Intravenous injection of apoptotic leukocytes enhances bone marrow engraftment across major histocompatibility barriers

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Cross-tolerization of T lymphocytes after apoptotic cell uptake by dendritic cells may be involved in self-tolerance maintenance. Furthermore, immunosuppressive properties are attributed to apoptotic cells. This study evaluated the consequences of apoptotic leukocyte administration in a restrictive engraftment model of murine bone marrow (BM) transplantation. Sublethally irradiated recipients received a limited number of allogeneic BM, with or without irradiated apoptotic leukocytes of different origins. No graft-versus-host disease was observed. Whereas only a low proportion of mice receiving BM cells alone engrafted, addition of apoptotic irradiated leukocytes, independently of the origin (donor, recipient, third-party mice, as well as xenogeneic peripheral blood mononuclear cells), significantly enhanced engraftment. Similar results were obtained after infusion of leukocytes rendered apoptotic by UVB irradiation or by anti-Fas monoclonal antibody stimulation, thus confirming the role of apoptotic cells in engraftment facilitation. Overall, these results suggest that apoptotic leukocytes can nonspecifically facilitate allogeneic BM engraftment. Such a simple approach could be of interest in BM transplantation settings involving an important HLA donor/recipient disparity, a T-cell–depleted graft, or reduced conditioning regimen intensity. (Blood. 2001; 98:224-230)

Introduction

Allogeneic hematopoietic stem cell (HSC) transplantation is a major therapeutic option to treat malignant and hereditary hematologic diseases. However, the high curative potential as well as the expansion of such a treatment modality are limited by the high rate of immunologic complications. Such side effects are mainly due to the presence of immunocompetent cells of both host and donor origins and the consequent alloreactive conflict. Graft rejection is mainly mediated by recipient T lymphocytes that resist the conditioning regimen. The main factors influencing the engraftment are the type and intensity of the myeloablative treatment, graft characteristics (number of stem cells and donor T cells) as well as the magnitude of the major histocompatibility complex (MHC) disparity between the donor and the recipient. Prevention of graft rejection by the use of T-cell–containing grafts is unfortunately associated with a higher incidence of graft-versus-host disease (GvHD). This complication occurs after the recognition of host alloantigens by mature donor T lymphocytes present in the graft, in the context of inflammatory cytokine release. An effective way to prevent GvHD is to deplete the donor T-cell population present in the graft, which is, however, associated with increased malignancy relapse and graft rejection. Therefore, the development of new strategies to modulate alloreactivity in the setting of allogeneic HSC transplantation is necessary.

An alternative approach to T-cell depletion is to selectively eliminate alloreactive T cells while retaining T-lymphocyte subsets with other specificities (eg, antiviral). Recently, this interesting approach has been used to anergize allogeneic T lymphocytes by blocking the CD28 interaction with B7 molecules. In that study, all recipients engrafted and experienced a lower than expected incidence of GvHD after haploidentical HSC. In addition to anergic donor T lymphocytes, a high number of apoptotic (or committed to apoptosis) cells were injected, as indicated by the increase of the absolute number of CD3+ cells infused. These apoptotic cells are not immunologically inert. Immunosuppressive cytokine production by phagocytes having engulfed apoptotic cells have been described. Furthermore, it has been recently suggested that the permanent uptake of apoptotic cells in the periphery allows dendritic cells (DCs) to induce and maintain tolerance to self after migration to draining lymph nodes.

Using a restrictive engraftment model of murine bone marrow transplantation (BMT), we therefore decided to evaluate the consequences of apoptotic leukocyte administration on the engraftment of an allogeneic BM graft. We found that apoptotic leukocytes co-infused with BM cells have a graft-facilitating effect without causing GvHD. This effect was not restricted to the donor origin of the apoptotic cells, because recipient, third-party, as well as xenogeneic apoptotic leukocytes facilitated engraftment. The graft-facilitating effect observed after the injection of apoptotic cells suggested a hyporeactivity against allogeneic donor BM cells but not apoptotic cells. The use of irradiated apoptotic cells could...
be a relatively simple way to modulate alloreactivity in HSC transplantation settings such as an important HLA donor/recipient disparity, a T-cell–depleted graft, or reduced conditioning regimen intensity (eg, older patients, nonmalignant disorders, or tolerance induction for organ transplantation).

Materials and methods

Mice

Pathogen-free, male, 5- to 6-week-old FVB (H-2q), C57BL/6 (H-2b), and BALB/c (H-2q) mice were obtained from IFFA-Credo (L’Abresle, France) and kept in quarantine for at least 1 week before BM transplantation. Mice were given ad libitum access to food and water. Neomycin sulfate (1.1 g/L; Demavic, Longvic, France) was added to water from day -1 of bone marrow transplantation (BMT) to reduce the risk of infection.

Bone marrow transplantation

Donor bone marrow (BM) cells from C57BL/6 or FVB mice were flushed from tibiae and femora in RPMI 1640 (BioWhittaker, Verviers, Belgium). Donor spleens were removed and homogenized, and single cell suspensions were obtained. After erythrocyte lysis using a buffered ammonium chloride solution, BM cells and splenocytes (SCs) were resuspended in phosphate-buffered saline (PBS; BioWhittaker). Human peripheral blood mononuclear cells (PBMCs) were prepared from freshly collected blood from 4 different healthy donors after Ficoll (Sigma, Saint Quentin Fallavier, France) density gradient centrifugation, washed twice, and resuspended in PBS. After the isolation procedures, viability of BM cells, SCs, and PBMCs was always more than 90% by trypan blue dye exclusion. BALB/c and C57BL/6 mice, used as recipients, received a total body irradiation (TBI) 16 hours before BMT. A 6- or 7-Gy single dose TBI was applied with a dose rate of 2.7 Gy/minute. Recipients then received a single intravenous (in a lateral tail vein) injection (300 μL) containing BM cells with or without apoptotic leukocytes from different origins. No immunosuppressive agents were given.

Apoptosis induction

Apoptosis was induced either by γ- or UVB-irradiation or by exposure of activated SCs to a lytic Anti-Fas monoclonal antibody (mAb). SCs and PBMCs were adjusted to 2 × 10^6 cells/mL (in PBS) and submitted to γ-irradiation (40 Gy) 6 hours before injection of the mice to allow apoptotic changes to occur. Alternatively, SCs were dispersed into Petri dishes (100-mm diameter) and exposed to 200 J/m² UVB radiation (Sankyo Denki, Tokyo, Japan). For Fas-induced apoptosis, SCs were adjusted to 2 × 10^6 cells/mL and activated by the addition of 2.5 μg/mL concanavalin A (Con-A, Seromed, Berlin, Germany) and recombinant human interleukin-2 (IL-2; 500 IU/mL; Cetus, Rueil-Malmaison, France) in Dulbecco modified Eagle medium (BioWhittaker) culture medium supplemented with 10% heat-inactivated fetal calf serum (Boehringer Mannheim, Meylan, France), 5 × 10^{-3} M 2-mercaptoethanol, 2 mM L-glutamine, 100 IU/mL penicillin, and 100 μg/mL streptomycin (Sigma) (complete medium) and incubated at 37°C in a humidified atmosphere with 5% CO₂. After 3 days of activation, apoptosis was induced by the addition of a lytic anti-Fas mAb (1 μg/mL, clone Jo2; Pharmingen, San Diego, CA) during an additional culture period of 6 or 24 hours in the absence of IL-2. The cells were then washed in PBS and adjusted to the desired concentration before injection. At the time of injection, only a small proportion of the leukocytes were in an advanced death stage as determined by trypan blue exclusion (≤ 10%). No aggregation was observed.

Flow cytometry analysis for the detection of engrafment

Detection of the engrafment was performed between day 45 and day 50 post-BMT. H-2 phenotypes of SCs from recipient mice were determined by flow cytometry using fluorescein isothiocyanate (FITC)-labeled anti-H-2¹ (KH114, mouse immunoglobulin G2a [IgG2a]) and phycoerythrin (PE)-labeled anti-H-2² (SF1-1.1, mouse IgG2a) antibodies (Pharmingen). Analysis was performed on a FACS Calibur (Becton Dickinson, Mountain View, CA) using CellQuest software (Becton Dickinson). Engraftment was routinely evaluated in spleen cells and circulating leukocytes. In one experiment, engrafment was determined in additional immunologic sites: thymus, BM, and cervical lymph node. Engraftment was considered as positive if at least 15% of recipient cells had the BM donor H-2 phenotype.15 Engraftment in different lineage (lymphoid or myeloid) was determined using forward light scatter and side light scatter gating.

GVHD evaluation

In each experiment, GVHD was assessed by body weight loss (weekly) and skin lesions (daily). In killed animals, GVHD was also evaluated at day 45 to day 50 histologically as described.13 The following organs were systematically examined: stomach, small and large intestines, rectum, and skin (neck and abdominal wall) as targets for GVHD. Liver, heart, lungs, spleen, kidney, testis, and brain were evaluated for tissue injuries due to irradiation.

Apoptosis detection by Annexin-V staining

Following induction of apoptosis, SCs were resuspended at 2 × 10^6 cells/mL in complete medium and dispensed into 24-well plates. After different periods of culture at 37°C and 5% CO₂, 5 × 10^6 cells were harvested, washed twice in cold PBS, and resuspended in cold Annexin-V buffer (Immunotech, Marseille, France). The cells were then stained with FITC-conjugated Annexin-V (Immunotech) for 10 minutes and analyzed using a FACS Calibur flow cytometer. In some experiments, detection of secondary necrotic cells was assessed using propidium iodide (Sigma) staining.

Ex vivo splenocyte interferon-γ production in response to alloantigens

Specificity of the induced tolerance was determined by using a one-way mixed leukocyte reaction. At day 45 to day 50 post-BMT, splenic T cells from naive BALB/c and FVB mice, as well as recipient BALB/c mice were purified by passage through nylon wool columns (> 91% CD3⁺) and then used as responders (2 × 10^5/well in round-bottom 96-well plates) against γ-irradiated (20 Gy) mature DCs generated from BM (×10^5/well). Mature DCs were derived from naive C57BL/6 mice as described.14 Mixed T lymphocyte/DC cultures were maintained in complete medium at 37°C in 5% CO₂. Supernatants were collected from 48-hour cultures. Mouse interferon (IFN)-γ production was measured using an enzyme-linked immunosorbent assay kit (R&D Systems, Abingdon, United Kingdom) according to manufacturer’s instructions.

Statistical analysis

Statistical analysis was performed using SigmaStat and SigmaPlot software version 4.0 (Jandel Scientific, Erkrath, Germany). Chi-square and Fisher exact tests were used when indicated. P values < .05 were considered as statistically significant.

Results

Splenocytes irradiated at 40 Gy are apoptotic

Gamma-irradiation of murine SCs or human PBMCs leads to apoptotic death of such cells.20,15,17 Using FITC–Annexin-V staining and FACS analysis, we evaluated the kinetics of apoptosis induction by 40 Gy γ-irradiation in murine SCs. As soon as 2 hours after irradiation, 40% of irradiated cells were labeled by FITC–Annexin-V (Figure 1E), and by 6 hours after irradiation (when irradiated cells were injected in recipient mice), as much as 80% of cells were apoptotic (Figure 1F), indicating that a majority of irradiated SCs in our model were apoptotic at the time of injection.
Donor-irradiated apoptotic SCs enhance the engraftment of an allogeneic HSC graft

To study the host-versus-graft reactivity and modulation, we designed a restrictive murine BMT model. BALB/c mice (H-2d) were exposed to a 6-Gy TBI before receiving a limited number of 10^6 BM cells from FVB mice (H-2q). In this model, the low dose of irradiation allows the recipient immune system to reject the low number of allogeneic BM cells injected. An autologous hematopoietic recovery was observed, as only 11% of recipient mice engrafted under such conditions (Table 1). To evaluate the graft-facilitating potential of donor apoptotic SCs, 5 \times 10^6 irradiated SCs from FVB mice were added to the BM cell suspension. This addition of apoptotic cells resulted in a significant increase in the percentage of engrafted mice (49% versus 11%, P < .001; Table 1). Furthermore, a higher percentage of donor-type cells was found in the engrafted mice that received apoptotic SCs in both lymphoid and myeloid lineage (Figure 2 and Table 2). This favorable effect of donor apoptotic SCs was also observed when the number of BM cells was reduced (3 \times 10^5 instead of 10^6, P < .001; Table 1).

Donor apoptotic SCs were also capable of increasing the engraftment rate in another donor/recipient pair, in which only 3% of C57BL/6 (H-2b) mice irradiated at 7 Gy and injected with 3 \times 10^5 BM cells from FVB mice engrafted. This finding was in agreement with previous studies showing that donor apoptotic SCs enhance allogeneic engraftment.

### Table 1. Irradiated donor splenocytes co-infused with bone marrow cells enhance allogeneic bone marrow engraftment

<table>
<thead>
<tr>
<th>Recipient mice</th>
<th>BM cells</th>
<th>FVB (H-2b) irradiated SCs</th>
<th>Mice engrafted (H-2b phenotype) at day 45 to 50 post-BMT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 (H-2b)</td>
<td>3 \times 10^5</td>
<td>None</td>
<td>3 (1/28)</td>
</tr>
<tr>
<td></td>
<td>3 \times 10^5</td>
<td>5 \times 10^6</td>
<td>57 (15/26)*</td>
</tr>
<tr>
<td>BALB/c (H-2d)</td>
<td>3 \times 10^5</td>
<td>None</td>
<td>2 (1/44)</td>
</tr>
<tr>
<td></td>
<td>3 \times 10^5</td>
<td>5 \times 10^6</td>
<td>36 (17/44)*</td>
</tr>
<tr>
<td>BALB/c (H-2d)</td>
<td>10^6</td>
<td>None</td>
<td>11 (5/44)</td>
</tr>
<tr>
<td></td>
<td>10^6</td>
<td>5 \times 10^6</td>
<td>49 (25/51)*</td>
</tr>
</tbody>
</table>

BM, bone marrow; SCs, splenocytes; BMT, bone marrow transplantation.
*Pooled results of 3 to 5 independent experiments. Number of engrafted mice/number of analyzed mice at day 45 to day 50 post-BMT are indicated in parentheses.
†P < .001.
A recent report also described that engraftment of allogeneic BM cells plus 5 × 10^5 irradiated FVB SCs (P < .001; Table 1). Administration of irradiated FVB SCs alone (with no BM cells) to BALB/c mice did not result in donor hematopoietic reconstitution, therefore excluding the possibility that our results were due to the presence in the splenic cell suspension of HSCs resistant to γ-irradiation. Last, GVHD (clinically and histologically evaluated) was never observed after administration of irradiated SCs.

Graft-facilitating effect of irradiated SCs is not mediated by residual antirecipient cytotoxic activity retained by such cells.

To confirm that the apoptotic status of the administered SCs was indeed responsible for the observed graft-facilitating activity, alternative stimuli (lytic anti-Fas mAb Jo2 treatment and UVB-irradiation) were used to induce apoptosis. As shown in Figure 3, 80% and 100% of recipient mice that received BM cells plus donor SCs exposed to Jo2-mAb- and UVB-irradiated cells, respectively. In contrast, only 10% of recipient mice engrafted after administration of BM cells alone (P < .001; Figure 3). Furthermore, the addition of UVB-irradiated apoptotic SCs to BM cells also resulted in graft facilitation (40% engraftment versus 14% in the absence of apoptotic cells, P = .10). These results further support the demonstration that apoptotic cells have graft-promoting effects.

Apoptotic third-party SCs or xenogeneic human PBMCs retain graft-facilitating effects.

We then tested whether apoptotic SCs needed to be MHC-matched to BM cells or recipient mice to exert their graft-facilitating effect. We found that the addition of irradiated SCs from third-party C57BL/6 (H-2q) mice to FVB (H-2q) BM cells also increased the proportion of recipient BALB/C (H-2b) mice with FVB donor-type phenotype (Tables 2 and 3). To confirm that this effect was independent of MHC matching between BM cells and co-injected irradiated cells, a pool of γ-irradiated human PBMCs was infused with the BM cell suspension. The lack of influence of MHC specificity in this model was supported by the findings that co-injection of FVB BM cells plus a pool of 40 Gy irradiated human PBMCs similarly increased the proportion of recipient
BM cells + irradiated PBMCs

BM cells alone

% of mice engrafted

0 20 40 60 80 100 120

Figure 4. Graft-facilitating effect of irradiated human PBMCs. BALB/c (H-2d) mice were submitted to 6-Gy TBI and grafted 24 hours later with 10^6 BM cells from FVB (H-2b; panel A, 2 independent experiments with 12 mice per group) or C57BL/6 (H-2b; panel B, one experiment with 12 mice per group) mice alone or with 10^5 irradiated human PBMCs. Between day 45 and day 50 post-BMT, engraftment was measured by FACS analysis. Results are expressed as previously described in Figure 3. **P < .005.

Sublethally irradiated BALB/c (H-2d) recipient mice were grafted with 10^6 bone marrow (BM) cells from FVB (H-2b) mice alone, with irradiated (Irrd.) leukocytes from different origins or with a limited number (10^4 or 10^5) of viable donor T lymphocytes purified from spleen using nylon wool columns. Two concentrations of viable donor alloreactive T lymphocytes were shown as control: the highest one favored a persistent full-donor phenotype, the lowest concentration induced only a transient mixed chimerism. At day 45 to day 50 post-BMT, the percentage of donor cells (including both lymphocytes and granulocytes + monocytes) in the different immunologic organs was determined by flow cytometry. Results are from individual recipients.

Table 4. Irradiated leukocytes co-infused with bone marrow cells enhance the engraftment in different immunologic organs

<table>
<thead>
<tr>
<th>Cells added to BM</th>
<th>Thymus</th>
<th>Bone marrow</th>
<th>Lymph node</th>
<th>Spleen</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0, 0, 0, 0, 0</td>
<td>0, 0, 0, 0, 0</td>
<td>0, 0, 0, 0, 0</td>
<td>0, 0, 0, 0, 0</td>
</tr>
<tr>
<td>Irrd. FVB</td>
<td>100, 100, 100, 0, 0</td>
<td>100, 100, 98, 0, 0</td>
<td>100, 100, 100, 0, 0</td>
<td>100, 100, 100, 0, 0</td>
</tr>
<tr>
<td>Irrd. BALB/c</td>
<td>100, 0, 0, 0, 0, 0</td>
<td>100, 0, 0, 0, 0, 0</td>
<td>100, 0, 0, 0, 0, 0</td>
<td>100, 0, 0, 0, 0, 0</td>
</tr>
<tr>
<td>Irrd. BM</td>
<td>99, 100, 100, 100</td>
<td>98, 100, 100, 100</td>
<td>100, 100, 100, 100</td>
<td>100, 100, 100, 100</td>
</tr>
<tr>
<td>Viable T lymphocytes</td>
<td>90, 0, 0, 19, 9</td>
<td>0, 0, 0, 12, 9</td>
<td>0, 11, 15, 91, 33</td>
<td>0, 0, 0, 78, 33</td>
</tr>
<tr>
<td>10^4</td>
<td>100, 100, 100</td>
<td>100, 100, 100</td>
<td>100, 100, 100</td>
<td>100, 100, 100</td>
</tr>
</tbody>
</table>

Discussion

Increased use of allogeneic HSC transplantation is limited by the high toxicity of this approach. The complex immunologic setting of allogeneic HSC transplantation is due to the possibility of direct...
Figure 5. IFN production of ex vivo T lymphocytes in response to allogeneic third-party DCs. Purified spleen T lymphocytes were obtained from naive BALB/c (H-2b, black triangles), FVB (H-2b, open squares) mice, or BALB/c mice grafted with 10^6 BM cells from FVB mice plus apoptotic SCs of C57BL/6 (H-2b) mice (noted TP, black circles) at day 45 to day 50 post-BMT. Four individual mice were tested in each group. A bold bar indicates the mean for each group. P = .739.

and/or indirect presentation of allogeneic peptides by antigen-presenting cells (APCs) of donor and host origins to T cells of both origins. Interrelated consequences of this immunologic reactivity, such as graft rejection, GvHD, and leukemic relapse, significantly affect survival after HSC transplantation. The intensity of the pretransplant conditioning regimen has an important influence on the outcome of HSC transplantation, because of its toxicity and immunologic consequences. Reduction of the conditioning regimen is associated with increased graft rejection. In contrast, increased intensity of conditioning regimen can favor the GvHD occurrence, in part by the release of pro-inflammatory cytokines. Here we demonstrate that, in the context of suboptimal TBI the GvHD occurrence, in part by the release of pro-inflammatory cytokines such as transforming growth factor β (TGF-β) or IL-10 might be involved. Interestingly, phagocytosis of apoptotic cells by APCs can induce secretion of such cytokines by these cells. Furthermore, apoptotic cells can themselves produce high quantities of IL-10. Cross-reactivity because of amino acid sequence conservation between mouse and human TGF-β and IL-10 (nearly 100% and 73%, respectively) could at least in part explain our results with human irradiated PBMCs. We did not detect an up-regulation of IL-10 or TGF-β production by irradiated cells (results not shown), but we cannot exclude the possibility of immunosuppressive cytokine production after apoptotic cell phagocytosis in vivo.

Cells rendered apoptotic by different stimuli also had a graft-facilitating effect, implying that the apoptotic status of cells co-injected with BM cells plays a significant role in our findings. Therefore, an additional (and possibly synergistic) mechanism is the cross-tolerance of host antidonor T cells by APCs after a massive infusion of apoptotic cells. This process mimics the successive physiologic events limiting inflammation during the elimination of unwanted cells in tissue homeostasis. It has been shown that DCs can engulf, process apoptotic cells, and, under certain conditions, tolerate T cells, and, more specifically, induce alloantigen-specific hyporesponsiveness in vitro and in vivo. An immature DC subset trafficking through diverse tissues can continuously phagocytose cells undergoing normal turnover by apoptosis and then induce tolerance of autoreactive naive T cells in draining lymph nodes. One can therefore hypothesize that massive infusion of apoptotic leukocytes can overcome a DC stimulatory signal (inflammation induced by TBE31,34 with a tolerogenic signal favoring recipient antidonor T cells cross-tolerance. Whether this DC-mediated donor hyporeactivity would be mediated directly by donor DCs present in the BM or indirectly by recipient DCs is a question that remains to be answered.

A further explanation for these results is the potential immunosuppressive properties of MHC-derived peptides. Apoptotic cells co-injected with BM cells are a potential source of relative high quantities of MHC peptides. Peptides derived from the polymorphic or nonpolymorphic regions of MHC class I and II molecules have been described to possess immunomodulatory capacities both in vitro and in vivo. Human HLA-DQ1-derived peptide can indeed inhibit CD4+ lymphocyte rat alloimmune responses, supporting the immunomodulatory potential of xenogenic MHC peptides.

In conclusion, our findings show that it is possible to overcome the MHC barriers and to easily facilitate allogeneic HSC engraftment by the addition of apoptotic cells to the BM inoculum. Such an approach could be used to reduce the conditioning regimen intensity and its associated toxicity in some settings in which it is not desirable (ie, older patients, nonmalignant disorders). This approach would also permit the expansion of HSC transplantation to tolerance induction protocols for solid organ transplantation. Further studies are necessary to evaluate other important issues such as possible impairment of the graft-versus-leukemia response or triggering of autoimmunity after the infusion of high quantities of apoptotic cells in the setting of HSC transplantation.

Acknowledgments

We thank Dany Trestchenkoff for help in preparing the manuscript, Eric Robinet and David E. Chalmers for critical reading of the manuscript, and Dominique Paris for his expertise in animal care and management.
References


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