Autologous apoptotic cells preceding transplantation enhance survival in lethal murine graft-versus-host models

Mareike Florek, Emanuela I. Sega, Dennis B. Leveson-Gower, Jeanette Baker, Antonia M. S. Müller, Dominik Schneidawind, Everett Meyer, and Robert S. Negrin
Graft-versus-host disease (GVHD)
Medical cause

• Major cause of morbidity and mortality
  • After allogeneic transplantation
  • Not in autologous type
Bone marrow transplantation (BMT)

- Autologous bone marrow transplant
  - = self
  - Remove before treatment

- Allogeneic bone marrow transplant
  - = other
  - Good match between donor and recipient

- Umbilical cord blood transplant
  - Type of allogeneic transplant
  - Less need for perfect matching
Medical cause of GVHD

- Major cause of morbidity and mortality
  - After allogeneic transplantation
  - Not in autologous type
- Recipient has tissue antigens that are not present in the transplant donor
- Donor T cells recognize and respond to proteins on host cell
- Less occurrence when match is close
  - 30-40% in relatives
  - 60-80% in non relatives
Subtype of GVHD

• Acute GVHD (aGVHD)
  • Before day 100 post-transplant

• Chronic GVHD (cGVHD)
  • After day 100 post-transplant
Acute GVHD

• Symptoms include three main organs
  • Skin
  • Liver
  • Gastrointestinal tract

• Overall grades
  • I – mild
  • II – moderate
  • III – severe
  • IV – very severe
Chronic GVHD

• Major cause death after BMT
• Development can be progressive, quiescent or de novo
• Risk factors: age and history of aGVHD

• Symptoms
  • Dry mouth
  • Dry eyes, vision changes
  • Fatigue, muscle weakness
  • Skin rash with raised, discolored areas
  • Lung damage
  • Weight loss
  • Resemble autoimmune syndromes
Pathophysiology of GVHD

Three sequential steps

1. Activation of APCs
2. Donor T-cell activation, proliferation, differentiation and migration
3. Target tissue destruction
The third effector phase of the graft-versus-host process (figure 3) is a complex cascade of cellular mediators (such as cytotoxic T lymphocytes and natural killer cells) and soluble inflammatory agents (eg, TNFα, interferon γ, interleukin 1, and nitric oxide).

These molecules work synergetically to amplify local tissue injury and further promote inflammation and target tissue destruction. The cellular effectors of acute GVHD are mainly cytotoxic T lymphocytes and natural killer cells.

Cytotoxic T lymphocytes that prefer to use the Fas and FasL pathway of target lysis seem to predominate in GVHD liver damage (hepatocytes express large amounts of Fas) whereas cells that use the perforin and granzyme pathways are more important in the gastrointestinal tract and skin.

Chemokines direct migration of donor T cells from lymphoid tissues to the target organs in which they cause damage. Macrophage inflammatory protein 1α and other chemokines (such as CCL2–CCL5, CXCL2, CXCL9, CXCL10, CXCL11, CCL17, and CCL27) are overexpressed and enhance homing of cellular effectors to target organs during experimental GVHD.

Expression of integrins, such as α4β7 and its ligand MADCAM1, is also important for homing of donor T cells to Peyer’s patches during intestinal GVHD.

Microbial products such as lipopolysaccharide, which leak through damaged intestinal mucosa or skin, can stimulate secretion of inflammatory cytokines through Toll-like receptors. The gastrointestinal tract is especially susceptible to damage from TNFα, and the gastrointestinal tract has a major role in amplification and propagation of the cytokine storm characteristic of acute GVHD.

TNFα can be produced by both donor and host cells and it acts in three different ways: (1) it activates APCs and enhances alloantigen presentation; (2) it recruits effector cells to target organs via induction of inflammatory chemokines; and (3) it directly causes tissue necrosis (as its name suggests).

Prevention of GVHD
On the basis of evidence from animal models for the central role of T cells in initiation of GVHD, many clinical studies of T-cell depletion as prophylaxis for the disease were undertaken in the 1980s and 1990s. Three main depletion strategies were studied: (1) negative selection (1) Host APC activation; (2) Donor T-cell activation; (3) Cellular and inflammatory effectors.

Figure 3: Pathophysiology of acute GVHD, Lancet 2009; 373: 1550–61
IL 1=interleukin 1. IFN γ=interferon γ. LPS=lipopolysaccharide. Treg=regulatory T cell. Th1=T-helper 1 cell. CTL=cytotoxic T lymphocyte.
Involved cells in GVHD

• T-cell subsets
  • CD4+ Tcells & CD8+ Tcells

• T-regulatory cells (CD4+/CD25+ Treg)
  • Essential role in immune suppression following ECP

• Antigen presenting cells (APCs)
  • Main types: dendritic cells (DCs), macrophages, B cells
  • Initiation phase of aGVHD

• Natural killer cells
  • Mediated cell death by Fas-Fas-ligand-mediated apoptosis and perforin-granzime-B-mediated cytolyses

• NF-kB
  • Important for immune and inflammatory responses
  • Produce by activation of IL-1 and TNF-α
Prevention of GVHD

• Focus primarily on the effector phase
• Immunosuppressive drugs
  • Significant toxicity
  • Risk for opportunistic infections
• Donor T-cell depletion
• Increase of regulatory T cells (Tregs)
• Targeting antigen-presenting cells (APCs), especially Dendritic cells (DCs)
• Extracorporeal photopheresis (ECP)
Potential targets for cellular immunotherapies in GVHD
Extracorporeal photopheresis in GVHD

• Induction of immunotolerance without general immunosuppression
• No increase of opportunistic infections
• Low risk of side effects
• Procedure: Cells exposure with 8-Methoxypsoralen (8-MOP) and UV-A light
• Process causes cellular apoptosis
• Maintain of T- and B-cell responses to novel and recall antigens
Materials and methods

- BALB/c mice
- BM transplantation
- ECP treatment
- Cell isolation and flow cytometry reagents
- Phospho flow
- In vitro mixed lymphocyte reaction (MLR) culture
- In vivo Bioluminescent imaging (BLI)
- CFSE labeling and pulsed in vivo BrdU labeling
- Cytokine analysis
- Cytotoxicity assay and in vivo tumor model
- In vivo Bcl1 tumor model
Results
Fig. 1 Host-type apoptotic cells infused prior to transplantation prolong survival

A) C57B6 → Balb/c

B) C57B6 → Balb/c

C) AKR/J → BalbK

For comparative purposes, we injected donor-type ECP-treated splenocytes into recipients (Figure 2A). We next examined trogocytosis, a phenomenon characterized by cell-to-cell transfer of cell surface antigens (supplemental Figure 2). Because nuclear factor-κB (NF-κB) plays an important role in APC maturation, we addressed whether NF-κB expression is altered in host-type DCs and served as stimulators in an MLR. Allogeneic T-cell response to apoptotic cells was significantly lower expression in host DCs in ECP-treated mice as compared with untreated mice (Figure 2A). Furthermore, both the frequency of DCs and the uptake of host MHCII (IAd) but not donor MHCII (IAb) was significantly lower 3 days post-BMT in ECP-treated mice (Figure 2D). ECP-treated mice showed less weight loss associated with GVHD and a significant improvement in survival (Figure 1B). To exclude strain-specific effects, we also studied the effect of ECP treatment in a syngeneic model (Figure 1A). Together, the exposure to a tolerogenic environment from non-ECP-treated mice after allogeneic and syngeneic BMT from ECP-treated mice was significant.

Historically, in the absence of ECP, mice underwent BMT. ECP-treated mice had immune suppression, and diminished trogocytosis in donor T cells (supplemental Figure 1A, see supplemental Data available at the www.bloodjournal.org). The ECP-treated group similarly showed survival, 8 vs 37 days). Significance was assessed using the log-rank test.

To investigate whether the tolerance-inducing effect of apoptotic cells can be exploited to reduce GVHD, BALB/c recipients received 10^7 ECP-treated BALB.K cells (data not shown). When apoptotic cells were cleared from recipients 48 hours prior to BMT, survival was significantly improved (Figure 1B). BALB/c mice treated with ECP injection of ECP-treated BALB.K cells (data not shown). When apoptotic cells were cleared from recipients 48 hours prior to BMT, survival was significantly improved (Figure 1B). BALB/c mice treated with ECP injection of ECP-treated BALB.K cells (data not shown). When apoptotic cells were cleared from recipients 48 hours prior to BMT, survival was significantly improved (Figure 1B).
Figure 2. Uptake of apoptotic cells reduces NF-κB activation and costimulatory molecule expression in host DCs and diminishes MHCII uptake in donor T cells.
D  day+3

CD40

CD80

MHCII

WT, no Tx

Tcon

ECP

E

pLN

p = 0.006

p = 0.001

host MHCII on donor CD4+

donor MHCII on donor CD4+

31%

5%

69%

95%
host Tregs from ECP and untreated mice did not proliferate after hypothesized that the increase in host Tregs after BMT re
donor IL10 could contribute to ECP protection.

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BALB/c IL-10-KO were used as recipients (Figure 5E). These

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unclear.

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treatment.

Although we could not detect differences in serum

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accelerated death due to DT toxicity, however, the survival bene

failed to improve survival as compared with the Tcon group.

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To determine the functional impact of host Tregs, we applied

displayed a higher suppressive capability than WT Tregs in an MLR

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using the ECP approach was associated with a clear survival

was maintained in the ECP group. Although induction of Tregs

in strain as recipients (Figure 6F), where Foxp3

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on January 18, 2017.

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host Treg speci
Figure 4. CD4+ T cells in ECP-treated mice show reduced expression of homing and activation markers.

<table>
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<tr>
<th>Day 4</th>
<th>Spleen</th>
<th>pLN</th>
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**p-values:**
- Spleen: **p < 0.0001**
- pLN: **p = 0.0007**
- Spleen: **p = 0.004**
- pLN: **p = 0.0006**
- mLN: **p = 0.03**
Figure 5. ECP treatment reduces proinflammatory cytokine secretion in vitro and in vivo and requires host type IL-10 for its beneficial effect.

A  
*in vitro*

<table>
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<tr>
<th>Cytokine</th>
<th>Supernatants of MLR cultures</th>
<th>Purified DCs</th>
<th>Freshly isolated allogeneic Tcons</th>
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<tr>
<td>IFN-γ</td>
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<td>TNF-α</td>
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<tr>
<td>IL-2</td>
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B  
*in vivo*

<table>
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<tr>
<th>Cytokine</th>
<th>Serum of mice treated with ECP</th>
<th>Intracellular IFN-γ production</th>
<th>Serum analysis of IL-10</th>
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<tr>
<td>IFN-γ</td>
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Don, donor; Rec, recipient.
Figure 5. ECP treatment reduces proinflammatory cytokine secretion in vitro and in vivo and requires host type IL-10 for its beneficial effect.

(A) Proinflammatory cytokine secretion into supernatants of MLR cultures is reduced in cells cocultured with apoptotic cells. Purified DCs were cultured with or without apoptotic cells for 48 hours, stimulated with LPS (2 μg/mL) and cocultured with freshly isolated allogeneic Tcons. Supernatants were harvested after 96 hours.

(B) Proinflammatory cytokines are reduced in serum of mice treated with ECP, although statistical significance was only reached for INFγ and IL-2. Serum was obtained 5 days post-BMT.

(C) Intracellular INFγ production is reduced in mice treated with ECP 4 days after transplantation. Single-cell suspension from LN and spleen were restimulated for 5 hours with PMA and Ionomycin in the presence of Monensin.

(D) Serum analysis of IL-10 at day 15 shows marginal increase in ECP-treated group.

(E) Host-type IL-10 is required for beneficial effect of ECP treatment. WT BALB/c recipients received either WT donor C57BL/6 Tcon (WT-Tcon, WT-ECP) or IL10-deficient donor C57BL/6 Tcon (Don IL10<sup>2/-</sup> ECP) and IL10-deficient BALB/c recipient mice received WT C57BL/6 donor Tcon (Rec IL10<sup>2/-</sup> Tcon; Rec IL10<sup>2/-</sup> ECP). ECP was performed with WT BALB/c splenocytes. Rec IL10<sup>2/-</sup> ECP group showed no beneficial effect of ECP as compared with WT-Tcon or Rec IL10<sup>2/-</sup> Tcon group. Don IL10<sup>2/-</sup> Tcon and Don IL10<sup>2/-</sup> ECP showed accelerated death as compared with all other groups.

Results were done in triplicates (A) and are representative of 3 individual experiments or (B,D) are representative of 2 individual experiments with 10 mice per group or 4 mice per group (C) or are representative of 2 independent experiments with n = 5 mice per group (E). Don, donor; Rec, recipient.
Figure 6. ECP treatment induces host-type Foxp3^+ Tregs that substantially contribute to but are not solely responsible for improved outcome.
Preemptive ECP treatment induces host-type Foxp3+ Treg to responder ratios is shown, treatment specifically increases host-type Tregs in C57BL/6 transplanted mice received BrdU injection (1 mg per mouse per IP) every second day followed by pLN harvest at day 2. No proliferation of host-type Treg was observed in C57BL/6-Foxp3DTR recipients were injected with 50 μg/kg DT at day 6. Mice transplanted with FVB Tcon (Foxp3DTR Tcon, control for DT toxicity) 1 group received 50 μg/kg DT at day 2, and transplanted with FVB Tcon (Foxp3DTR Tcon, control for DT toxicity) 1 group received 50 μg/kg DT at day 6. Mice injected with DT showed exacerbated GVHD due to DT toxicity in all groups. ECP group Foxp3DTR-C57BL/6. WT-C57BL/6 recipients received FVB Tcon and followed by BLI imaging. Data are representative (A,B,C,F,G) of at least 2 individual experiments with 3 to 10 mice per group or (D) are a composite of 2 experiments with 10 mice per group. 

Tregs originated either from WT BALB/c (WT Treg) or from mice treated with ECP (ECP Treg). Recipients were transplanted with C57BL/6-Foxp3DTR recipients were injected with 50 μg/kg DT at day 6 and transplanted with FVB Tcon (Foxp3DTR Tcon, control for DT toxicity) 1 group received 50 μg/kg DT at day 2. Specific depletion of host Tregs prior to BMT in FVB Tregs that substantially contribute to but are not solely responsible for improved outcome.

1. From (C): Percentage of CD4+/Foxp3 in LPS and ECP conditions (p=0.037).
2. From (D): CTLA4 expression on CD4+ cells in LPS and ECP conditions (p=0.01).
3. From (D): CTLA4 expression on CD4+Foxp3+ cells in LPS and ECP conditions (p=0.03).

E: Proliferation capacity of Treg after BMT. Tregs from ECP-treated mice had significantly higher levels of CTLA4 expression within the CD4+ T-cell subpopulation, compared with untreated mice challenged with LPS only. (E) Treg from ECP-treated mice more actively suppressed T-cell proliferation than WT Tregs. 

- ECP-treated mice had significantly higher levels of CTLA4 expression.
- WT Tregs did not exhibit significant CTLA4 expression.
- Recipient mice received 10^7 T cells from mice in panel C were evaluated for surface CTLA4 expression.
- ECP-treated mice had significantly higher levels of CTLA4 expression within the CD4+ T-cell subpopulation.

- Recipient mice were transplanted with TCD-BM plus Tcon (left, middle). In mice receiving TCD-BM alone, host and donor-Treg proliferated (right).
- ECP increases Tregs within 48 hours.
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**Figure 6.** ECP treatment induces host-type Foxp3+ T-cell proliferation than WT Tregs.

Transplanted mice received BrdU injection (1 mg per mouse per IP) every second day followed by pLN harvest at day 2 and transplanted with FVB Tcon (Foxp3DTR Tcon, WT Tcon + DT, WT ECP + DT). Tcon and followed by BLI imaging. Data are representative (A,B,C,F,G) of at least 2 individual experiments with 3 to 10 mice per group, or (D) are a composite of 2 experiments with 10 mice per group.

From BALB/c. FACS gating strategy for Tregs isolated at day 1 (WT Tcon, ECP Treg, Tcon Foxp3DTR, ECP Foxp3DTR).

- **F**
  - **Graph**: Percent survival vs days after transplant.
  - **Legend**:
    - BM
    - WT Tcon
    - WT ECP
    - WT Tcon + DT
    - WT ECP + DT
    - Tcon Foxp3DTR
    - ECP Foxp3DTR

- **G**
  - **Graph**: Photon counts/second.
  - **Legend**:
    - Tcon
    - WT Treg
    - ECP Treg

**Note:**
- Mice injected with DT showed exacerbated GVHD due to DT toxicity in all groups.
Figure 7. GVT effect is maintained after ECP treatment.

(A) Specific killing of target (T) tumor cell lines A20 (left) and Bcl1 (right) by effector CD8+ T cells at an E:T ratio of 20:1 and 5:1. Specific killing occurred in ECP-treated (ECP) or nontreated (Tcon) mice 10 days after transplantation (day 32). (B) GVT effects in vivo are maintained in mice transplanted with IL-10 derived IL-10 to augment BM engraftment by ECP.
Summary of results I

• host-type ECP-treated cells prior to transplantation diminishes GVHD and significantly improves survival
• ECP treatment at day -5 did not improve outcomes
• Depends to apoptotic cells that reduce NF-kB and inhibit maturation of host DCs
• Inhibition of NF-kB activation in DCs reduce T-cell activation in GVHD
• Reduce donor T-cells contribute to reduce proinflammatory signals
Summary of results II

- Tregs increased in patients with acute GVHD when successfully treated with ECP
- Tregs inhibit T-cell activation by inhibition of CD28 signaling
- Increase suppressive capacity by expression of CTLA4
- IL10 are required in the recipient at the time of ECP
- lower MHCII expression on donor T cells in ECP-treated mice
- T- and B-cell responses to novel and recall antigens remained intact
- Prophylactic ECP delayed the induction of GVHD
Conclusion

• prophylactic ECP prior to BMT reducing transplant complications and improved survival in a murine BMT model

• ECP treatment is safer than many other immunosuppressive approaches

Critical review

• long term impact of ECP?

• Exact immunological effect of ECP?