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ORIGINAL ARTICLE

Flt3L-mobilized dendritic cells bearing H2-K^{bm1} apoptotic cells do not induce cross-tolerance to CD8$^+$ T cells across a class I MHC mismatched barrier

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Summary

Tolerization of allogeneic CD8$^+$ T cells is still a pending issue in the field of transplantation research to achieve long-term survival. To test whether dendritic cells (DC) bearing allogeneic major histocompatibility complex (MHC) class I mismatched apoptotic cells could induce cross-tolerance to alloreactive CD8$^+$ T cells, the following experimental strategy was devised. Rag2/γ_c KO B6 mice were treated with Fms-like tyrosine kinase 3 ligand (Flt3L)-transduced B16 melanoma cells to drive a rapid expansion and mobilization of DC *in vivo*. Of all DC populations expanded, splenic CD11c$^+$CD103$^+$CD8α^+ DC were selectively involved in the process of antigen clearance of X-ray irradiated apoptotic thymocytes *in vivo*. Considering that CD11c$^+$CD103$^+$CD8α^+ DC selectively take up apoptotic cells and that they are highly specialized in cross-presenting antigen to CD8$^+$ T cells, we investigated whether B6 mice adoptively transferred with Flt3L-derived DC loaded with donor-derived apoptotic thymocytes could induce tolerance to bm1 skin allografts. Our findings on host anti-donor alloresponse, as revealed by skin allograft survival and cytotoxic T lymphocyte assays, indicated that the administration of syngeneic DC presenting K^{bm1} donor-derived allopeptides through the indirect pathway of antigen presentation was not sufficient to induce cross-tolerance to alloreactive CD8$^+$ T cells responding to bm1 alloantigens in a murine model of skin allograft transplantation across an MHC class I mismatched barrier.

Introduction

Dendritic cells (DC) constitute a heterogeneous population of professional antigen presenting cells that derive from multiple different lineages and have the potential to prime efficiently naïve T cells with two distinct outcomes: the induction of tolerance or the generation of a protective immune response [1–3]. Apoptotic cells resulting from cellular turnover in the peripheral tissues are an excellent source of self-antigen that can potentially induce autoimmune disorders if they are not efficiently cleared. On the other hand, auto-reactive T cells that escape thymic deletion need to be silenced in the periphery to prevent undesirable autoimmune responses. In the absence of acute inflammation or infection, immature DC traffic through tissues, take up apoptotic cells resulting from cellular turnover and migrate constitutively via lymphatic vessels to lymph nodes (LN) to silence autoreactive T cells through the induction of anergy/deletion or by promoting the development of regulatory T cells [4–8].

Intravenous administration of donor-derived cells undergoing early apoptosis has been successfully used to deliver the entire repertoire of donor alloantigens together with immuno-regulatory signals to recipient's DC without causing their activation [9]. This strategy enhances hematopoietic cell engraftment [10], prolongs heart survival [11–13] and delays the onset of graft versus host disease [14].

The low numbers of DC that can be isolated from mouse spleen often limit their use for *in vivo* functional studies. For that reason, several groups have made use of the hematopoietic growth factor Fms-like tyrosine kinase 3 ligand (Flt3L) to expand the absolute number of peripheral DC in mice and humans [15,16]. It has been reported that Flt3L-mobilized DC display low levels of costimulatory molecules and therefore they are able to foster tolerogenic responses following mucosal administration of antigen [17] and to delay allograft rejection in distinct transplantation settings [18,19].

As $CD11c^+CD103^+CD8\alpha^+$ DC is the main subset involved in the uptake of apoptotic bodies [20] and exhibit a specialized and efficient function in presenting antigen to $CD8^+$ T cells [21–25], we hypothesized that targeting allogeneic apoptotic bodies to this antigen presenting cell type would lead to tolerization of alloreactive $CD8^+$ T cells. With this aim, we designed an experimental skin graft transplantation setting across an major histocompatibility complex (MHC) class I mismatched barrier (bm1 into C57Bl/6), in which rejection is mediated by alloreactive $CD8^+$ T cells, to investigate the role of Flt3L-mobilized splenic DC loaded with allogeneic apoptotic cells in the induction of cross-tolerance to alloreactive $CD8^+$ T cells by the indirect pathway of antigen presentation [26–28]. We used Rag2/γ chain double knockout (Rag2/γ_c KO) mice, which lack B and T lymphocytes as well as NK cells. These mice were treated with Flt3L to expand DC numbers greatly for functional *in vitro* and *in vivo* assays. Flt3L treatment expands massively different populations of DC including $CD11c^+CD103^+CD8\alpha^+$ DC, which were the DC population involved in the process of clearance of syngeneic (B6) and allogeneic (bm1) apoptotic cells *in vivo*. Despite $CD11c^+CD103^+CD8\alpha^+$ DC selectively take up syngeneic and allogeneic apoptotic cells, presentation of donor-derived peptides through the indirect pathway did not promote donor-specific cross-tolerance to allogeneic $CD8^+$ T-cell recognizing K^{bm1}-derived peptides in an MHC class I mismatched skin graft transplantation model.

Taken together, our results indicate that allopeptides generated from donor-derived apoptotic cells presented through the indirect pathway of allorecognition to host alloreactive $CD8^+$ T cells were insufficient to abrogate $CD8^+$ T cell alloresponses against bm1 antigens expressed on skin allografts transplanted across an MHC class I mismatched barrier.

Material and methods

Mice

Eight- to twelve-week-old C57Bl/6 ($H2\text{-}K^b$, B6) mice were purchased from Charles River (Barcelona, Spain). B6.C-H-2^{bm1}/By ($H2\text{-}K^{bm1}$, from now on bm1 mice) and Rag2/γ_c KO mice in B6 background were obtained from The Jackson Laboratory. Mice were bred at the Central Animal Facility of the University of León (Spain). All experiments with rodents were handled and cared for in accordance with the Ethical Committee for Animal Research of the School of Veterinary Medicine (University of León) and the European Guidelines for Animal Care and Use of Laboratory Animals.

In vivo expansion of DC in Flt3L-treated Rag2/γ_c KO mice

The Flt3L-transduced B16 melanoma cells were cultured in complete medium (RPMI 1640, 10% FCS, 2 mM L-Glutamine, 1 mM sodium piruvate, 10 mM HEPES, 50 μg/ml Gentamycin and 5×10^{-5} M β-mercaptoethanol) and 1×10^6 cells were subcutaneously (s.c.) injected into Rag2/γ_c KO mice [29]. After 10–12 days, mice were sacrificed and the spleens were cut into small fragments and digested into single-cell suspensions using phenol red free RPMI 1640 containing 25 μg/ml DNAse I (Roche, Mannheim, Germany), 10% FCS, 25 mM HEPES and 500 μg/ml collagenase A (Roche) for 40 min at 37 °C, as previously described [22,30]. Collagenase-digested spleens were filtered through a nylon mesh and red cells were subsequently lysed in ACK buffer (Bio-Whittaker, Walkersville, MO, USA).

Flow cytometry

CD11c (HL3), CD11b (M1/70.15), CD8α (53-6.7), CD103 (M290, 2E7), B220 (RA3-6B2), Ly6C (AL-21), CD80 (16-10A1), CD86 (RMMP-1), CD40 (HM40-3), MHC class II (AF6-120.1, IA^b), CD205 (DEC-205), ICOS-L (HK5.3), BTLA (4G12b, [31]), PD-L1 (MIH3), PD-L2 (TY25), B7x (clone 9), 4-1BBL (TSK-1), CD83 (Michel-19), Tim-3 (B8.2C12), PD-1 (4F10, [32]), OX40 (OX-86), CD137 (17B5), CCR7 (4B12) and rat isotype control (R35-95) mAbs were purchased from Biolegend or BD and used in this study. Anti-$H\text{-}2K^b$ (5F1.2.14) mAb, which binds to $H\text{-}2K^b$ but does not detect $H\text{-}2K^{bm1}$ variant, was used to discriminate between B6 ($H\text{-}2K^b$) and bm1 ($H\text{-}2K^{bm1}$) cells [33]. Fc receptors were blocked by incubating cell suspensions with 2 μg/ml of blocking anti-FcγR mAb (2.4G2) to reduce nonspecific binding before adding the specified mAbs [34]. Dead cells and debris were excluded from acquisition by staining with 4′, 6′-diamido-2-phenylindole hydrochloride (DAPI). Flow cytometry acquisition was conducted on a Cyan 9 cytometer (Beckman Coulter, Miami, FL, USA) and data analysis was performed using WinList version 6.0 (Verity Software House, Topsham, ME, USA).

Adoptive transfer of CFSE-labeled apoptotic cells into Rag2/γ KO mice

For the induction of apoptosis, B6 and bm1 mice were X-ray irradiated (9 Gy) (Maxishot 200, YXLON International, Denmark) and thymocyte suspensions were minced through a nylon mesh and labeled with CFSE (Molecular Probes). Briefly, cells were incubated in Dulbecco's PBS (D-PBS) containing 5 μM CFSE at 37 °C for 10 min. The reaction was stopped by adding two volumes of cold RPMI 1640 containing 10% FCS followed by two washes in D-PBS. For adoptive transfers, $80–100 \times 10^6$ CFSE-labeled B6 or bm1 thymocytes undergoing apoptosis were injected into the tail vein of Rag2/γ_c KO recipients. Eleven hours later, recipient mice were sacrificed and splenic CFSE-labeled B6 or bm1 DC were isolated and analyzed by FACS.

Skin graft transplantation

Allogeneic skin graft transplantation and transplantation surgery were carried out as previously described [35]. Briefly, B6 or bm1 skin graft beds were prepared on the posterolateral thorax of B6 recipients under ketamine/xylazine anesthesia. Grafts were covered with Vaseline gauze and a Band-Aid, which were removed on day 8. Grafts were considered rejected when less than 10% of the graft bed contained viable skin.

In vivo cytotoxic T lymphocyte (CTL) assay

Spleens from B6 and bm1 mice were harvested and single-cell suspensions were prepared in RPMI 1640 complete medium. Target cells were washed twice in D-PBS and labeled with CFSE at 5 μM (B6) or 1 μM (bm1) for 10 min at 37 °C. The reaction was stopped by adding two volumes of cold RPMI 1640 containing 10% FCS followed by two washes in D-PBS. $20–30 \times 10^6$ of each target cell (B6 and bm1 cells) were mixed at 1:1 ratio and were subsequently intravenously (i.v.) injected into B6 recipients at day 25 postskin graft transplantation. Forty-eight hours later, recipient mice were euthanized and specific killing of target cells was monitored in host spleen and pLNs (pool of axillar and inguinal LN). The percentage of specific target lysis was calculated by comparing the survival of each target population with the survival of syngeneic population according to the following equation: % specific killing of target cells = 100 − [(% of target population in experiment/% of syngeneic population in experiment)] × 100 [36,37].

Statistical analysis

Comparisons of continuous variables between groups and statistical significance were assessed using the parametric unpaired two-tailed Student's *t*-test. Results are expressed as mean ± SD. A value of $P < 0.05$ was considered statistically significant.

Skin graft survival was calculated by using the Kaplan–Meier life table method and statistical analysis for the comparison of the survival curves was performed by the log rank test. The statistical analysis was performed using Graphpad Prism 5.0 software (Graphpad Software, Inc, Lo Jolla, CA, USA).

Results

Experimental design

To test whether antigen presentation by syngeneic immature DC loaded with allogeneic MHC class I mismatched apoptotic bodies could induce cross-tolerance to allogeneic $CD8^+$ T cells through the indirect pathway, the following experimental strategy was designed. 1×10^6 Flt3L-transduced melanoma cells were s.c. injected into Rag2/γ_c KO B6 recipients. Ten to twelve days later, mice were i.v. injected with either irradiated (9 Gy) syngeneic (H2-K^b, B6) or allogeneic (H2-K^{bm1}, bm1) apoptotic thymocytes, as described in Material and Methods section. Eleven hours later, Flt3L-expanded Rag2/γ_c B6 KO DC bearing donor-derived B6 or bm1 allopeptides were adoptively transferred into B6-naïve mice and 7 days later, recipient mice were challenged with either syngeneic (B6) or allogeneic (bm1) skin grafts. Skin graft survival together with host CTL activity against donor-type bm1 alloantigen was monitored to assess the extent of tolerization accomplished (Fig. 1).

Flt3 ligand drives the expansion of splenic $CD11c^+CD103^+$ and $CD11c^+CD103^-$ dendritic cells *in vivo*

Flt3L is a well-characterized hematopoietic growth factor capable of expanding DC numbers [15,16,29,38]. To characterize the different DC populations expanded after Flt3L treatment, we took advantage of Rag2/γ_c KO mice, which lack T, B and NK cells, to promote the expansion of a large number of Flt3L-mobilized DC that can be subsequently used in *in vivo* experiments of tolerance induction. Optimal DC expansion was observed 10–12 days after the s.c. injection of 1×10^6 Flt3L-transduced B16 melanoma tumor cells into Rag2/γ_c KO recipients, in which about 58% of cells isolated from the spleen of Flt3L-treated mice were $CD11c^+$ DC expressing

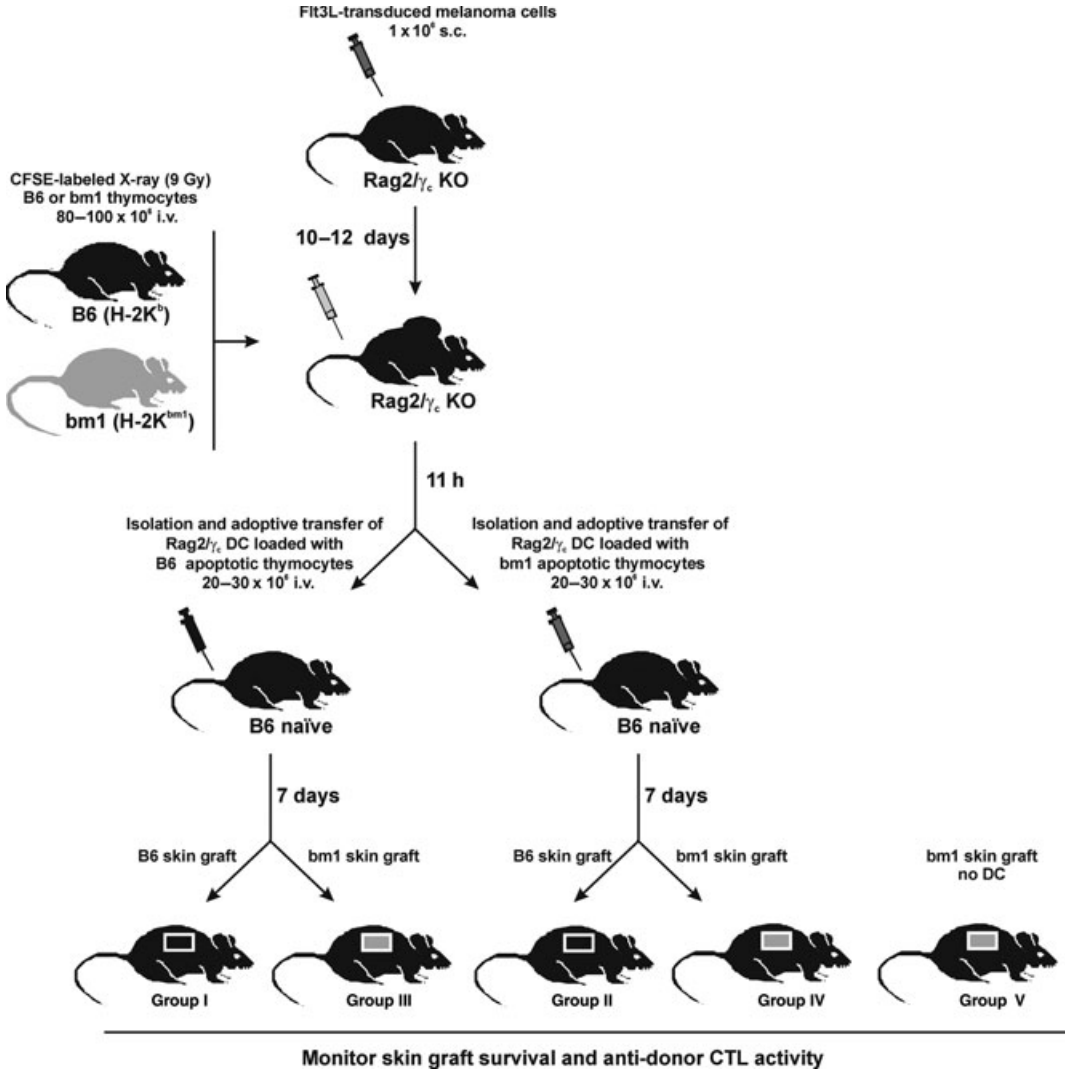


Figure 1 Experimental design. Rag2/γ_c KO mice were treated with Flt3L to expand DC numbers. Ten to 12 days later, DC were loaded *in vivo* with either syngeneic (B6) or allogeneic (bm1) irradiated thymocytes undergoing apoptosis, which were i.v. infused into B6-naïve mice. Mice were then challenged with B6 or bm1 skin grafts 7 days later. Skin graft survival and anti-donor CTL activity were monitored.

intermediate levels of MHC class II molecules, whereas in untreated recipients, only 21% of the spleen cells were $CD11c^+$ IAb^+ (Fig. 2a, left panel). Applying a gating strategy to select $CD11c^+$ DC, we found that the proportion of $CD8\alpha^+$ $CD103^+$ DC in Flt3L-treated mice was 29% compared to 12% in untreated mice (Fig. 2a, middle panel). The percentage of plasmacytoid DC ($CD11c^+B220^+Ly6C^+$, pDC) in Flt3L-treated Rag2/γ_c KO mice was similar to that in untreated controls (Fig. 2a, right panel).

The analysis of the absolute number of mobilized DC revealed a 70-fold expansion after Flt3L treatment (untreated, 1.5 ± 0.77 vs. Flt3L-treated mice, 67.3 ± 14.1; $P < 0.0005$, Fig. 2b). A more detailed analysis of the Flt3L-mobilized DC populations indicated that $CD103^-CD8\alpha^-$ DC were the predominant population isolated from the spleens of these mice (untreated, 1.0 ± 0.55 vs. Flt3L-treated mice, 38.6 ± 13.3; $P < 0.0005$). Other DC subsets expanded under the influence of Flt3L were as follows: $CD103^+CD8\alpha^+$ (untreated, 0.028 ± 0.012 vs. Flt3L-treated mice, 9.68 ± 2.91; $P < 0.0005$), $CD103^+CD8\alpha^-$ (untreated, 0.003 ± 0.001 vs. Flt3L-treated mice, 7.3 ± 3.13; $P < 0.0005$), $CD103^-CD8\alpha^+$ DC (untreated, 0.2 ± 0.12 vs. Flt3L-treated mice, 11.3 ± 0.98; $P < 0.0005$) and pDC (untreated, 0.27 ± 0.24 vs. Flt3L-treated mice, 12.12 ± 2.15; $P < 0.0005$) (Fig. 2c).

Collectively, these results confirm the role of Flt3L in fostering the expansion of distinct subpopulations of $CD11c^+$ DC *in vivo*, including the $CD11c^+CD103^+$ $CD8\alpha^+$DC population.

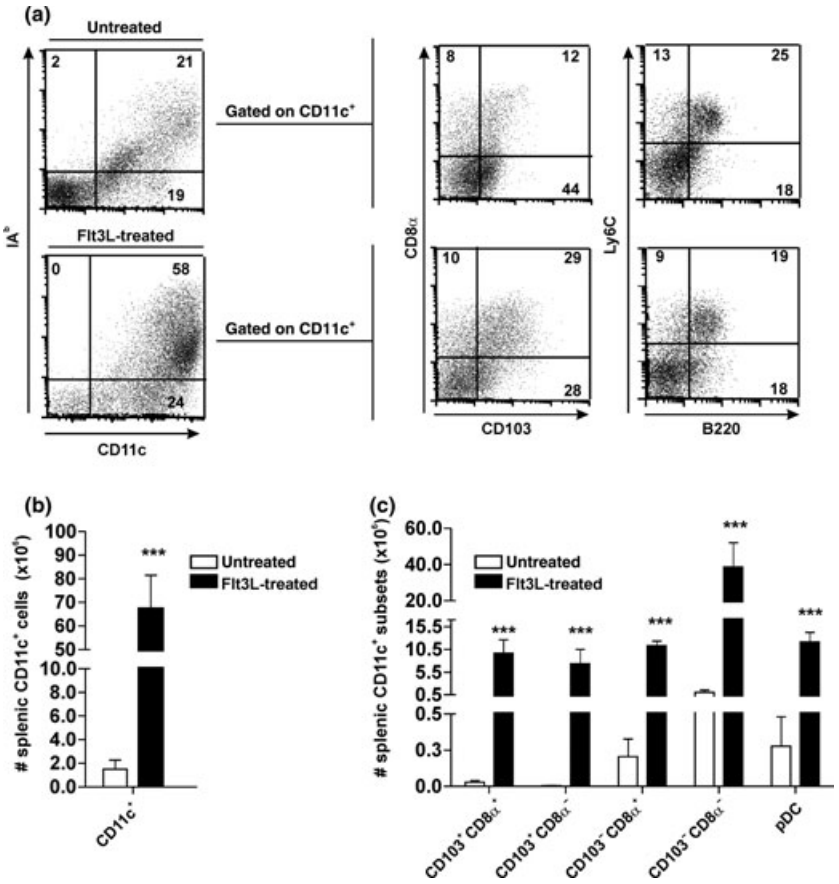


Figure 2 Flt3L-transduced melanoma cells transplanted into Rag2/γc KO drives the expansion of $CD11c^+CD103^+$ dendritic cells. Splenic DC expansion was assessed in untreated- or B16 Flt3L melanoma-treated Rag2/γ_c KO mice. Isotype-matched control mAbs were used to set the quadrant lines to calculate the percentage of $CD11c^+$ cells expressing IA^b surface marker (a, left panel). The percentage of $DAPI^-CD11c^+$ cells expressing CD8α, and CD103 molecules (a, middle panel), as well as pDC ($DAPI^-CD11c^+B220^+Ly6C^+$), are also depicted (a, right panel). Absolute number of splenic $CD11c^+$ cells (b) as well as $CD11c^+CD8\alpha^+$, $CD103^+CD8\alpha^-$, $CD103^-CD8\alpha^+$, $CD103^-CD8\alpha^-$ DC and pDC (c) is displayed. Data shown are mean ± SD from two independent experiments with five mice per group; $^{***}P < 0.0005$, paired Student's *t*-test.

Circulating irradiated syngeneic and allogeneic apoptotic cells are selectively taken up by Flt3L-mobilized $CD11c^+CD103^+CD8\alpha^+$ dendritic cells

Cells undergoing programmed cell death are highly susceptible to be phagocytosed, and represent an essential source of antigen in tumor biology and immune-related diseases such as autoimmunity and transplantation [39–43]. The direct consequence of the uptake of apoptotic cells under noninflammatory conditions by steady-state DC migrating from peripheral tissues to the T-cell area of draining LN is the induction of tolerance to cell-associated antigens [4,5,44,45]. It has been recently reported that blood-borne cells undergoing apoptosis are preferentially captured by a subpopulation of $CD11c^+CD8\alpha^+$ DC that resides in the marginal zone of the spleen, which can be further characterized by the expression of the α_E integrin marker (CD103) [20,21]. To investigate the DC populations involved in the uptake of syngeneic vs. allogeneic apoptotic cell bodies *in vivo*, irradiated CFSE-labeled syngeneic (B6) or allogeneic MHC class I mismatched allogeneic (bm1) thymocytes were used as a source of apoptotic bodies that were i.v. injected into Rag2/γ_c KO recipients that had received Flt3L-transduced melanoma B16 cells 10–12 days earlier. After 11 h, mice were euthanized and splenic DC were isolated by collagenase digestion and analyzed by FACS. Strikingly, Flt3L-derived $CD11c^+$ DC cells positive for CD103 and the CD8α markers were selectively involved in the process of antigen clearance of cell-associated antigens undergoing apoptosis, as they became positive for the intravital dye CFSE, whereas pDC were not directly implicated in the uptake of apoptotic bodies. Of note, Flt3L-mobilized $CD11c^+CD103^+CD8\alpha^+$ phagocytosed to similar extent

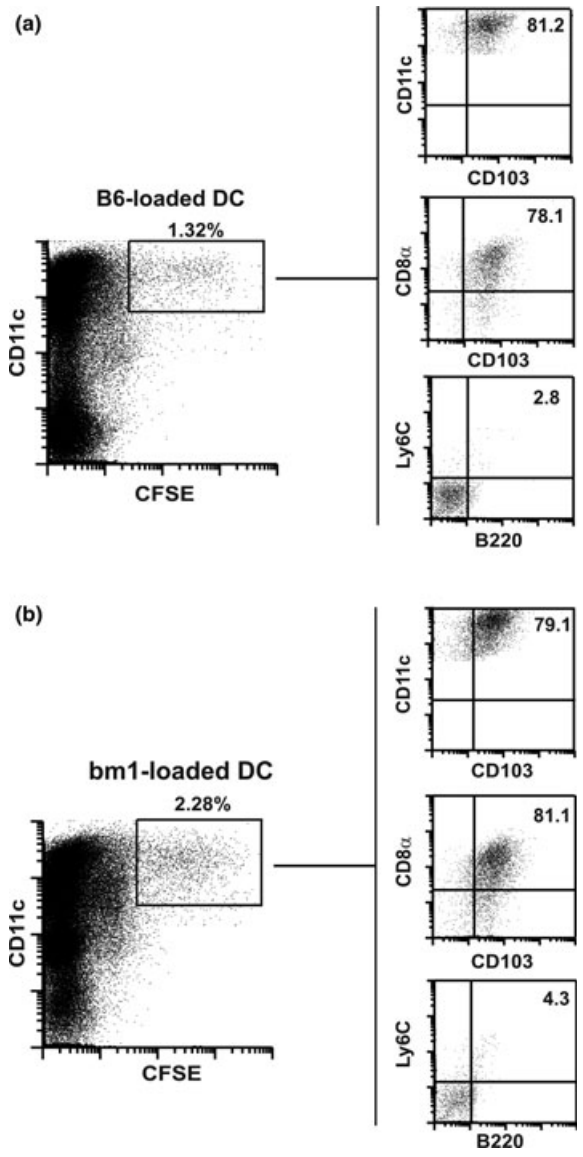


Figure 3 Flt3L-mobilized CD11c$^+$CD103$^+$ dendritic cells from Rag2/γ_c KO mice selectively take up syngeneic and allogeneic apoptotic thymocytes. CFSE-labeled apoptotic thymocytes (80–100 × 10^6) harvested from B6 or bm1 mice were i.v. injected into Rag2/γ_c KO mice that received Flt3L-transduced B16 melanoma cells 10–12 days before. After 11 h, spleens were removed, digested with collagenase A and cells were gated on DAPI$^-$CFSE$^+$CD11c$^+$ to calculate the percentage of DC which have taken up irradiated CFSE-labeled apoptotic cells from B6– (a) or bm1– (b) donor mice. Splenic DC were further stained with CD8α and CD103 mAbs to calculate the percentage of CD11c$^+$ cells expressing CD103 and CD8α DC that engulf CFSE-labeled apoptotic cells. pDC were phenotypically identified as double positive for B220 and Ly6C. Data are representative of three independent experiments with three mice per group.

apoptotic thymocytes from either syngeneic (Fig. 3, upper panel) or allogeneic (Fig. 3, lower panel) irradiated thymocytes.

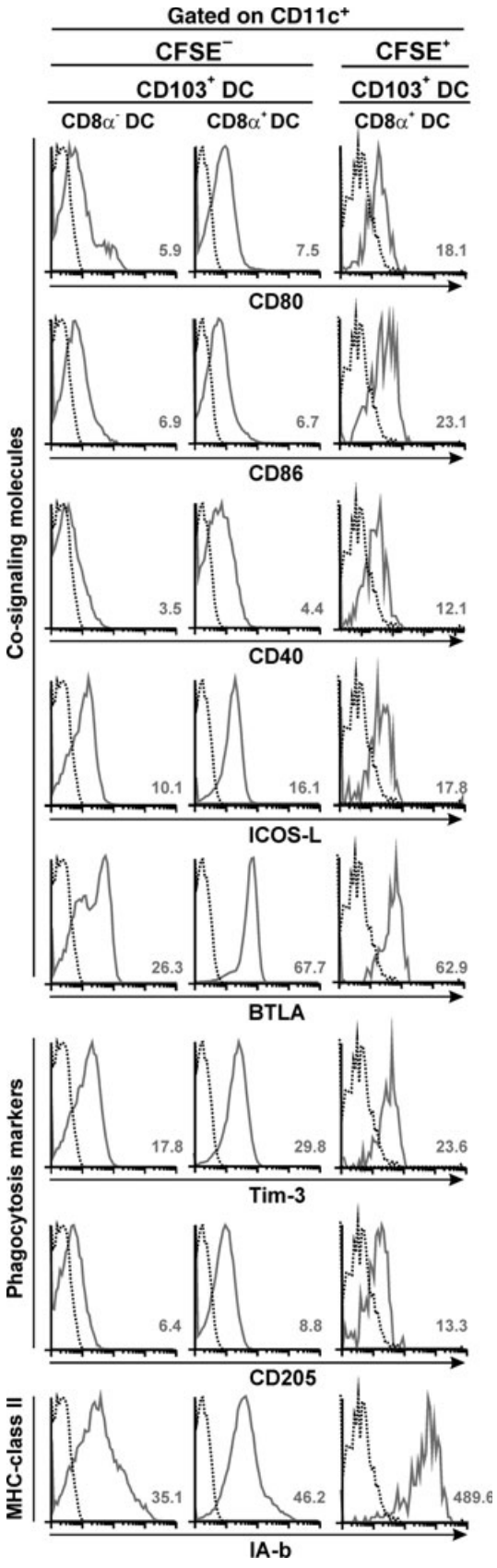
In summary, of all DC populations that are expanded under the influence of Flt3L, only CD11c$^+$CD103$^+$CD8α$^+$DC are particularly involved in the uptake of cells undergoing apoptosis.

Flt3L-derived CD11c$^+$CD103$^+$CD8α$^+$ dendritic cells bearing K^{bm1} apoptotic cell bodies exhibit a stage of differentiation intermediate between immature and semimature DC

There is accumulating evidence that antigen uptake, processing and tolerogenic cross-presentation of apoptotic material imply DC maturation to certain extent [46]. To monitor the extent of maturation and functional status of Flt3L-mobilized DC, the expression of co-signaling, surface molecules involved in phagocytosis and MHC class II molecules before and after the phagocytosis of CFSE-labeled allogeneic apoptotic thymocytes was assessed. CD103$^+$ CD8α$^+$ DC carrying CFSE-labeled apoptotic cells exhibited a significantly enhanced expression of co-signaling surface molecules as well as MHC-class II molecules (IA^b), compared with CFSE$^-$CD103$^+$CD8α$^+$ DC that did not take up apoptotic thymocytes (CD80, $P < 0.05$; CD86, $P < 0.05$; CD40, $P < 0.005$ and IA^b, $P < 0.005$). Other surface markers, such as ICOS-L, BTLA, Tim-3, with the exception of CD205 ($P < 0.05$), were expressed to a similar extent in CD103$^+$CD8α$^+$ DC as in CFSE$^-$CD103$^+$CD8α$^+$ DC regardless of their involvement in the uptake of CFSE-labeled apoptotic thymocytes (Fig. 4 and Table 1). The significant rise in the expression of costimulatory molecules on CFSE$^+$CD103$^+$CD8α$^+$ DC compared with CFSE$^-$CD103$^+$ CD8α$^+$ DC indicated that phagocytosis of apoptotic bodies progressed at the same time with DC maturation to certain extent.

On the other hand, CFSE$^-$CD103$^+$CD8α$^+$ DC, which were not implicated in phagocytosis, exhibited a significant augmentation in the expression of ICOS-L ($P < 0.05$), BTLA ($P < 0.005$) and CD205 ($P < 0.05$) when compared with CFSE$^-$CD103$^+$CD8α$^-$ DC. The expression of costimulatory molecules, MHC class II and Tim-3, was detected to a similar extent in these two DC populations. Finally, there was no detectable expression of PD-L1, PD-L2, PD-1, OX40, Qa-1, B7x, CD137, CD83, 4-1BBL or CCR7 in any of these DC populations (data not shown).

Overall, our data emphasize the fact that CD11c$^+$CD103$^+$CD8α$^+$ DC capture syngeneic or allogeneic apoptotic cells with a similar efficiency, and that the process of phagocytosis induced DC maturation that led to an intermediate stage of differentiation between immature and semimature DC.



Injection of syngeneic Flt3L-expanded DC loaded with allogeneic K^{bm1} apoptotic bodies did not result in a significant K^{bm1} skin allograft survival

We next investigated whether targeting bm1 apoptotic allogeneic cells to Flt3L-expanded syngeneic DC would induce cross-tolerance to allogeneic $CD8^+$ T cells and provide protection against allograft rejection in a murine model of skin graft transplantation across an MHC class I-mismatched barrier.

Syngeneic B6 skin grafts placed on naïve B6 mice receiving DC loaded with either B6 (group I) or bm1 (group II) apoptotic cells survived more than 120 days, as we expected (Fig. 5). Remarkably, the course of bm1 allograft rejection in B6 mice treated with DC-bearing B6 apoptotic thymocytes (group III) was equivalent to those recipients that were adoptively transferred with bm1-loaded DC (group IV) or those that were grafted with allogeneic bm1 skin without DC (group V), suggesting that this experimental approach did not lead to the induction of cross-tolerance to alloreactive $CD8^+$ T cells responding to bm1 alloantigens (Fig. 5).

These results indicate that syngeneic DC loaded with K^{bm1} donor-derived apoptotic cells do not promote tolerance to allogeneic $CD8^+$ T cells by the indirect pathway of antigen presentation or prolong MHC class I mismatched skin allograft survival.

Flt3L-expanded B6 dendritic cells bearing K^{bm1} donor-derived apoptotic thymocytes did not induce cross-tolerance to alloreactive cytotoxic T cells *in vivo*

To examine the extent of cross-tolerization to H-$2K^{bm1}$ antigen after skin graft transplantation, an *in vivo* CTL killing assay was performed to measure host CTL activity against donor-type alloantigens. For this purpose, syngeneic B6 and allogeneic bm1 target splenocytes were labeled with CFSE at different concentrations (B6, 5 μM

Figure 4 Phagocytosis of allogeneic apoptotic thymocytes by host Flt3L-expanded $CD11c^+CD103^+CD8\alpha^+$ DC exhibit a stage of differentiation intermediate between immature and semimature DC. Flt3L-treated Rag2/γ_c KO mice were i.v. injected with 80–100 × 10^6 CFSE-labeled bm1 thymocytes. Spleens were removed 11h later, digested with collagenase A and cells were then gated on $CD11c^+DAPI^-$ to assess the expression of co-signaling molecules, molecules involved in phagocytosis as well as MHC-class II expression on Flt3L-mobilized $CFSE^-CD103^+$ (composed by $CD8\alpha^-$ and $CD8\alpha^+$DC) and $CFSE^+CD103^+$ (mostly $CD8\alpha^+$) DC populations. Grey solid lines indicate the expression of surface molecules, whereas black dotted lines represent their respective isotype controls. Mean Fluorescence Intensity is depicted in each panel. One representative experiment of three with similar results is shown.

Table 1. Statistical analysis of co-stimulatory, phagocytic receptors, and major histocompatibility complex class II expression of $CFSE^{-}CD103^{+}CD8\alpha^{+}$ DC population compared with $CFSE^{-}CD103^{+}CD8\alpha^{-}$ and $CFSE^{+}CD103^{+}CD8\alpha^{+}$ DC.

	$CFSE^{-}CD103^{+}$ $CD8\alpha^{+}$		$CFSE^{-}CD103^{+}$ $CD8\alpha^{-}$			$CFSE^{-}CD103^{+}$ $CD8\alpha^{+}$		$CFSE^{+}CD103^{+}$ $CD8\alpha^{+}$		
Surface markers	Mean	SD	Mean	SD	*P*-value	Mean	SD	Mean	SD	*P*-value
Isotype control	1.697	0.52	2.3	0.79	NS	1.697	0.52	5.35	2.54	NS
CD80	9.88	2.94	7.01	3.28	NS	9.88	2.94	21.08	2.85	$*P = 0.0091$
CD86	9.99	3.35	8.42	1.67	NS	9.99	3.35	30.69	6.57	$*P = 0.0083$
CD40	6.71	2.31	4.08	0.93	NS	6.71	2.31	16.26	3.65	$*P = 0.0188$
ICOS-L	15.67	1.13	10.28	2.40	$*P = 0.0247$	15.67	1.13	18.71	2.06	NS
BTLA	61.68	7.16	26.58	5.58	$**P = 0.0026$	61.68	7.16	50.75	3.69	NS
Tim-3	26.67	6.80	14.53	6.83	NS	26.67	6.80	24.74	5.44	NS
CD205	10.08	1.40	5.72	1.34	$*P = 0.0177$	10.08	1.40	16.08	2.44	$*P = 0.0211$
IA^{b}	75.66	36.25	62.17	26.30	NS	75.66	36.25	490.2	84.07	$**P = 0.0014$

$*P < 0.05$; $**P < 0.005$.

Mean represents mean fluorescence intensity values of three experiments; SD, standard deviation; NS, nonsignificant.

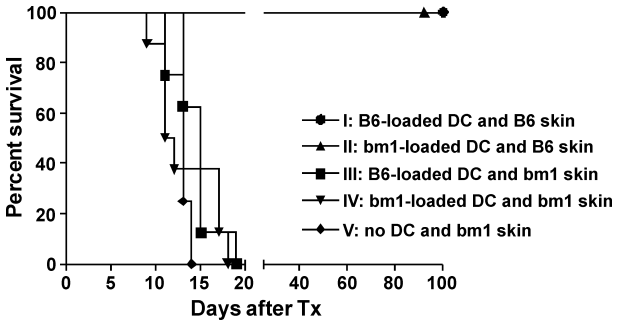


Figure 5 Flt3L-derived DC bearing K^{bm1} apoptotic cells do not stimulate cross-tolerance to allogeneic $CD8^{+}$ T cells across an MHC class I mismatched barrier. B6 or bm1 apoptotic thymocytes ($80–100 \times 10^{6}$) were i.v. injected into Rag2/γ_c KO mice, which received B16 Flt3L-secreting melanoma cells 10–12 days before. After 11 h, $20–30 \times 10^{6}$ Rag2/γ_c KO DC cells bearing B6 or bm1 apoptotic cells were i.v. injected into B6-naïve mice. Seven days later, B6 naïve mice were challenged with syngeneic B6 or allogeneic bm1 skin allografts and graft survival was monitored. Five experimental groups were established. B6 skin grafted mice that received Rag2/γ_c KO DC loaded with either B6 (group I, ●) or bm1 (group II, ▲) apoptotic thymocytes; bm1 skin-grafted mice receiving B6- (group III, ■) or bm1 loaded Rag2/γ_c KO DC (group IV, ▼) and group V, bm1 skin-grafted mice that did not receive antigen-loaded Rag2/γ_c KO DC (♦). Skin graft survival was calculated by using the Kaplan–Meier life table method.

and bm1, 1 μM) and then were adoptively transferred into B6 recipients that had been previously injected with Flt3L-derived DC loaded with syngeneic or allogeneic apoptotic thymocytes. This differential CFSE labeling of target cells in combination with the staining for $H\text{-}2K^{b}$ (clone 5F1.2.14), which does not recognize $H\text{-}2K^{bm1}$ cells, allowed us to distinguish clearly the two target populations *in vivo* in host spleen and pLNs by flow cytometry 48 h later (Fig. 6a). As expected, recipient mice challenged with B6 skin that had previously received either B6- (group I) or bm1 (group II)-loaded DC, modestly rejected bm1 targets to a similar extent, despite the exposure of group II to DC-bearing bm1 apoptotic bodies (Fig. 6a, left panel). In contrast, B6 recipients grafted with bm1 skin that were adoptively transferred with DC-bearing B6 (group III) or bm1 (group IV) apoptotic thymocytes (Fig. 6a, middle panel) or those recipients that did not receive antigen-loaded DC (group V) (Fig. 6 a, right panel) rejected more efficiently bm1 targets than groups I and II, suggesting that only allogeneic skin graft rejection led to sensitization of the recipients, but syngeneic DC loaded with allogeneic apoptotic thymocytes did not stimulate a cross-priming reaction against donor-type antigens.

We next quantified the percentage of specific lysis of CFSE-labeled bm1 targets in host spleen and pLNs, as described in the material and methods section. The killing activity of host anti-donor $H\text{-}2K^{bm1}$ targets observed in host spleens was similar to that calculated in pLNs. The extent of killing of bm1 targets injected in mice grafted with B6 skin that were adoptively transferred with either B6 (group I) or bm1 (group II) irradiated thymocytes was equivalent in both groups of mice not only in the spleen (group I, 24.7 ± 7.38; group II, 29.8 ± 11.56) but also in pLNs (group I 28.8 ± 12.12; group II, 39.1 ± 9.27) (Fig. 6b) indicating that the exposure of recipient mice to syngeneic DC loaded with allogeneic apoptotic bodies did not lead to sensitization of the host. The percentage of killing of bm1 targets in the spleen and pLNs of mice grafted with bm1 skin receiving DC bearing B6 apoptotic cells (group III) was 95.1 ± 2.19 and 99.3 ± 0.3, respectively. No additional sensitization of the recipients against donor-type cells was observed, as group IV rejected bm1 targets in the spleen (95.6 ± 1.92) and pLNs

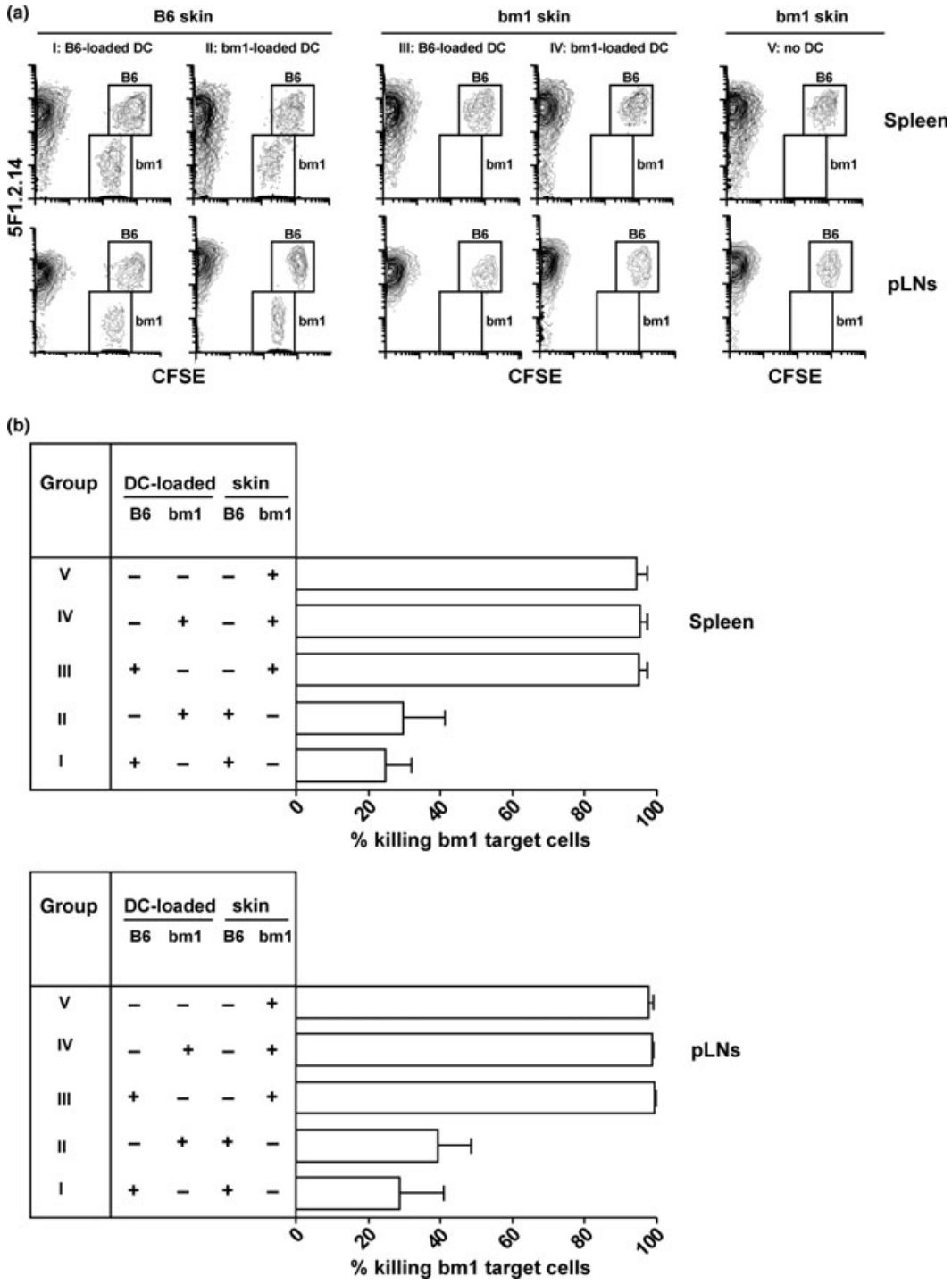


Figure 6 Flt3L-mobilized B6 DC loaded with K^{bm1} donor-derived apoptotic bodies do not induce cross-tolerization to allogeneic $CD8^+$ T cells. Twenty-five days after B6 or bm1 skin graft transplantation, B6 recipients were i.v. injected with a 1:1 ratio of CFSE-labeled B6 (5 μM) and bm1 (1 μM) target splenocytes. The survival of each target population was analyzed 48h later in host spleen and pLNs. Staining with anti-Kb (5F1.2.14), which does not label K^{bm1} cells, in combination with CFSE, allowed us to distinguish clearly the two different target populations. (a) Representative density dot plots illustrate B6 and bm1 CFSE-labeled targets that were injected into B6 recipients and analyzed in host spleen (upper panel) and host pLNs (lower panel). (b) The percentage of specific lysis in the spleen (upper panel) and pLNs (lower panel) was calculated comparing the survival of the each target population to the survival of the syngeneic population according to the equation described in Material and methods section. Data representative of two independent experiments with three mice per group are shown.

(98.8% ± 0.4) to an extent similar to that of mice from group III or those mice that only received bm1 skin grafts without being injected with antigen-loaded DC (group V) (spleen, 94.4 ± 3.18; pLNs, 97.8 ± 1.37) (Fig. 6b).

Altogether, these *in vivo* data support the notion that syngeneic DC loaded with K^{bm1} donor-derived apoptotic cells were not sufficient to induce cross-tolerance to cytotoxic T cells responding to K^{bm1}-derived allopeptides.

Discussion

Apoptosis constitutes a mechanism of cell death that takes place physiologically without inducing inflammation. Once apoptotic cells are recognized and processed by antigen presenting cells, antigenic-derived peptides gain access to the cytosol and are incorporated to the groove of MHC-class I molecules that will be presented to $CD8^+$ T cells by the indirect pathway of antigen presentation [47–49]. Antigen presentation by this pathway in the absence of danger signals is a crucial step for the initiation and maintenance of central and peripheral tolerance. As phagocytosis of apoptotic cells delivers inhibitory signals and prevents DC maturation and T-cell activation upon antigen presentation because of the low levels of costimulatory molecules, targeting donor alloantigen to syngeneic immature DC *in vivo* represents an interesting approach for the immune regulation of anti-donor T-cell responses, the promotion of heart allograft survival and the amelioration of allograft vasculopathy, although these results have only been accomplished in certain mouse strain combinations, particularly when C3H or B10 mice were used as recipients [9,11–13,50].

The marginal zone of the spleen clears blood-borne microorganisms, debris, old erythrocytes and dying cells. Indeed, it is well established that circulating apoptotic cells are captured and cleared from circulation by two populations of macrophages named marginal zone macrophages and metallophilic macrophages [51,52]. Along macrophages, CD8α DC reside beneath the ring of red pulp and above the metallophilic macrophages of the marginal zone and constitute the major population involved in the process of uptake of apoptotic cells [42,53]. Marginal zone $CD11c^+CD103^+CD8\alpha^+$ DC migrate to the T-cell zone after the uptake of apoptotic bodies where they may participate in promoting tolerance to self antigens because of their preferential ability to capture, process and present antigens of dying apoptotic cells particularly to $CD8^+$ T cells [20,23,24].

On the basis of highly specialized functional activity of $CD11c^+CD103^+CD8\alpha^+$ DC, we hypothesized that targeting allogeneic MHC class I mismatched apoptotic thymocytes to Flt3L-mobilized syngeneic DC would lead to indirect antigen presentation of allopeptides and tolerance induction to host $CD8^+$ alloreactive T cells, which are the main effector mechanism of cellular rejection across an MHC class I barrier. We herein demonstrate that $CD11c^+CD103^+CD8\alpha^+$ DC were selectively implicated in capturing cell-associated antigen-derived from syngeneic and allogeneic apoptotic dying thymocytes *in vivo*, which is in agreement with the role of CD103 DC subset in capturing apoptotic cells induced to die through Fas/FasL interaction [20]. The role of pDC in the internalization of apoptotic cells *in vivo* was insignificant compared with the other two major populations of splenic DC, as previously shown by others investigators [9,13,20,42]. Despite the specificity of the targeting approach to guide apoptotic alloantigen into Flt3L-mobilized $CD11c^+CD103^+$ $CD8\alpha^+$ DC, the chosen experimental strategy did not result in prolongation of skin allograft survival. In an attempt to circumvent this obstacle and try to enrich the population of DC taking up allogeneic apoptotic bodies, we sorted Flt3L-mobilized $CD11c^+CD103^+$ DC and observed that when isolated *ex vivo* and placed under culture conditions, they did not survive long (data not shown). This fragile behavior of $CD11c^+CD103^+$ DC discouraged us from pursuing this approach.

The inefficacy of Flt3L-mobilized DC in the induction of donor-specific tolerance to $CD8^+$ T cells reactive against bm1 antigens could be explained by the fact that apoptotic bodies captured by syngeneic Flt3L-expanded DC are processed and presented via the indirect pathway of allorecognition, in which the repertoire of allogeneic peptides derived from the sequence that distinguishes $H\text{-}2K^b$ and $H\text{-}2K^{bm1}$ may generate a limited heterogeneity of allopeptides. This may not be sufficient to instruct the whole repertoire of alloreactive $CD8^+$ T cells, particularly those alloreactive CD8 T cells recognizing foreign MHC class I (K^{bm1}) by the direct pathway of antigen presentation would escape from this tolerizing approach. An alternative explanation is that only 1–2% of the DC were CFSE positive, despite the large number of CFSE-labeled apoptotic cells injected into recipient mice. Moreover, among all splenic phagocytic DC cell subsets, phagocytosis was mainly restricted to $CFSE^+CD103^+CD8\alpha^+$ DC population that was only detectable at early time points postadoptive transfer, which is in agreement with similar findings reported in the literature [42]. Iyoda *et al.* applying a similar experimental strategy demonstrated that less than 5% of splenic $CFSE^+CD11c^+CD8\alpha^+$ DC bearing apoptotic bodies could be detected 10 h after i.v. injection of CFSE-labeled apoptotic cell bodies. More importantly, they could show that those low DC numbers capturing dying cells were able to present efficiently those antigens *in vitro* and *in vivo* in the context of both MHC class I and MHC class II to antigen-specific T cells [42]. We conceive that other phagocytic cells may be involved in

the clearance of apoptotic cell bodies, but remain undetectable because of a more active processing and destruction of the CFSE fluorochrome upon apoptotic cell uptake within their cytoplasm. The findings, however, indicate that the majority of splenic DC may not bear allopeptides and therefore are insufficient in cell numbers to instruct properly alloantigen-specific CD8 T cells not to reject bm1 skin allografts.

This strategy was, however, free of side effects, as syngeneic DC loaded with allogeneic apoptotic cells did not sensitize recipient T cells against donor MHC alloantigens, because no acceleration of skin graft rejection or augmented host anti-donor CTL activity was observed.

Distinct strategies for the induction of donor-specific tolerance to vascularized solid organs and cellular allografts have been proposed operating through different mechanisms of action. The administration of allogeneic immature DC has been reported to prolong allograft survival. This experimental approach allows antigen presentation via the direct pathway of allorecognition in the absence of proper co-stimulation, and seems to abrogate allogeneic T cell activation and clonal expansion, driving responding T cells toward a state of unresponsiveness [18,54,55]. Another efficient strategy to prolong allograft survival across fully MHC barriers is the administration of donor-specific transfusion of splenocytes [56]. Finally, delivery of alloantigen in the form of apoptotic bodies has been shown to delay graft rejection [9,12]. These strategies alone or in combination with costimulation blockade (anti-CD40L mAb and/or CTLA4-Ig) have proven very efficient in prolonging fully MHC mismatched allografts in certain donor/host strain combinations and particularly in those less immunogeneic strains of mice such as C3H or B10, in which $CD8^+$ T cell tolerization is not required for transplantation tolerance and host anti-donor alloreactive $CD8^+$ T cell frequency is substantially lower than that in B6 mice [57]. In these murine models of vascularized organ transplantation, allograft survival is further increased when immature allogeneic DC are coadministered with anti-CD40L and/or CTLA4-Ig [12,19], because under this conditioning regimen, donor allogeneic immature DC are protected from rejection and can persist longer in the circulation exerting their anergizing action. Besides, costimulation blockade with anti-CD40L and CTLA4-Ig exerts an intrinsically protecting immunosuppressive effect on general alloreactive responses, particularly on $CD4^+$ T cell-mediated allogeneic responses [58–61].

Our findings demonstrate that syngeneic DC loaded with K^{bm1} donor-derived apoptotic bodies do not induce cross-tolerance through the indirect pathway of allorecognition to K^{bm1}-derived allopeptides in a murine model of skin allograft transplantation across an MHC class I mismatched barrier. Tolerization of allogeneic $CD8^+$ T cell-mediated responses to achieve long-term survival is still a pending issue in the field of transplantation research, and novel approaches for the induction of tolerance within the $CD8^+$ T compartment need to be further explored.

Authorship

MLDR: performed experiments and wrote the manuscript. JCS: analyzed the data and contributed with valuable suggestions. JIRB: designed the study and wrote the manuscript.

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Disclosures

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