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SIROLIMUS ENHANCES THE EFFECT OF APOPTOTIC CELL INFUSION ON HEMATOPOIETIC ENGRAFTMENT AND TOLERANCE INDUCTION

Letter to the Editor

Allogeneic hematopoietic cell transplantation (AHCT) is an efficient and widely used treatment for various hematological diseases. However, the potential benefit of AHCT may be offset by graft-versus-host disease (GVHD) as well as other transplant-related toxicities. The development of the so-called non-myeloablative or reduced intensity conditioning regimens (RICR) has widened the indications of AHCT to a broader spectrum of elderly patients usually not candidate for standard myeloablative AHCT and diseases other than acute leukemia.¹ However, and despite some initial enthusiasm,¹ GVHD remains a matter of concern after RICR AHCT. Moreover, highly immunosuppressive regimens associated with the use of RICR may significantly increase the rate of infectious complications and delay immune reconstitution.^{2,3} Alternative approaches to favor engraftment and limit long-term post-transplant immunosuppression are still needed.

Intravenous (i.v.) apoptotic cell infusion can modulate alloreactivity by interacting with particular dendritic cell subsets.⁴ We have previously established that i.v. apoptotic cell infusion facilitates engraftment –when administrated with allogeneic bone marrow (BM) transplants– without triggering autoimmunity or GVHD.⁵ This effect relies on a TGF- β -dependent mechanism.⁵ Furthermore, i.v. apoptotic cell infusion is associated with a CD4⁺CD25⁺ regulatory T cell (Treg) increase early after BM transplantation (BMT) (day 6 and 8).⁵ These Tregs expressed high levels of *Foxp3* transcripts and exerted a potent *ex vivo* suppressive activity through a cell-to-cell contact mechanism.⁵ Nonetheless, such results⁵ were achieved without administration of immunosuppressive drugs. In human settings, immunosuppressive treatments are mandatory, at least during the early phase of AHCT, until tolerance is fully established. Since immunosuppressive drugs are capable of interfering with long-term tolerance induction,⁶ we investigated whether the widely used immunosuppressive drugs (cyclosporin A [CsA],

mycophenolate mofetil [MMF], or sirolimus [SRL]) can modulate the effect of apoptotic cells co-administrated with a hematopoietic graft in a murine BMT model.

BMT was performed, as described,⁵ according to institutional guidelines and our local ethic committee. Six gray (Gy) sub-lethally irradiated BALB/c (H-2^d, Thy-1.2) recipient mice (Janvier, Le Genest Saint Isle, France) were grafted with allogeneic FvB (H-2^q, Thy-1.1) BM (10⁶ cells), with or without FvB apoptotic splenocytes (5.10⁶ cells; apoptosis was induced by a 40 Gy γ -irradiation),⁵ and received immunosuppressive treatment for the next 14 days.

Immunosuppressive drugs were administered intra-peritoneally as follows: CsA (Novartis Pharma, Rueil-Malmaison, France) 50 mg/kg/d from day 0 to 14,⁷ diluted in sterile saline solution; SRL (Wyeth Research, Monmouth Junction, NJ) 1.5 mg/kg/d from day 0 to 14, diluted in sterilized medium consisting of 160 mg of carboxy-methyl-cellulose (Sigma Aldrich, Saint Louis, MO) in 80 ml of sterile water,⁷ MMF (Roche, Mannheim, Germany) 20 mg/kg/d from day 0 to 14, diluted in sterile saline solution.⁶ These immunosuppressive regimens were selected as the standard ones used for *in vivo* immunosuppression in other mouse models.^{6,7} Engraftment was evaluated on peripheral blood cells by flow cytometry analysis (CyAn^{LX} cytometer, DakoCytomation, Fort Collins, CO) at day 30 post-BMT using H-2- or Thy1-specific antibodies (BD Biosciences PharMingen, Le Pont de Clay, France).⁵ Engraftment was considered to be achieved if at least 15% of recipient peripheral leukocytes had the BM donor H-2^q or Thy1.1 phenotype.⁵ After confirming the enhancing effect of apoptotic cell infusion on engraftment (40% of engrafted mice vs. 5% without apoptotic cell infusion, $p=0.02$), we investigated the interactions with the immunosuppressive drugs (Table 1). CsA treatment did not increase BM engraftment in mice receiving BM alone (14% of engrafted mice in the CsA group vs. 5% in the control group, $p=0.76$). However, in mice receiving both BM and apoptotic cells, CsA antagonized the favorable effect of apoptotic cells (6% of engrafted mice in the CsA group vs. 40% without CsA; $p=0.04$). Similarly to CsA, MMF did not significantly promote engraftment in mice receiving BM alone (7% of engrafted mice vs. 5% without MMF, $p=0.52$). However, and in

contrast to CsA, engraftment after BM and apoptotic cells infusion was comparable between recipients treated with MMF or not treated (40% vs. 40% of engrafted mice, $p=0.78$). On the other hand, SRL dramatically increased hematopoietic engraftment in the group of mice grafted with BM alone (93% engrafted mice vs. 5% without treatment, $p<0.0001$), as well as in the group grafted with BM and apoptotic cells (100% of engrafted mice vs. 40 % without treatment; $p<0.0001$). Engraftment was not significantly different between the group of mice grafted with BM alone (93% of engrafted mice) and those grafted with both BM and apoptotic cells (100% of engrafted mice, $p=0.78$) in the presence of SRL (Table 1).

In order to decipher a possible synergistic effect of SRL on apoptotic cell-induced engraftment, further experiments were performed with a lower number of BM cells ($5 \cdot 10^5$ cells/mouse).

Despite this low amount of BM cells, SRL treatment still allowed engraftment in 80% of recipient mice after infusion of BM alone (0% of engraftment in the absence of SRL, $p<0.0001$), and in 100% of recipients receiving BM plus apoptotic cells infusion (as compared to 25% without SRL, $p<0.0001$)(Table 1). Most importantly, the combination of such a low number of BM cells, SRL treatment, and apoptotic cell infusion resulted in a $93 \pm 2\%$ of circulating donor-derived cells in the peripheral blood 30 days post-transplantation (as compared to $57 \pm 11\%$ with BM alone and SRL treatment, $p<0.0001$, Table 1). To further appreciate the effect of SRL on apoptotic cell-induced engraftment, pre-transplant irradiation was reduced from 6 to 5 Gy. Similar results were obtained: after a 5 Gy conditioning regimen, SRL treatment allowed engraftment in 6 recipient mice out of 10 after infusion of BM cells alone whereas addition of apoptotic cells to the graft induced engraftment in all the mice. Thus, SRL exerts an additional beneficial effect on apoptotic cell-induced engraftment.

To appreciate the impact of immunosuppressive drugs on apoptotic cell-induced engraftment, we analyzed splenic T-cell subsets in recipient mice at day 15 (i.e., the end of immunosuppressive treatment). No significant difference was observed in CD4/CD8 ratio whatever the analyzed group (Table 1). Treatment with CsA –but not with other drugs–

significantly decreased the absolute numbers of CD4 and CD8 T cells in the spleen of mice receiving BM and apoptotic cells (Table 1). Since i.v. infusion of apoptotic cells in addition to a BM graft can induce a Treg increase in the spleen,⁵ we therefore asked whether Treg expansion after apoptotic cell infusion may be influenced by exposure to immunosuppressive drugs. Splenic Tregs (defined as CD4⁺CD25⁺Foxp3⁺ T cells) were evaluated by cytometry using anti-Foxp3 mAb (FJK-16s eBioscience, San Diego, CA) at day 15 post-BMT. CsA treatment resulted in a significant decrease of splenic Tregs after apoptotic and BM cell infusion (2.4%±0.3 % vs. 5.3±0.6% of CD4⁺CD25⁺Foxp3⁺ T cells without CsA, $p<0.001$). MMF did not significantly modify apoptotic cell-induced splenic Tregs (6.7±0.7% of CD4⁺CD25⁺Foxp3⁺ T cells). In contrast, SRL treatment combined with apoptotic cell administration, significantly increased splenic Tregs (7.5±0.6 vs. 5.3±0.6%, [$p=0.013$] of CD4⁺CD25⁺Foxp3⁺ T cells) (Figure 1 A-B).

The above results prompted us to analyze long-term engraftment and tolerance to donor BM allo-antigens. This was assessed by a skin graft model. Full-thickness skin grafts (approximately 1 cm², from the tail) obtained from FvB (BM donor) and C57BL/6 (third-party, H-2^b) donor mice were grafted on the lateral thorax of BM (with or without apoptotic cells) recipient BALB/c mice and secured with a bandage for 10 days. Grafts were then monitored daily for the first 2 weeks, then twice a week. Rejection was defined as the complete loss of viable donor graft tissue. Skin graft tolerance was found to be closely linked to BM engraftment (Figure 2). Recipient mice treated with CsA and receiving BM cells alone or with apoptotic cells quickly rejected BM donor origin skin graft in the same way as naive mice. In contrast, BM engrafted mice that received BM and apoptotic cells and MMF (n=5) or SRL (n=10) or without immunosuppressive regimen (n=15), tolerated skin grafts from BM donor origin for up to 400 days (Figure 2). These mice kept the ability to reject a C57BL/6 third party skin graft (Figure 2), suggesting a long-term tolerance to BM donor Allo-Ag, when MMF or SRL were used as a transient immunosuppressive regimen together with apoptotic cell infusion.

The use of donor apoptotic cell infusion can be proposed as an "alternative" cell-based approach to favor hematopoietic engraftment after RICR, modulate alloreactivity and limit excessive post-transplant immunosuppression. Here, we studied the effect of the most commonly used immunosuppressive drugs on i.v. apoptotic cell infusion-induced engraftment. Our results establish that immunosuppressive drugs such as the calcineurin inhibitor CsA, commonly used in clinical transplantation, can interfere with apoptotic cell infusion-induced engraftment and long-term tolerance. In contrast, other drugs, such as SRL, can exert a positive effect, adding to the growing evidence on the differential impact of immunosuppressive drugs on tolerance induction.^{6, 7}

Induction of T cell tolerance requires TCR triggering.⁸ We have previously shown that, i.v. apoptotic cell infusion induces a TGF- β -dependent increase of Tregs.⁵ Addition of CsA after apoptotic and BM cell infusion resulted in a significant decrease of splenic Tregs, suggesting that CsA may prevent TGF- β -dependent conversion of Tregs induced by apoptotic cells. Moreover, CsA-dependent inhibition of IL-2 production may also interfere with apoptotic cell-induced Treg expansion, since IL-2 is critical for the development and maintenance of Tregs *in vivo*.⁷ In contrast to CsA, SRL –targeting the mammalian Target of Rapamycin (mTOR) and acting downstream the IL-2 receptor– enhances Treg expansion,⁷ thereby amplifying the tolerogenic environment generated by apoptotic cell infusion at the time of BMT. Our current findings highlight a novel role for SRL in the apoptotic cell infusion-induced modulation of alloreactivity.

From the practical standpoint, our findings constitute a step forward towards the use of i.v. donor apoptotic cell infusions to enhance engraftment in various clinical settings. Although CsA may exert a beneficial effect on GvHD, it is likely that CsA should be avoided with apoptotic cell infusion, as it may abrogate its favorable effect. Alternatively, MMF, which is commonly administrated in clinical practice for calcineurin inhibitor-intolerant hematopoietic cell recipients,⁹ can be employed without interference with the action of apoptotic cells. Most interestingly, and

despite some adverse effects,¹⁰ SRL-based immunosuppressive regimens that are increasingly used in the solid transplant field can probably represent an attractive setting for the design and testing of tolerance induction in conjunction with donor apoptotic cell infusion. Moreover, i.v. infusion of apoptotic cells may mimic extracorporeal photochemotherapy (ECP), a therapeutic approach used to treat severe chronic or acute GvHD. Indeed, significant numbers of apoptotic leukocytes are generated post-ECP prior their re-infusion.¹¹ In addition, Treg have been shown to be induced after ECP in a murine model.¹² Whether CsA may interfere with ECP-induced Treg is an interesting question to address. Finally, based on the data reported here, SRL-based immunosuppressive regimens can be proposed as an interesting way to favor hematopoietic engraftment after RICR.

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References

1. Mohty M, Nagler A, Killmann NM. Reduced-intensity conditioning allogeneic stem cell transplantation: hype, reality or time for a rethink? *Leukemia* 2006; **20**: 1653-4.
2. Maris M, Boeckh M, Storer B, Dawson M, White K, Keng M et al. Immunologic recovery after hematopoietic cell transplantation with nonmyeloablative conditioning. *Exp Hematol* 2003; **31**: 941-952.
3. Maris MB, Niederwieser D, Sandmaier BM, Storer B, Stuart M, Maloney D et al. HLA-matched unrelated donor hematopoietic cell transplantation after nonmyeloablative conditioning for patients with hematologic malignancies. *Blood* 2003; **102**: 2021-2030.
4. Morelli AE. The immune regulatory effect of apoptotic cells and exosomes on dendritic cells: its impact on transplantation. *Am J Transplant.* 2006; **6**: 254-61.
5. Kleinclauss F, Perruche S, Masson E, de Carvalho Bittencourt M, Biichle S, Remy-Martin JP et al. Intravenous apoptotic spleen cell infusion induces a TGF-beta-dependent regulatory T-cell expansion. *Cell Death Differ* 2006; **13**: 41-52.
6. Blaha P, Bigenzahn S, Koporc Z, Schmid M, Langer F, Selzer E et al. The influence of immunosuppressive drugs on tolerance induction through bone marrow transplantation with costimulation blockade. *Blood* 2003; **101**: 2886-2893.
7. Zeiser R, Nguyen VH, Beilhack A, Buess M, Schulz S, Baker J et al. Inhibition of CD4+CD25+ regulatory T-cell function by calcineurin-dependent interleukin-2 production. *Blood* 2006; **108**: 390-399.
8. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N et al. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 2003; **198**: 1875-1886.
9. Dvorak CC, Callard E, Agarwal R. Use of intravenous mycophenolate mofetil for graft-versus-host disease prophylaxis in an allogeneic hematopoietic stem cell transplant recipient with an allergic reaction to cyclosporin and tacrolimus. *Bone Marrow Transplant* 2006; **38**: 253-254.

10. Cutler C, Henry NL, Magee C, Li S, Kim HT, Alyea E, et al. Sirolimus and thrombotic microangiopathy after allogeneic hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* 2005; **11**: 551-557.
11. Plumas J, Manches O, Chaperot L. Mechanisms of action of extracorporeal photochemotherapy in the control of GVHD: involvement of dendritic cells. *Leukemia* 2003; **17**: 2061-2.
12. Maeda A, Schwarz A, Kernebeck K, Gross N, Aragane Y, Peritt D, et al. Intravenous infusion of syngeneic apoptotic cells by photopheresis induces antigen-specific regulatory T cells. *J Immunol* 2005; **174**: 5968-76.

TABLE 1. Effect of immunosuppressive drugs on hematopoietic engraftment and on spleen T cell subsets after simultaneous infusion of BM and apoptotic cells

BMT (6 Gy γ -irradiation)	IST	% of engrafted mice (n) *	p value [#]	% donor derived cells in engrafted mice [§] (mean \pm SEM [range])	CD4/CD8	CD4 T cells (x 10 ⁶)	CD8 T cells (x 10 ⁶)	CD4 T cell subsets [£] (%): naive memory activated		
BM 5.10 ⁵	none	0% (0/20)		0	ND	ND	ND	ND	ND	ND
BM 5.10 ⁵ +Apo	none	25% (5/20)		67 \pm 14 [27–98]	ND	ND	ND	ND	ND	ND
BM 10 ⁶	none	5% (1/20)		79	1.0 \pm 0.3	6.8 \pm 1	6.8 \pm 0.4	ND	ND	ND
BM 10 ⁶ +Apo	none	40% (10/25)		85 \pm 6 [15–100]	2.4 \pm 0.9	11.0 \pm 1.3	6.1 \pm 1.3	25 \pm 5	21 \pm 4	50 \pm 5
BM 10 ⁶	CsA	14% (2/14)	0.76	71.5 \pm 26.5 [45–98]	3.6 \pm 1.4	7.4 \pm 0.4	2.3 \pm 0.3	ND	ND	ND
BM 10 ⁶ +Apo	CsA	6% (1/16)	0.04	100	4.6 \pm 2.3	6.2 \pm 1.3 [‡]	1.9 \pm 0.5 [‡]	21 \pm 5	15 \pm 3	62 \pm 9
BM 10 ⁶	MMF	7% (1/14)	0.52	54	1.5 \pm 0.6	10.1 \pm 1.3	6.9 \pm 0.5	ND	ND	ND
BM 10 ⁶ +Apo	MMF	40% (6/15)	0.78	87 \pm 7 [60–100]	1.6 \pm 0.9	7.6 \pm 1.2	5.6 \pm 1.2	27 \pm 7	26 \pm 8	45 \pm 13
BM 10 ⁶	SRL	93% (13/14)	<0.0001	84 \pm 4 [55–98]	2.9 \pm 1	9.3 \pm 0.7	3.9 \pm 0.7	ND	ND	ND
BM 10 ⁶ +Apo	SRL	100% (25/25)	<0.0001	80 \pm 3 [46–98]	2.7 \pm 1	10.9 \pm 1.3	4.4 \pm 0.5	33 \pm 8	21 \pm 2	42 \pm 8
BM 5.10 ⁵	SRL	80% (8/10)	<0.0001	57 \pm 11 [19–82] [†]	ND	ND	ND	ND	ND	ND
BM 5.10 ⁵ +Apo	SRL	100% (10/10)	<0.0001	93 \pm 2 [86–98] [†]	ND	ND	ND	ND	ND	ND

Abbreviations used: Apo, donor apoptotic splenocytes; IST, Immunosuppressive treatment; ND, not determined. *Engraftment was determined by flow cytometry analysis in peripheral blood 30 days after BMT as described in the text. [#]p values obtained when compared with the same group without IST. [§]% of donor-derived cells detected by cytometry. [†]p<0.001. T cell subsets in the spleen (mean \pm SEM) were analyzed in 8-9 mice/group. [‡]p<0.05 when compared with no IST. [£]naive (CD44^{low}/CD62L^{high}), activated (CD44^{high}/CD62L^{high}), memory (CD44^{high}/CD62L^{low}) CD4 T cells were determined at day 15 using CD62L (Mel-14) and CD44 (IM7, BD Biosciences PharMingen) expression. Student's t-test was used for group comparison. Pooled results from 2 to 4 independent experiments.

FIGURE LEGEND

Figure 1. Effects of i.v. donor apoptotic cell infusion and immunosuppressive drugs on Foxp3⁺ Tregs.

BALB/c mice were sub-lethally irradiated (6 Gy) and grafted with 10⁶ FvB BM cells with or without 5x10⁶ FvB apoptotic cells. CD4⁺CD25⁺Foxp3⁺ T cells were determined by flow cytometry in the spleen of recipient mice at day 15 (the day after the last infusion of immunosuppressive drug). (A) CsA inhibits i.v. apoptotic cell infusion-induced Tregs. Recipients grafted with BM cells plus apoptotic cells were treated for 14 days with the following immunosuppressive drugs: CsA (■, n=12), MMF (Δ, n=9), SRL (●, n=9). Mice that received BM plus apoptotic cells were used as controls (▲, n=12). Pooled results of 2 to 4 independent experiments are expressed as mean ± SEM of the percentage of CD4⁺CD25⁺Foxp3⁺ cells among CD3⁺ T cells. Bars correspond to the mean value of each group. (B) Representative dot plots showing CD25 and Foxp3 expression in the spleen of BALB/C mice on day 15 after BMT, having received BM cells plus apoptotic cells (BM + Apo) in conjunction with CsA, MMF or SRL. Note that CD3⁺CD4⁺CD25⁺Foxp3⁻ cells are only detected in mice that did not receive immunosuppressive drugs. Student's t-test was used for group comparison.

Figure 2. Effects of i.v. donor apoptotic cell infusion and immunosuppressive drugs on long-term tolerance to donor bone marrow allo-antigens.

Sub-lethally irradiated BALB/c recipient mice received an FVB BM allograft, with donor apoptotic leukocytes. Engrafted (■□) and non-engrafted (▲Δ) BALB/c mice given donor FVB apoptotic and BM cells with or without immunosuppressive drugs were grafted with a donor (FVB, ▲■) and a third party (C57BL/6, □Δ) skin graft 30 day post-BMT. Rejection was defined as the complete necrosis of graft tissue. (A) Engrafted BALB/c recipients after donor BM and apoptotic cell infusion tolerated a skin graft provided by BM donors (■), while retaining the capacity to reject a third-party skin graft (□). In contrast, non engrafted BALB/c mice rejected both donor (▲)

and third party (Δ) skin graft. n = 15 mice/group from 3 independent experiments. **(B)** CsA prevents long-term tolerance to donor BM skin graft. The two mice (\blacksquare) considered to be engrafted at day 30 post-BMT rejected their skin grafts in the same way as non engrafted BALB/c mice (n= 5; \blacktriangle). Hematopoietic engraftment after CsA treatment was transient and lost at the time of skin graft, since donor-derived cells were not detectable at time of donor skin graft rejection in the 2 mice considered to be engrafted. In contrast, MMF **(C)** (n = 5 mice/group) or SRL **(D)** (n= 10 mice/group) treatment does not alter long-term tolerance to donor BM skin graft. Survival data were analyzed using Kaplan-Meier survival analysis.

Figure 1

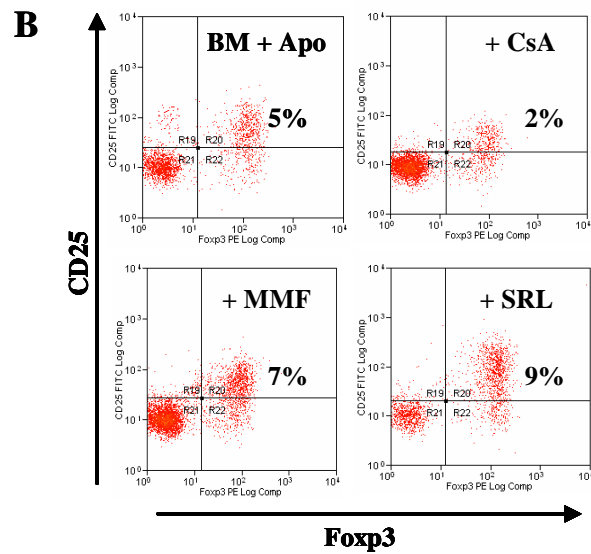
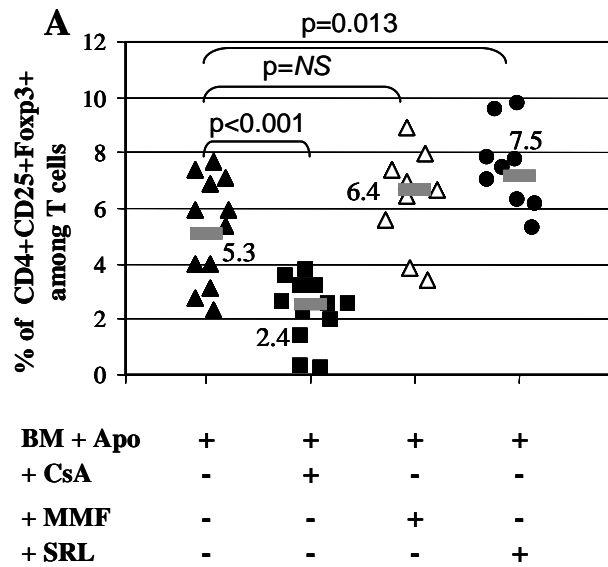


Figure 2

