous (iv) early apoptotic cell infusion as an alternative cell-based therapeutic approach in allogeneic hematopoietic cell transplantation, initially conceptually [1], and then experimentally [2]. In this review, we will introduce immunomodulatory mechanisms associated with early apoptotic cells. Then, we will report and discuss data obtained in preclinical experimental models, before proposing future perspectives for such therapeutic approach.

### **Immunomodulatory properties associated with early apoptotic cells**

- An immunosuppressive/anti-inflammatory microenvironment created by the apoptotic cell death

Apoptosis is a cell death mechanism occurring in different physiological situations, including development, or during normal cell turn over with elimination of unwanted or undesirable cells. For instance, more than half of the cells are eliminated by apoptosis in the mammalian brain during development[3]. More than one hundred billion of neutrophils die daily by apoptosis[3]. Elimination of apoptotic neutrophils by macrophages following tissue injury—a process called efferocytosis[4]—is critical for the resolution of inflammation, as well as initiation of tissue repair[5]. In all these situations, apoptotic cells are quickly removed after apoptosis has been triggered [6]. This

elimination takes place before the apoptotic cells become completely dislocated [6], and is estimated to occur less than 3 hours after the apoptotic signal initiation [7]. This suggests initially that apoptotic cell death is a silent immune mechanism [8, 9]. Apoptotic cells are preferentially uptaken by professional phagocytes, such as macrophages [10-16], microglia in the brain [17-20], and/or some dendritic cells (DC) subsets [14, 15, 21-23] that move to the sites where cells die in response to specific signals, called “find-me” signals [24] (Figure 1). In general, these “find-me” signals correspond to the translocation of intracellular structures to the apoptotic-cell surface that engage with phagocytes via their multiple cognate receptors (Figure 1). Then, professional phagocytes can release after apoptotic cell removal immunosuppressive cytokines that neutralize subsequent immune responses (Figure 2). Thus, both macrophages [25, 26], microglia [27] and human monocyte-derived DC [28]—a model for inflammatory conventional DC [29]—have been reported to produce interleukin (IL)-10 after apoptotic cell uptake. Interactions of apoptotic cells with monocytes induce IL-10 secretion after lipopolysaccharide (LPS) stimulation [30]. Secretion of TGF- $\beta$  after apoptotic cell removal by macrophages [11, 31], microglia [27] or conventional DC [31] has also been described. Secretion of these anti-inflammatory cytokines can occur spontaneously after apoptotic cell encounter or is observed after stimulation with Danger signals mimicked *in vitro* by TLR ligands (LPS or zymosan, for TLR4 and TLR2, respectively) (Table 1). Regulatory factors other than immunosuppressive cytokines, such as retinoid acid [32], prostaglandin-E2 (PGE2) [11, 27], enhanced tryptophan catabolism via stimulation of indoleamine 2,3-dioxygenase (IDO) enzymatic activity [33, 34], and/or specialized pro-resolving lipid mediators (e.g., resolvins) [35], are also produced by professional phagocytes eliminating apoptotic cells (Figure 2). Thus, immunosuppressive cytokine-independent mechanisms induced by apoptotic cells have been also reported [33-38]. For instance, nitric oxide (NO) is spontaneously produced by mouse macrophages after interactions with early apoptotic cells [37, 38]. This prevents the release of MIP-2, the murine homolog of IL-8, which plays a role in neutrophil recruitment [38]. Synthesis of NO after apoptotic cell removal by murine macrophages inhibits also the production of the chemokine KC [37]. Moreover, uptake of apoptotic cells by professional phagocytes

is associated with a decreased capacity to produce pro-inflammatory cytokines, including:TNF, IL-1 $\beta$ , IL-6, IL-8,GM-CSF, IL-12 or IL-23[11, 20, 23, 28, 31, 39-41], or inflammatory factors, such as,reactive oxygen species (Figure 2). Again, as observed for anti-inflammatory cytokines, decrease of pro-inflammatory factor synthesis can occur spontaneously after apoptotic cell interactions in pathological situations where pro-inflammatory cytokine production is exacerbated. Prior apoptotic cell removal prevents also *ex vivo*pro-inflammatory cytokine secretion by TLR stimulation (Table 1).A decrease of pro-inflammatory cytokine secretion after apoptotic cell exposure can be a direct effect on pro-inflammatory cytokine genes, independent of IL-10 and TGF- $\beta$  [39, 41].Thus, the immunosuppressive microenvironment created by apoptotic cells themselves[42-44]is transiently amplified by professional phagocytes after apoptotic cell removal.

- A possible transfer of the immunomodulatory message to adaptive immune cells by antigen-presenting cells

The contact of conventional DC with apoptotic cells blocks their ability to mature and produce inflammatory cytokines, while their homing capacity might be stimulated. Hence, conventional DC acquire the expression of CCR7 [45, 46] –that in response to CCL19 and CCL21 gradients– guides DC to the draining lymph nodes. In the draining lymph nodes, conventional DC exposed to apoptotic cells can interact with naive T cells and deliver a “tolerogenic” signal favoring T cell commitment into a regulatory phenotype, such as peripherally-derived Foxp3<sup>+</sup> CD4<sup>+</sup> regulatory T cells (abbreviated pTreg according to a recent nomenclature [please refer to Abbas AK et al., Nat Immunol., 2013]) or IL-10-producing Tr1 cells[14, 31, 36, 47, 48]. Macrophages after apoptotic cell removal may also possess the ability to migrate to draining lymph nodes [5, 49]. However, this function may be restricted to “satiated” macrophages (*i.e.*, stuffed macrophages with apoptotic cells) [50]. Since macrophages usually degrade completely engulfed antigens due to their contents in proteolytic enzymes [51], no antigen presentation to T cells is considered to occur[52]. However, one may imagine that emigrating macrophages release immunosuppressive cytokines in the draining lymph

nodes leading to a transient local non-specific immunosuppression which may affect DC/T cell/B cell interactions.

■ A focus on the role of professional phagocytes in apoptotic cell-induced immunomodulation

Macrophages and DC represent both heterogeneous cell subsets with each cell subset dedicated to distinct functional properties. Significant improvements have been recently performed on the functional characterization and the ontogeny of macrophage [53] and conventional DC subsets [54]. Among these new findings [53, 54], some may impact on apoptotic cell removal and its functional consequences. Concerning macrophages, characterization of M1/M2 phenotypes, macrophage origin (tissue-resident *versus* blood monocyte-derived macrophages) and macrophage location may influence apoptotic cell elimination with some macrophages more dedicated to this job. This includes, for instance, the following subsets: IL-10-producing alternatively activated M2 macrophages [55], or glucocorticoid and M-CSF, or IL-10 and M-CSF-derived M2 macrophages [16]. Furthermore, tissue resident macrophages may prevent apoptotic cell removal by inflammatory monocyte-derived macrophages in a 12/15-lipoxygenase-dependent manner [56]. Marginal zone macrophages of the spleen are specialized in the uptake of blood-borne apoptotic leukocytes [12, 13]. In addition, marginal zone macrophages can secrete after apoptotic cell removal CCL22 to recruit specifically Treg [57]. Recent data describe the relationship between dedicated antigen-presenting cell (APC) subsets and the induction of pTreg, one of the main mechanisms involved in peripheral tolerance. This is true for macrophage subsets in the lung [58] or the intestine [59], as well as for DC subsets both in Human [52, 60] and mouse [61]. The recent characterization of migratory *versus* lymphoid-resident conventional DC can be useful in the future to better define the role of distinct APC subsets in apoptotic cell-induced tolerance and to target preferentially tolerogenic APC. While migratory conventional DC take up antigens from peripheral tissues, lymphoid-resident DC capture antigens from the blood or the lymph. Thus, blood-borne apoptotic leukocytes are rather eliminated by marginal zone CD8 $\alpha$ <sup>+</sup> conventional DC [23, 62]. In contrast, migratory DC arise in peripheral tissues and then migrate into the lymph nodes. In steady state (*i.e.*,

in absence of Danger signals or an ongoing inflammatory response), peripheral tolerance related to pTreg induction is rather induced by migratory conventional DC [61, 63] –as observed in the skin [61, 64], the lung [61], and the gut [65]– than lymphoid-resident CD8<sup>+</sup> DC, as initially thought [66]. In rats, a conventional DC subset dedicated to transport continually apoptotic cell-derived materials to T cell areas of the draining lymph nodes (*i.e.*, mesenteric lymph nodes) has been described in the intestine [21]. Mouse migratory CD103<sup>+</sup> conventional DC acquire apoptotic cell-derived antigens in the lung under steady state and present them to T cells in the draining lymph nodes [67]. In addition to peripheral tolerance, central tolerance to antigens from cells dying in the periphery can also be achieved, since peripheral conventional DC [68] as well as PDC [69] can transport antigens from the periphery to the thymus and may participate in antigen-reactive thymocyte deletion. Understanding the fine mechanism responsible for tolerance to apoptotic cell-derived antigens and more precisely the identification of APC subsets can be harnessed to favor or restore tolerance in transplantation settings or during inflammatory/autoimmune disorders, respectively.

■ A neglected/an emerging role of non-professional phagocytes?

In addition to professional phagocytes, non-professional phagocytes (*i.e.*, neighboring cells) can also uptake apoptotic cells (Table 2). The process of apoptotic cell removal by *amateur* phagocytes is usually slower [70-72], requires apoptotic cells in a more advanced stages [70] and is limited to subcellular fragments (*i.e.*, a smaller “prey”) rather than whole dying cells [71, 73]. Secondary necrosis due to delayed or impaired apoptotic cell removal leads to the release of toxic intracellular compounds that corresponds to Danger signals, named damage-associated molecular patterns (DAMP) [74]. These DAMP activates APC that, in turn, initiate efficient T cell responses through Th1, Th2 or Th17 differentiation profile. Moreover, in certain conditions, pro-inflammatory chemokines (e.g., IL-8, MCP-1/CCL2 and RANTES/CCL5) are released by these non-professional phagocytes [75, 76], leading to inflammatory cell recruitment. On the other hand, in certain tissues where professional phagocytes have limited access in steady state (e.g., the retina or the kidney), neighbor *amateur* phagocytes participate efficiently to the quiet elimination (*i.e.*, without



inflammation) of apoptotic cells[77, 78]. Thus, depending on the cells involved in apoptotic cell removal and on the speed/kinetics of this removal, apoptotic cells may block or initiate immune responses. One has to evoke the status of another APC that interacts with T cells: plasmacytoid dendritic cells (PDC), a particular DC subset that differ from conventional DC (for review [79]). No doubt exists that PDC can uptake dying cell materials[80, 81], as almost each cell type seems to be able to do it (Table 2). It remains to be determined whether PDC correspond to real professional or non-professional phagocytes. Several arguments suggest that PDC are non-professional phagocytes, including: *i*) delayed apoptotic cell elimination[82], *ii*) subcellular fragment uptake as attested by endocytosis of apoptotic cell-derived membrane microparticles or apoptotic bodies[82, 83] but not whole apoptotic cells [84, 85], *iii*) the release of pro-inflammatory cytokines (e.g., IFN- $\alpha$ , IL-6)[83, 86]. However, despite limited capacity to uptake apoptotic cells, PDC may play a role in apoptotic cell-induced immunomodulation by interacting with other immune cells: macrophage and pTreg[84].

#### ■ Receptors and soluble factors involved in apoptotic cell removal

Finally, the capacity to eliminate apoptotic cells and to be a professional or an *amateur* phagocyte may depend on engaged receptors involved in apoptotic cell uptake, as well as the possibility to secrete soluble proteins enhancing apoptotic cell removal (e.g., milk fat globule EGF factor 8 protein [MFG-E8] called also lactadherin[87], growth arrest-specific 6 [Gas6][88], or defense collagens including the complement component C1q, mannose-binding lectin, pulmonary surfactant protein A and D or adiponectin[89]). As stated before, apoptotic cell removal implicates cognate receptors on phagocytes and specific ligands displayed by apoptotic cells. These ligands are called either “eat-me” signals or apoptotic cell-associated molecular pattern (ACAMP) in reference to pathogen-associated molecular pattern (PAMP) expressed by pathogens (Figure 1). Interactions between phagocytes and apoptotic cells implicate multiple ligand/receptor pairs. Identification and characterization of these receptors and their ligands is an active research field (for review [90]). Whether the preferential implication of a particular receptor or of a set of receptors in apoptotic cell removal is associated with a particular function of phagocytes remains to be determined. Nevertheless, recognition of

phosphatidylserines –expressed early after apoptosis– is associated with anti-inflammatory cytokine release[11, 91]. Preventing phosphatidylserine recognition on apoptotic cells shifts the immune response from tolerance to immunogenicity[92, 93]. The same observation has been performed with the genetic invalidation of the Mer tyrosine kinase (Mertk)[94] –a phagocyte receptor that binds the soluble phosphatidylserine ligand, Gas6–, or of MFG-E8 soluble factor [95]–that links phosphatidylserines expressed by apoptotic cells to phagocyte  $\alpha_v\beta_3/\beta_5$  integrins. Moreover, in the absence of functional complement protein C1q in mouse models or in patients, failure to remove apoptotic cells is associated with lupus-like systemic autoimmune diseases[89]. C1q deficiency is an illustrative example for the major role of soluble factors in apoptotic cell-induced immunomodulation. Indeed, C1q participates in the classical complement-dependent engulfment of apoptotic cells [23, 96, 97], but is also able to bind directly phosphatidylserine residues of these dying cells [98] and to stimulate the expression of Mertk and Gas6 that, in turn, increase the capacity of macrophages to remove apoptotic cells [99]. Furthermore, C1q affects macrophage polarization during apoptotic cell uptake by inhibiting IL-1 $\beta$  secretion [100]. Finally, the classical complement-dependent elimination of apoptotic cells occurs mainly through IgM opsonisation[89]. This explains why IgM may be required for modulation of immune responses by apoptotic cells [36]. A better characterization of these receptor/ligand interactions will permit in the future to optimize apoptotic cell-based therapies and maybe to treat autoimmune diseases associated with a defect of apoptotic cell removal, such as lupus.

■ Toward the use of the immunomodulatory properties of apoptotic cells in clinical settings

In conclusion, several factors influence the immune response following apoptotic cell removal (Figures 1 and 2). This includes: *i*) which cell type dies[35], *ii*) the phagocyte cell type (macrophages *versus* DC *versus* non-professional phagocytes) which can be linked to the site where the cell dies, *iii*) the settings in which the cell dies (that is associated or not with Danger signals, either DAMP, or PAMP), *iv*) the factors released by phagocytes and apoptotic cells, and *v*) the cause of cell death. Indeed, apoptosis was initially defined morphologically[101], but different biochemical pathways in

response to different apoptotic stimuli lead to diverse apoptotic cell deaths with distinct immune consequences (tolerance *versus* immunogenicity)(for review on molecular definitions of apoptotic cell death, please refer to [102]; please also refer to a review dealing with immunogenic and tolerogenic cell death [103]). In a recent review [104], the 5 factors evoked above were called the 5 Ws of dying cells (for: Who, Where, Why, What and When). This enables to predict the immunological outcome of apoptotic cell death. Considering apoptotic cell-based therapy to limit inflammation and control undesirable immune responses, the following criteria have been selected: early apoptotic mononuclear cells (*i.e.*, mainly lymphocytes), intravenous infusion to target preferentially splenic professional phagocytes (marginal zone DC and/or macrophages that deal each day with billions of dying leukocytes), and induction of tolerogenic apoptosis (see next paragraph).

#### **Preclinical data using early apoptotic cell infusion in experimental models**

As discussed in the first part of this review, apoptotic cells are able to modulate the functions of several immune cells, including: macrophages, DC, NK cells, or CD4<sup>+</sup> T cells. The functions of these cells are deregulated in several inflammatory or autoimmune diseases. This is the case, for instance, of deregulated inflammatory cytokine secretion in sepsis or in RA. Based on their immunomodulatory or transient immunosuppressive properties described above, apoptotic cell administration has been performed in different experimental models of inflammatory and/or immune disorders (Table 3). From these studies, it is possible to better understand how apoptotic cells interact with the immune system, but also to propose the design of future clinical approaches. In this part of the review, we will analyze the different preclinical studies (Table 3) to select critical parameters to exert therapeutic effects, or in the contrary, to exclude factors that lead to no or opposite effects. Most of the studies report the prevention of the disease; that means that, apoptotic cells were infused at the triggering of the disease (*i.e.*, at immunization with auto-antigens, or at time of transplantation) or before the disease occurred. This is true for: type 1 diabetes in NOD mice [47], experimental autoimmune encephalomyelitis (EAE) [12, 62, 105], arthritis [36, 106, 107], fulminant hepatitis [108], contact hypersensitivity [109-111], acute and chronic graft rejection [85, 112-114], hematopoietic cell

engraftment[2, 14, 84, 115, 116], acute graft-versus-host (GvHD) disease [14]and reduction of infarction size after acute myocardial infarction[117, 118]. Thus, it is difficult to transpose this in the clinical setting where, in general, the disease is already present when a therapeutic solution has to be found. Nevertheless, one may propose that apoptotic cell infusion can be useful in disease prevention associated with a high risk of morbidity/mortality, such as GvHD (please refer to a recent review on the use of apoptotic cells in allogeneic hematopoietic cell transplantation [119]), a high risk of graft rejectionor in prevention of diabetes in the pre-diabetic stage. The use of apoptotic cell infusion in the prevention of complications associated with transplantation (*i.e.*, graft rejection or GvHD) is conceivable, since, in general, the transplantation process is scheduled, and so, apoptotic cell infusion can be performed before or at time of the transplantation, before the complications occurs. However, it is difficult to apply to cadaveric solid organ transplantation where the transplantation procedure is not predictable. Moreover, apoptotic cell administration has also been tested in experimental models to treat ongoing active diseases (as in the “real life”), such as acute heart graft rejection[114], chronic GvHD(our unpublished data) or sepsis[91, 120]. This latter pathology is interesting, since administration during the disease protects mice from sepsis-induced death[91, 120],while infusion 5 days before sepsis worsens mice survival, possibly by decreasing the capacity to secrete IFN- $\gamma$ [121]. Thus, this opens the way to use apoptotic cell administration to cure ongoing diseases, such as acute graft rejection or chronic GvHD without specific side effects (e.g., auto-antibody production)[122].

How clinical trials using apoptotic cells should be designed? In other words, we will now address from the experimental data the critical parameters for the future clinical studies, namely: *i*) the route of administration; *ii*) the number (schedule) of administration; *iii*) the adequate apoptotic cell typeto be used and the apoptotic signal. Most of the studies (91%, 20 out of 22) used the intravenous (iv) route of administration (Table 3). Consequently, the spleen and its marginal zone APC (lymphoid resident CD8 $\alpha^+$  DC[62, 85, 114], B cells [105, 106]or macrophages[12, 14, 84]) are critical for the modulation of the immune responses, since the spleen is the main filter for blood-borne apoptotic

cells[123]. In contrast, 4 studies reported apoptotic cell infusion either intra-peritoneally (ip)[106, 107], intra-tracheally[91] or intra-myocardially[118]. Some studies compared these routes of administration and iv route; no difference was observed[106, 118]. These particular routes were evaluated, since the diseases were induced through the same routes[91, 107]. Thus, this may theoretically present the advantage of targeting the affected organ (e.g., the myocardium[118], the lung or the peritoneum[91]) without generating a systemic effect. Apoptotic cell-based therapies can be useful to treat systemic diseases, and in this case, the iv route has to be preferred. On the other hand, this approach can be used in local diseases by injecting closed to the affected organs/tissues. The infused apoptotic cell number and the schedule of infusion are relatively homogeneous among the different studies:  $10^5$  to  $5 \times 10^7$  in mice[2, 12, 14, 36, 47, 62, 84, 85, 91, 105, 106, 108-116, 120, 121], or  $8 \times 10^6$  to  $10^8$  in rats[107, 117, 118]. In general, only one infusion was performed (86%, 18 out of 21). Prevention of type 1 diabetes [47] and arthritis [36, 106] may require repeated infusion. Thus, the iv route is interesting in a therapeutic point of view since it is easily available and it is also tolerogenic. The number of infused cells is compatible with cytopheresis when this will be transposed to Human (see below).

Now, considering the apoptotic cell type, and the apoptotic signal, the analysis of the different studies (Table 3) allows us to identify common requirements: *i*) cells rendered apoptotic are mainly leukocytes at early apoptotic stage (including: mouse spleen cells in 13 studies[2, 14, 84, 85, 105, 109-116], rat PBMC in two studies[117, 118], mouse T cells in two studies[12, 62], human Jurkat T cell line in one[91] and rat or mouse thymocytes in 3 studies[36, 106, 107], while mouse neutrophils [120] and a mouse beta cell line [47] were used in one study, respectively); *ii*) tolerogenic apoptotic stimuli used are the following:  $\gamma$ -irradiation in 10 studies [2, 14, 84, 107, 112, 115-118, 121], UVB irradiation in 7 [47, 85, 91, 108, 109, 113, 114], Fas-mediated death in 3[2, 12, 62], spontaneous apoptosis in culture due to survival factor deprivation in 2[106, 120], and dexamethasone treatment in one study[36]. Few studies compared apoptotic stimuli, but when UVB and  $\gamma$ -irradiation or Fas-mediated apoptosis, UVB-,  $\gamma$ - and X ray-irradiation were compared: no differences were observed[2,

84, 113]. Thus, in order to develop a therapeutic approach, one may propose to use PBMC collected by cytopheresis, expose them to one of the apoptotic signals described above and to control that PBMC are at early stage of apoptosis as attested by Annexin-V staining with limited propidium iodide or 7-AminoActinomycin D (7-AAD) staining.

Finally, analysis of antigen dependency (*i.e.*, whether apoptotic cells should carry the antigen of interest or whether this effect is observed whatever the apoptotic cell origin) is useful to shed light on disease mechanisms and to specify how apoptotic cell infusion interferes with the disease, as well as in a practical point of view to design the future clinical trials. Thus, experimental models can be divided in two concerning antigen dependency (Table 3), with some experimental models requiring apoptotic cells bearing the antigen of interest (*i.e.*, either donor or auto-antigens), such as: diabetes[47], EAE[12, 62, 105], contact hypersensitivity [109-111] or transplantation[85, 112-114]. In contrast, arthritis[36, 106, 107], sepsis[91, 120], fulminant hepatitis [108] and allogeneic hematopoietic cell transplantation [2, 14, 84, 115, 116] are controlled independently of the apoptotic cell origin (Table 3). So, one may speculate that some diseases need tolerance induction against pathogenic auto- or allo-antigens while other implicate a non-specific down-regulation of inflammation or transient immune suppression (Figure 3). In line with this hypothesis, splenic macrophages [14, 120] or liver Kupffer cells [108] have been shown to play a critical role in diseases modulated by the anti-inflammatory effects of apoptotic cell infusion, whereas splenic conventional DC –that are more critical for antigen presentation to T cells than macrophages [29, 51, 52]– are necessary for tolerance to donor allo- or auto-antigens[62, 85, 110, 111, 114]. Some of these studies evaluated functionally the role of APC phagocytosing infused apoptotic cells by *in vivo* depletion using either clodronate loaded liposomes[14, 84], gadolinium chloride[108, 113] or transgenic mice expressing diphtheria toxin receptor under the control of macrophage- or DC-specific promoter rendering macrophages or DC sensitive to diphtheria toxin administration [12, 62, 85]. In addition, *ex vivo* functions of macrophages were also tested after apoptotic cell infusion with reduced secretion of pro-inflammatory cytokine secretion, such as TNF[107] (see also Table 1). Schematically,

inflammatory diseases controlled by apoptotic cell infusion exhibit –after this infusion– a decrease in circulating inflammatory cytokines [107, 120] associated with an increased production of anti-inflammatory cytokines (*i.e.*, IL-10 [36, 106, 108] and/or TGF- $\beta$  [91, 107, 108]), a modification of macrophage or Kupffer cell function [107, 108, 120] (see also Table 1), and a decrease of neutrophil infiltrates [91, 120]. Neutralization of TGF- $\beta$  prevents the beneficial effect of apoptotic cell infusion [14, 84, 116]. This confirms that apoptotic cell infusion controls some inflammatory diseases by exerting anti-inflammatory effects. In addition to anti-inflammatory cytokines, pro-angiogenic factors (*e.g.*, VEGF) and metalloproteinases can also be secreted favoring the resolution of inflammation [117, 118]. One may imagine that down-regulation of inflammation by targeting macrophage functions can be sufficient to affect pathogenic T cell or antibody responses, even though apoptotic cells do not interact directly with DC/T cell/B cell crosstalk, as attested by down-regulation of allo- [116] or auto-antibodies [106], or pathogenic Th17 T cells [36]. In contrast, diseases treated with antigen-bearing apoptotic cells (*i.e.*, requiring tolerance to allo- or auto-antigens) involve splenic lymphoid resident conventional DC [62, 85, 114]. These DC dedicated to antigen presentation to T cells may then participate in the induction of regulatory immune cells, including: Tr1 [36, 47, 106, 111], FoxP3<sup>+</sup>Treg [14, 47, 105, 112, 114], regulatory CD8<sup>+</sup> T cells [109] or regulatory B cells (Breg) [36, 106]. Alternatively, tolerance induction by antigen-bearing apoptotic cell administration may reflect the inhibition of pathogenic Th1 or Th17 cells [12, 36], anti-donor T cells secreting IFN- $\gamma$  [85, 114], anti-donor allo- [85, 114] or auto-antibodies [47]. Overall, apoptotic cell administration is able to control inflammatory disorders by diminishing the pro-inflammatory state and to induce or restore tolerance to donor allo-antigens or auto-antigens by inhibiting pathogenic T or B cell responses and inducing pro-tolerogenic/regulatory cells (Figure 3).

In a practical point of view, preclinical models are useful to determine antigen dependency as reported in Table 3. Then, if a disease requires a particular antigen, this antigen needs to be expressed by the apoptotic cells, or at least locally present when apoptotic cells are eliminated. This leads to select cells from a particular origin (*e.g.*, the donor in the case of transplantation) or to

engineer patient PBMC to express a nominal antigen as performed in autoimmune diseases[12, 62].

In contrast, if the disease does not need any specific antigen and is treated independently of apoptotic cell origin, PBMC can be obtained from healthy volunteers after written informed consent and a bank of PBMC can be established. Alternatively, PBMC can be obtained from the patient himself. This latter solution is the simplest, even if no lymphopenia, leukemic cells or infections are present.

The cumulative encouraging data obtained in preclinical models allow physicians to initiate clinical studies. This is particularly true for the prevention of acute GvHD. Acute GvHD—an inflammatory disorder associated with allogeneic hematopoietic cell transplantation—is due to the activation of host APC by the conditioning regimen that stimulates donor allogeneic T cells. These activated allogeneic T cells as well as factors released during this process (e.g., TNF) destroy healthy tissues from the recipients including: the liver, the gastro-intestinal tract and the skin[124]. In experimental models (Table 3), infusion of apoptotic cells at time of transplantation induces TGF-beta and increases regulatory T cells that prevent GvHD occurrence [14, 119]. Recently, a phase 1/2a clinical study has been registered in the ClinicalTrials.gov website (<http://clinicaltrials.gov>) under the number, # NCT00524784, and encouraging data have been presented recently in two meetings[125, 126]. In this study, iv donor early apoptotic cell infusion was performed the day before allogeneic hematopoietic cell transplantation in order to prevent acute GvHD occurrence (please see above). Thirteen patients have been treated. Cells were obtained from hematopoietic cell donors after cytopheresis. The cell number has been transposed from animal models and a “dose escalate” has been performed ranging from  $35 \times 10^6/\text{kg}$  to  $210 \times 10^6/\text{kg}$ . No specific toxicities have been associated with iv donor early apoptotic cell infusion. Comparison with historical data concerning acute GvHD occurrence in the same transplantation center or from the literature seems to show a reduction of high grade acute GvHD (grade >II). Interestingly, no severe GvHD was observed in the three patients receiving the highest apoptotic cell number [126]. This clinical study opens the way to apoptotic cell-based therapies in other clinical settings already assessed in experimental models (Table 3).



## Conclusion

In conclusion, early apoptotic cell infusion has been reported to treat or prevent experimental inflammatory/autoimmune disorders, as well as complications associated with transplantation (Table 3). A phase 1/2a clinical trial has been performed in 13 patients without specific toxicities and a good early outcome [126]. This confirms the feasibility of such cell-based therapy approach in Humans. Today, the major questions concern: *i*) the interactions of such therapy with other treatments received by patients, as for instance steroids (that increase apoptotic cell removal by macrophages [127, 128]) or methotrexate (that induce macrophage apoptosis [129] and in theory may delay apoptotic cell removal), *ii*) the possible autoimmunity due to delayed or impaired apoptotic cell clearance and *iii*) the potential overall immunosuppression that may alter beneficial immune responses against pathogens for instance, or leukemic cells in the settings of hematopoietic cell transplantation. We have already studied the interactions of apoptotic cell infusion with immunosuppressive drugs routinely used in allogeneic hematopoietic cell transplantation and rapamycin (sirolimus) has a synergic effect [115]. This kind of study has to be extended to other conventional drugs, such as steroids, or methotrexate for RA. Concerning other side effects (autoimmunity or global immunosuppression), careful selection and follow-up of patients may limit these effects. As performed for anti-TNF, patients with active or ongoing infections or a past of cancer should be excluded. In order to prevent autoimmune disease related to impaired apoptotic cell removal, factors known to play a role in this clearance such as C1q have to be analyzed before patient enrolment. Finally, instead of infusing apoptotic cells, apoptotic cell death may be induced *in vivo* using different approaches (*i.e.*, CD3 specific antibody [31]) and tolerance can be achieved with similar mechanisms (for review [119]).

## Future perspectives

How the fields described here (*i.e.*, the use of apoptotic cells and the understanding of mechanisms involved in apoptotic cell removal) will evolve in the next 5 to 10 years? We speculate that several phase 1/2a studies and even phase 2 studies, will be performed in different autoimmune diseases or

in the settings of transplantation. Other therapies will be derived from this approach to focus on the main mechanism(s) or factor(s) responsible for the therapeutic effects. For instance, apoptotic cell secretome (that is the factor released by cells undergoing apoptosis) has been shown to exert similar effect on acute myocardial infarction than apoptotic cell infusion [130]. Liposomes expressing phosphatidylserines mimicking an “eat-me” signal associated with anti-inflammatory properties [11, 91] can also be infused in the femoral vein to improve acute myocardial infarction [131]. These two recent publications [130, 131] confirm that the non-specific anti-inflammatory function of apoptotic cell infusion is sufficient to limit acute myocardial infarction. Phosphatidylserine-expressing liposomes are not a cell-based product. This has the advantage of being controlled or produced at large scale using pharmaceutical procedures and not to be dependent on cell therapy regulations. Other perspectives are linked to improvements performed in the understanding of APC subsets and on the characterization of receptors and ligands involved in apoptotic cell removal. One may imagine that the identification of receptor(s) expressed by tolerogenic professional phagocytes would be preferentially targeted to restore tolerance and cure inflammatory auto- or allo-immune diseases.

## **Executive summary:**

### ***Introduction***

- Improvement of existing treatment is needed for several inflammatory and/or immune-mediated disorders, including transplantation, acute or chronic inflammatory diseases by modulating the deregulated immune response.

### ***Immunomodulatory properties associated with early apoptotic cells***

- Apoptotic cells themselves, elimination of apoptotic cells by innate immune cells or interactions of these cells with apoptotic cells lead to an immunomodulatory microenvironment that may favor or restore tolerance.
- Several factors govern these immunomodulatory properties and mechanisms involved in this process are currently being deciphered.

### ***Preclinical data using early apoptotic cell infusion in experimental models***

- Intravenous early apoptotic cell infusion can be used for disease prevention, notably in diseases associated with high mortality/morbidity, for instance, acute graft-*versus*-host disease.
- Intravenous apoptotic cell infusion can be used for disease treatment, such as, sepsis or acute graft rejection.
- Critical aspects to consider to achieve a beneficial therapeutic effect include: *i*) the iv route of administration, but local administration (into the myocardium, lung or peritoneum) is also possible; *ii*) one infusion is often sufficient; *iii*) blood leukocytes is the easiest and major source of apoptotic cells; *iv*) a tolerogenic apoptotic signal inducing early apoptotic cells is required (e.g.,  $\gamma$ - or UVB-irradiation).
- The analysis of mechanisms involved in these experimental models suggests that iv apoptotic cell infusion acts by two distinct mechanisms: *i*) modification of the inflammatory microenvironment by targeting professional phagocytes and *ii*) induction of “specific” tolerance to a given antigen through regulatory cell commitment in the favorable microenvironment shaped by the professional phagocytes.

- A phase 1/2a clinical trial performed in the prophylaxis of graft-*versus*-host disease in thirteen patients show no specific side effects at early time point after apoptotic cell infusion. [Note added in proof: the study is now published: Mevorach D et al., Biol Blood Marrow Transplant. 2014 Jan;20(1):58-65.]

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## Reference annotations

Papers of special note have been highlighted as: \* of interest \*\* of considerable interest

\*2. Bittencourt MC, Perruche S, Contassot E et al.: Intravenous injection of apoptotic leukocytes enhances bone marrow engraftment across major histocompatibility barriers. *Blood* 98(1), 224-230 (2001).

This paper is the first manuscript reporting the beneficial use of apoptotic cell infusion in an experimental model of allogeneic hematopoietic cell transplantation.

\*\*11. Fadok VA, Bratton DL, Konowal A, Freed PW, Westcott JY, Henson PM: 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(4), 890-898 (1998).

This seminal paper is one of the first reporting the immunomodulatory functions of apoptotic cells, *via* TGF- $\beta$  secretion by macrophages phagocytosing apoptotic cells (see also #30)

\*\*12. Miyake Y, Asano K, Kaise H, Uemura M, Nakayama M, Tanaka M: Critical role of macrophages in the marginal zone in the suppression of immune responses to apoptotic cell-associated antigens. *J Clin Invest* 117(8), 2268-2278 (2007).

This paper is interesting since it extends the use of apoptotic cell infusion in another preclinical model of experimental autoimmune encephalomyelitis, EAE and it identifies also that splenic marginal zone macrophages are critical for the inhibition of immune responses against blood-borne apoptotic cell-associated antigens.

\*21. Huang FP, Platt N, Wykes M et al.: A discrete subpopulation of dendritic cells transports apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. *J Exp Med* 191(3), 435-444 (2000).

This paper describes, in rats, a subset of dendritic cells dedicated to transport continually apoptotic cell-derived antigens from intestine to T cell areas of secondary lymphoid organs.

**\*\*30.** Voll RE, Herrmann M, Roth EA, Stach C, Kalden JR, Girkontaite I: Immunosuppressive effects of apoptotic cells. *Nature* 390(6658), 350-351 (1997).

This outstanding paper is the first showing that interactions of apoptotic cells with antigen-presenting cells induce immunosuppressive cytokine secretion (here IL-10) and thus apoptotic cells possess transient immunosuppressive functions (see also #11)

**\*\*56.** Uderhardt S, Herrmann M, Oskolkova OV et al.: 12/15-lipoxygenase orchestrates the clearance of apoptotic cells and maintains immunologic tolerance. *Immunity* 36(5), 834-846 (2012).  
Beside the new role of 12/15-lipoxygenase, this paper reports that tissue resident macrophages may inhibit/control the recruitment of inflammatory monocyte-derived macrophages and then the engulfment of apoptotic cells by these inflammatory macrophages.

**\*70.** Parnaik R, Raff MC, Scholes J: Differences between the clearance of apoptotic cells by professional and non-professional phagocytes. *Curr Biol* 10(14), 857-860 (2000).

This paper is one of the first papers reporting the role of *amateur* phagocytes in apoptotic cell removal. In addition, it shows that amateur phagocytes need apoptotic cells in a more advanced stage and that they are less efficient.

**\*104.** Griffith TS, Ferguson TA: Cell death in the maintenance and abrogation of tolerance: the five Ws of dying cells. *Immunity* 35(4), 456-466 (2011).

This is an interesting and recent review describing how tolerance to apoptotic cell-derived antigens is maintained or broken. The authors use the investigative technique used by some detectives and called the 5 Ws (Who, Where, Why, What and When).

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## Tables

Table 1. Early apoptotic cells modulate cytokine/factor synthesis by phagocytes		
Nature of the phagocytes	Cytokines or factors* modulated after apoptotic cell interactions (spontaneously or after cell stimulation**)	Ref.
human monocytes	↑ IL-10, ↓ IL-1β, TNF, IL-12 (variable according to incubation time before LPS stimulation) (LPS)	[30]
human monocyte-derived macrophages	↑ TGF-β, ↑ PGE-2, ↓ TxB2, ↓ LKTC4 (spontaneously) ↑ TGF-β, ↓ GM-CSF, IL-1β, IL-8, IL-10, TNF-α (LPS, TLR4 ligand) ↑ TGF-β, ↓ GM-CSF, IL-10, TNF (zymosan, TLR2 ligand)	[11]
human monocyte-derived macrophages; human macrophage cell line RAW264.7; murine macrophage cell line J774	↓ IL-12 p35mRNA, ↓ IL-12 (IFN-γ + LPS). These effects are independent of IL-10 and TGF-β.	[39]
murine peritoneal macrophages; human monocytes; human monocyte-derived macrophages; human macrophage cell line RAW264.7	↑ IL-10 (spontaneously), but difference exists between the phagocyte tested IL-10 was the sole cytokine studied.	[25]
murine peritoneal macrophages	↑ TGF-β <i>in vitro</i> and <i>ex vivo</i> ( <i>in vivo</i> exposure) (spontaneously) TGF-β was the sole cytokine studied.	[91]
murine peritoneal macrophages	↓ TNF <i>ex vivo</i> (spontaneously, <i>in vivo</i> exposure in the setting of SCW-induced arthritis, and after <i>in vitro</i> LPS stimulation)	[107]
murine bone-marrow derived DC or macrophages	↓ IL-23 (LPS); IL-23 was assessed indirectly by a IL-17-dependent bioassay	[40]
murine bone-marrow derived DC or macrophages	↓ IL-12 but not TNF (LPS). These effects are independent of IL-10 and TGF-β.	[41]
human monocyte-derived dendritic cells	↓ IL-23, ↑ IL-10, no effect on IL-12p70 or on PGE-2 (zymosan) no effects (LPS)	[28]
murine immature bone marrow-derived CD8α <sup>-</sup> CD11b <sup>+</sup> DC	↓ IL-1α, IL-1β, IL-6, IL-12p35, IL-12p40, TNFmRNA & ↓ IL-1α, IL-1β, IL-6, TNF; no effect on TGF-β, IL-1Ra, MIF (spontaneously) ↓ IL-1α, IL-1β, IL-6, IL-12p70, TNF, ↑ TGF-β (LPS) IL-10 was not tested.	[23]
murine spleen DC	<i>in vitro</i> ↑ TGF-β (spontaneously) <i>in vivo</i> ↑ TGF-β requires both macrophages and immature DC	[31]
murine microglial cells	↓ IL-1β associated with apoptotic cells <i>in vivo</i> (other cytokines were not tested)	[20]
rat microglial cells	↑ TGF-β, HGF (spontaneously) ↓ NO, PGE-2, TNF, no effect on IL-10 (LPS)	[27]
<p>* In a given study, only the cytokines or factors affected by apoptotic cell exposure were mentioned after the following symbols: ↑ means increase; ↓ means decrease. However, readers have to consider that if a cytokine/factor is mentioned for an experimental condition but not for the other one, it means that this cytokine/factor is not affected in this particular condition; **the agent used to stimulate phagocytes is given. Abbreviations used: HGF, hepatocyte growth factor; LKTC4, leukotriene C4; TxB2, thromboxane B2;</p>		



**Table 2. "Non-professional" phagocytes involved in apoptotic cell removal are potential targets for apoptotic cell-based therapies.**

<b>Cells involved in removal</b>	<b>Comments</b>	<b>Ref.</b>
normal astrocytes	<i>in vitro</i> ; phosphatidylserine-dependent for review	[17] [132]
glioma cells (tumoralastrocytes)	<i>in vitro</i> ; phosphatidylserine-dependent	[17]
CNS glial cells	<i>in Drosophila</i>	[133]
neuronal progenitors	<i>in vivo</i> ; ELMO1/Rac1-dependent	[134]
neurons	for review	[132]
retina cells	<i>in vivo</i> ; during development	[135]
retinal pigment epithelial cells*	<i>in vivo</i> ; Mertk-dependent	[15]
	Gas6-dependent	[136]
	$\alpha v/\beta 5$ integrin-dependent	[137]
	these 3 pathways implicate phosphatidylserines	
lens epithelial cells	<i>in vitro</i> ; apoptotic cell removal is delayed compared with professional phagocytes and requires late apoptotic cells	[70]
kidney parenchymal cells	<i>in vivo</i> ; during development	[77]
baby hamster kidney cells	<i>in vitro</i> ; apoptotic cell removal is delayed compared with professional phagocytes and requires late apoptotic cells	[70]
kidney tubular epithelial cells	<i>in vivo</i> and <i>in vitro</i> ; <i>Kim-1-dependent</i>	[78]
mammary epithelial cells	<i>in vitro</i> and <i>in vivo</i> ; CD36-, vitronectin receptor $\alpha v/\beta 3$ -, CD91-dependent; associated with TGF- $\beta$ release	[138]
	<i>in vivo</i> ; MFG-E8-dependent; if altered, local inflammation	[95]
	Mertk-dependent; if altered, local inflammation	[139]
	VEGF secretion after apoptotic cell uptake	[140]
cell line HC11		
lung epithelial cells	<i>in vivo</i> ; Rac-1-dependent; associated with IL-10 secretion; if altered, IL-33 production and local inflammation as well as exacerbation of the Th2 response to allergens	[141]
fibroblasts	vitronectin receptor $\alpha v/\beta 3$ -, mannose/fucose specific lectin-dependent	[142]
	leads to malignant transformation via oncogene transfer	[143]
endothelial cells:	carbohydrate-specific receptor-dependent; increased removal	
Liver	by IL-1 $\beta$	[73]
HEV	<i>in vivo</i> ; <i>in vitro</i>	[144]
microvascular HMEC-1 and HUVEC	<i>in vitro</i> ; increase of <i>IL-8</i> and <i>MCP-1/CCL2</i> mRNA as well as IL-8 secretion; enhanced binding of leukocytes to phagocytosing endothelial cells	[75]
HUVEC	MFG-E8-dependent	[145, 146]
peritoneal mesothelial cells	limited to subcellular fragments, slow kinetics	[71]
mesenchymal stem cells (MSC)	<i>in vivo</i> in macrophage-less mice; slow kinetics enhanced MSC osteogenic differentiation; increase of CXCR4 and CXCR5 expression, IL-8, CCL2 and CCL5 secretion, as well as Th17 differentiation (via IL-6)	[72] [76]
skeletal myoblasts	<i>in vitro</i> ; <i>BAI-1-dependent</i>	[147]
tumor cells (gastric carcinoma, glioma)	for a recent review, please see Ref. [148]	[17, 148]
*according to Ref. [3]: "Retinal pigment epithelial cells phagocytose more material over a lifetime than any other cell in the body." Abbreviations : CNS, central nervous system; HEV, high endothelial venule cells; HUVEC, human umbilical vein endothelial cells; Mertk, Mer tyrosine kinase; VEGF, vascular endothelial growth factor.		

Table 3. Potential therapeutic use of apoptotic cell infusion based on experimental models		
Pathologies	Effects, route of administration, cell type and apoptotic stimulus, mechanism(s), models	Ref.
<b>Chronic inflammatory autoimmune diseases</b>		
Type I diabetes	- Prevention; iv; UVB-induced apoptotic beta cell line, NT1 ( $10^5$ weekly, 3 weeks); induction of Th2 response, IL-10-producing Tr1 cells and FoxP3 <sup>+</sup> Treg in the spleen; suppression of anti-beta cell auto-Abs; NOD mice - Same effect with UVB-induced apoptotic splenic stroma cells	[47] [149]
Experimental Autoimmune Encephalomyelitis (EAE)	- Prevention; iv; Fas ligand-induced apoptotic T cells ( $2 \times 10^7$ ); Ag-specific; implication of splenic marginal zone macrophages; diminution of MOG-specific Th1 and Th17 in the spleen; MOG <sub>35-55</sub> -induced EAE (C57BL/6) - Prevention; iv, Fas ligand-induced apoptotic T cells ( $2 \times 10^7$ ); Ag-specific; implication of splenic CD8 $\alpha^+$ CD103 <sup>+</sup> CD207 (langerin) <sup>+</sup> conventional DC; MOG <sub>35-55</sub> -induced EAE (C57BL/6) - Prevention; iv but not after sc or ip; apoptotic spleen cells ( $5 \times 10^7$ ); Ag-specific; implication of IL-10 producing splenic marginal zone PD-L1 <sup>+</sup> macrophages; long term maintenance by splenic Treg; B cells are not required; MOG <sub>35-55</sub> -induced EAE (C57BL/6) or PLP <sub>178-191</sub> -induced EAE (SJL)	[12] [62] [105]*
Arthritis	- Prevention; iv (but same data ip); spontaneous apoptotic thymocytes ( $2 \times 10^7$ in total, 3 consecutive days); protection mediated by IL-10 produced by both B and T cells; reduction of pathogenic anti-collagen II auto-Abs; collagen induced arthritis in DBA/1 mice but not in a passive antibody transfer model of arthritis in K/BxN mice - Prevention; ip with SCW immunization; 15 Gray $\gamma$ -irradiated thymocytes ( $2 \times 10^8$ ); TGF- $\beta$ increase; decrease of macrophage pro-inflammatory response (TNF, IFN- $\gamma$ ); increase of blood and draining lymph node Treg; SCW-induced arthritis in Lewis rats - After immunization; iv; dexamethasone or etoposide-induced apoptotic thymocytes ( $3 \times 10^7$ , 3 consecutive days); decrease of draining lymph node Th17 cells; increase of IL-10 producing splenic marginal zone B cells and of IL-10 producing T cells in the draining lymph node; natural IgM-dependent clearance; methylated BSA-induced arthritis in C57BL/6 mice	[106] [107] [36]
<b>Acute inflammatory diseases</b>		
Sepsis	- Resolution of acute inflammation; ip ( $4 \times 10^7$ , day 3) or endotracheal instillation ( $1.8-2 \times 10^7$ , 36-48 h after); UVB-induced apoptotic human Jurkat T cell line; phosphatidylserine-induced TGF- $\beta$ secretion; decrease of immunecell infiltrate; thioglycollate-stimulated peritoneum or LPS-stimulated lung - Increase of mice survival (day 0, or 1h, 3 h, 6 or 24 h after); iv; spontaneous apoptotic neutrophils ( $10^7$ ); reduction of circulating inflammatory cytokines (IL-12, TNF, IFN- $\gamma$ ); decrease of neutrophil infiltration in target organs; reduction of serum LPS levels; implication of macrophage phagocytosis; LPS-induced endotoxic shock in C57BL/6 mice and cecal ligation and puncture sepsis model in C57BL/6 mice - Worsen mice survival (day -5); iv; 10 Gy- $\gamma$ -irradiated spleen cells ( $5 \times 10^7$ ); lack of IFN- $\gamma$ production (which is protective); cecal ligation and puncture sepsis model in C57BL/6 mice	[91] [120] [121]
Fulminant hepatitis	- Prevention; iv (day-3 to day-7); UVB-induced apoptotic spleen cells ( $1.5 \times 10^7$ , $2 \times 10^8$ no effect); whatever the origin of apoptotic cells; dependent on Kupffer cells and IL-10 via membrane bound TGF- $\beta$ ; but not on CD25 <sup>+</sup> cells or CD11c <sup>+</sup> cells; reduction of NO and TNF <i>in vitro</i> ; LPS plus D-galactosamine-induced fulminant hepatitis in C57BL/6 or BALB/c mice	[108]
<b>Contact hypersensitivity</b>	- Prevention (day -7); iv; 3 Gy $\gamma$ -induced apoptotic spleen cells ( $1 \times 10^7$ ); Ag-specific; Induction of regulatory TRAIL <sup>+</sup> CD8 <sup>+</sup> T cells without activating CD4 <sup>+</sup> T cell help; delayed-type hypersensitivity using 2,4,6 trinitrobenzene sulfonic acid (TNBS)	[109]

	- Prevention; iv; UVA-irradiated and 8-methoxypsoralen (8-MP)-sensitized spleen cells**This induces apoptosis, but other changes specific to UVA and 8-MP may also occur. ( $5 \times 10^7$ ); Ag-specific; IL-10 dependent; dependent on CD11c <sup>+</sup> DC; contact hypersensitivity using dinitrofluorobenzene	[110, 111]
<b>Transplantation</b>		
<b>Cardiac allograft</b>		
Acute rejection	- Prevention (day-7 before Tx); iv; UVB- or 1.50 Gy-irradiated apoptotic spleen cells ( $5 \times 10^7$ ); donor-specific; requirement of phagocytosis (host macrophages) and phosphatidylserine recognition; different donor/recipient rat strain combination - Treatment (day 7 after Tx); iv; UVB-induced apoptotic spleen cells ( $10^7$ ); donor-specific; splenic host CD8 $\alpha^+$ DC; Intra-graft TGF- $\beta$ and IL-10 mRNA; Foxp3 <sup>+</sup> Treg present in the graft; suppression of systemic anti-donor allo-antibody response and IFN- $\gamma$ ; different donor/recipient mouse strain combination	[113] [114]
Chronic rejection	- Prevention (day-7); iv; UVB-induced apoptotic spleen cells ( $10^7$ ); donor-specific; splenic host CD11 <sup>high</sup> DC; suppression of systemic anti-donor allo-antibody response and IFN- $\gamma$ ; presentation of donor apoptotic cell-derived allopeptides declines 3 days after apoptotic cell infusion; intra-abdominal aortic transplantation (BALB/c into C57BL/6 mice)	[85]
Islet allograft	- Prevention (day -7); iv; 35 Gy- $\gamma$ -irradiated-apoptotic spleen cells ( $5 \times 10^6$ ); donor-specific; possible involvement of Treg	[112]
<b>Hematopoietic cell transplantation</b>		
Hematopoietic engraftment	- Prevention (day 0, the day of Tx); iv; 40 Gy-irradiated, UVB- or FasAb-induced apoptotic spleen cells ( $5 \times 10^6$ ); whatever the origin of apoptotic cells (donor, recipient, third party or xenogeneic [human]); different donor/recipient mouse strain combination - This graft facilitating effect is TGF- $\beta$ -dependent - This effect implicates host splenic macrophages –but not conventional DC; increase of Treg in the spleen - This effect implicates also donor bone marrow-derived PDC that induces Treg commitment via TGF- $\beta$ ; same effect with X ray-irradiated apoptotic spleen cells	[2] [116] [14] [84]
Acute GvHD	- Prevention (day 0); iv; 40 Gy $\gamma$ -irradiated donor apoptotic spleen cells ( $5 \times 10^6$ ); involvement of CD25 <sup>+</sup> cells	[14]
Chronic GvHD	- Treatment (week 6 after Tx); iv; X-ray-irradiated apoptotic spleen cells ( $5 \times 10^6$ ); DBA2 into B6D2F1 model	***
Allo-Ab after graft rejection	- Prevention (day 0); iv; 40 Gy-irradiated apoptotic spleen cells ( $5 \times 10^6$ ); whatever the origin of apoptotic cells; TGF- $\beta$ -dependent; same model as in [2, 14]	[116]
<b>Acute myocardial infarction</b>	- Prevention (onset of ischemia); iv; 45 Gy $\gamma$ -irradiated apoptotic PBMC ( $8 \times 10^6$ ); Attenuation of infarcted size and improvement of functional parameters assessed by echography; increased homing of endothelial progenitors; increase of IL-8, VEGF and MMP9 mRNA <i>in vitro</i> ; ligation the left anterior descending artery in rat - The same effect is observed after intramyocardial injection; increase of CD68 <sup>+</sup> cell infiltrate	[117] [118]
*The target antigen is coupled with syngeneic splenocytes using ethylene carbodiimide (ECDI). ECDI induces splenocyte apoptosis. ** This induces apoptosis but other changes specific to ECDI may also occur; ***our unpublished data. Abbreviations: Abs, antibodies; Ag, antigen; GvHD, graft-versus host disease; ip, intraperitoneal; iv, intravenous infusion; LPS, lipopolysaccharide; MOG <sub>35-55</sub> , myelin oligodendrocyte glycoprotein peptide; NO, nitric oxide; OVA, ovalbumine peptide; PBMC, peripheral blood mononuclear cells; PLP, proteolipid protein; sc, subcutaneous; SCW, streptococcal cell wall; Tx, transplantation; UV, ultraviolet; This table was updated and adapted from Ref. [119, 150].		



## Figure legends:

**Figure 1. Signals involved in apoptotic cell removal.** Different signals orchestrate apoptotic cell removal by neighbor cells (*amateur* phagocytes, see Table 1) or professional phagocytes, such as macrophages or conventional DC (cDC). These signals include: (1) the loss of “do not eat-me” signals; (2) the secretion of “find-me” signals that can be counterbalanced by “keep out” signals[151]; (3) the acquisition of “eat-me” signals. Soluble factors can participate to acceleratedelimination of apoptotic cells. Adapted from Ref. [150]. Abbreviations used:BAI-1, brain angiogenesis inhibitor-1;CRP, C reactive protein; CRT, calreticulin; C1q, complement component C1q; Gas6, Growth Arrest-specific 6; KIM-1, kidney injury molecule-1 (also known as TIM-1); MBL, mannose-binding lectin; Mertk, Mer tyrosine kinase; MFG-E8, milk fat globule EGF 8 protein called also lactadherin; PS, phosphatidylserines; SP-A, pulmonary surfactant A; SP-D, pulmonary surfactant D; TSP1, thrombospondin-1.

**Figure 2.Immunomodulatory microenvironment created by apoptotic cells.**During the apoptotic process, apoptotic cells can produce spontaneously anti-inflammatory factors, such as IL-10[44] or TGF- $\beta$ [43], or be a source of CCR5 chemokine receptor that neutralizes its ligands, CCL3/MIP-1 $\alpha$  or CCL5/RANTES and subsequently blocks immune cell migration[42]. Apoptotic cells interact with several innate immune cellsor antigen-presenting cells (APC), including: monocytes, macrophages (M $\Phi$ ), microglia in the brain, conventional dendritic cells (cDC), NK cells or B cells. Uptake of apoptotic cells or only the interaction with apoptotic cells lead to modulation of several factors in innate cells or APC. These factors are mentioned in boxes linked to each APC or innate cell subset. Adapted from Ref. [152]. Some of these factors are produced spontaneously after apoptotic cell encounter or increased or diminished after a subsequent stimulation by TLR ligands in the context of inflammation (see Table 1). \*Concerning NO synthesis, differences existbetween human and muse macrophages [153] and maybe also between *in vivo* and *in vitro* conditions[154]. Abbreviations used: AhR, aryl hydrocarbon receptor; GM-CSF, granulocyte and macrophage colony stimulating factor;

HGF, hepatocyte growth factor; IL, interleukin; IL-1RA, interleukin-1 receptor antagonist; iNOS, inducible nitric oxide synthase; LT4C, leukotriene C4; NGF, nerve growth factor; NO, nitric oxide; NOS2, nitric oxide synthase-2; PAF, platelet-activating factor; PGE-2, prostaglandin-E2, RvE, resolvin E; TXA2, thromboxane A2; VEGF, vascular endothelial growth factor A. ↓, decrease; ↑, increase; see also text.

**Figure 3. Anti-inflammatory effects *versus* tolerance induction after early apoptotic cell infusion.**

Apoptotic cell-induced immunosuppression is transient (limited to the time course of apoptotic cell removal and the persistence of immunosuppressive cytokines: mainly IL-10 and TGF- $\beta$ , see also Figure 2), localized to the site where the cells are dying and/or being eliminated (*i.e.*, the spleen after iv infusion), and non-specific (*i.e.*, all the cells sensitive to immunosuppressive cytokines at the site of apoptotic cell removal are affected). Experimental models of pathological disorders have been shown to respond to this anti-inflammatory effect, including arthritis, sepsis, fulminant hepatitis, allogeneic hematopoietic cell engraftment, or acute myocardial infarction (see text). In contrast, apoptotic cell-induced tolerance is related to antigen (Ag)-specific regulatory cell induction. This antigen-specific tolerance may persist (according to the life span of the induced pTreg) and may not be restricted to the site of apoptotic cell clearance (depending on the migratory properties of the generated pTreg). Experimental models of pathological diseases, such as: type 1 diabetes, EAE, contact hypersensitivity, prevention or treatment of acute graft rejection, prevention of chronic graft rejection or of acute GvHD (aGvHD) have been shown to require infusion of apoptotic cells bearing specific auto- or donor allo-antigen(s). After iv infusion, splenic macrophages are rather involved in anti-inflammatory effect of apoptotic cells, whereas splenic lymphoid resident conventional DC are implicated in tolerance induction.