Published in final edited form as:
Transplantation. 2014 September 15; 98(5): 563–568. doi:10.1097/TP.0000000000000097.

Langerhans cell homeostasis and turnover after nonmyeloablative and myeloablative allogeneic hematopoietic cell transplantation

Marco Mielcarek[1,2], **A. Yasmine Kirkorian**[3,4], **Robert C. Hackman**[1,2], **Jeremy Price**[3,4], **Barry E. Storer**[1,2], **Brent L. Wood**[2], **Marylene Leboeuf**[3], **Milena Bogunovic**[3,4], **Rainer Storb**[1,2], **Yoshihiro Inamoto**[1], **Mary E. Flowers**[1,2], **Paul J. Martin**[1,2], **Matthew Collin**[5], and **Miriam Merad**[3,4]

[1]Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA

[2]University of Washington, Seattle, WA

[3]Immunology Institute, Mount Sinai Medical Center, New York, NY

[4]Tisch Cancer Institute, Mount Sinai Medical Center, New York, NY

[5]Institute of Cellular Medicine, Newcastle University, UK

Abstract

Background—Langerhans cells (LCs) are self-renewing epidermal myeloid cells that can migrate and mature into dendritic cells. Recipient LCs that survive cytotoxic therapy given in preparation for allogeneic hematopoietic cell transplantation may prime donor T cells to mediate cutaneous graft-versus-host disease (GVHD). This possible association, however, has not been investigated in the setting of nonmyeloablative allografting.

Methods—We prospectively studied the kinetics of LC-chimerism after sex-mismatched allogeneic hematopoietic cell transplantation with nonmyeloablative (n=23) or myeloablative (n=25) conditioning. Combined XY-FISH and Langerin-staining was used to assess donor LC-

Address for Correspondence: Marco Mielcarek, MD, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue N.,Mail Stop D1-100, PO Box 19024, Seattle, WA 98109. mmielcar@fhcrc.org.

Authors' Contributions:
Marco Mielcarek designed the study, analyzed and interpreted data, and wrote the manuscript.
A. Yasmine Kirkorian performed LC chimerism analysis
Robert C. Hackman performed histopathological analysis of skin biopsies
Jeremy Price assisted with data interpretation and edited manuscript
Barry E. Storer performed statistical analysis
Brent L. Wood performed cell sorting of dendritic cells
Marylene Leboeuf performed cell sorting of dendritic cells
Milena Bogunovic performed LC chimerism analysis
Rainer Storb assisted with data interpretation and edited manuscript
Yoshihiro Inamoto assisted with data interpretation and edited manuscript
Mary E. Flowers assisted with data interpretation and edited manuscript
Paul J. Martin assisted with data interpretation and edited manuscript
Matthew Collin performed LC chimerism analysis, analyzed and interpreted data, and wrote the manuscript.
Miriam Merad designed the study, analyzed and interpreted data, and wrote the manuscript.

Financial Disclosure Statement: The authors of this manuscript have no conflicts of interest to disclose as described by the journal *Transplantation*.

chimerism in skin biopsies obtained on days 28, 56 and 84 after transplant. The degree of donor LC-chimerism was correlated with the development of skin GVHD.

Results—We observed significantly delayed donor LC-engraftment after nonmyeloablative transplantation compared with other hematopoietic compartments and compared with LC-engraftment after myeloablative conditioning. In most recipients of nonmyeloablative transplants, recipient LCs proliferated *in situ*, recruitment of donor-LCs was delayed by two months, and full donor LC-chimerism was only reached by day 84 after transplant. Although persistence of host LCs on day-28 after transplant was not predictive for acute or chronic skin GVHD, the recruitment of donor-derived LCs was associated with non-specific inflammatory infiltrates (p=0.009).

Conclusions—These results show that LCs can self-renew locally but are replaced by circulating precursors even after minimally toxic nonmyeloablative transplant conditioning. Cutaneous inflammation accompanies donor LC-engraftment, but differences in LC conversion-kinetics do not predict clinical or histopathological GVHD.

Keywords

Antigen presentation; engraftment kinetics; GVHD; hematological malignancies; stem cell transplantation

INTRODUCTION

Langerhans cells (LCs) are myeloid dendritic cells (DCs) of the epidermis that have local self-renewal capacity in mice (1,2) and humans (3,4). Persistent recipient LCs were originally proposed to cause graft-versus-host disease (GVHD) after allogeneic hematopoietic cell transplantation (allo-HCT) with myeloablative (MA) conditioning in humans (5,6). Murine models of allo-HCT have confirmed that recipient LCs can persist for prolonged periods after cytotoxic conditioning therapy (1,2) and are sufficient to induce cutaneous GVHD in recipients of MHC-mismatched (2) and MHC-matched grafts (7). In mixed chimeras receiving donor-lymphocyte infusion, LCs promote GVHD by licensing donor CD8+ T cells infiltrating inflamed skin (8). In these models, inflammation also plays a key role in depleting recipient LCs and attracting monocyte-derived cells to form new donor-derived LCs in the epidermis (9).

Human studies of allo-HCT with reduced-intensity conditioning regimens, compared to those with MA conditioning regimens, have demonstrated that the survival of recipient LCs is significantly prolonged and that full donor engraftment of LCs is associated with GVHD (10). However, the ability of recipient LCs to proliferate and survive has not been explored after nonmyeloablative (NA) allo-HCT with fludarabine and low-dose (2–3 Gy) TBI conditioning, in which acute GHVD has been shown to be significantly delayed (11). Furthermore, the relationship between cutaneous inflammation and engraftment of donor LCs has not been explored at the histological level.

In this study, we therefore examined LC homeostasis in relation to hematopoietic cell chimerism, clinical and histopathological GVHD, and skin inflammation after NA or MA allo-HCT. Our results show that recipient LCs proliferate *in situ* and persist for prolonged periods after NA conditioning. Repopulation with donor LCs occurs in association with

cutaneous inflammation but does not correlate with histopathological skin GVHD or clinical diagnosis of GVHD.

RESULTS

Forty-eight patients who had biopsies at one or more of 3 time-points after sex-mismatched allo-HCT were included in the analysis (day 28, n=40; day 56, n=12; day 84, n=22). Twenty-three patients had received NA conditioning, and 25 had received MA conditioning (Table 1). Since Langerin is a stable antigen that resists heat denaturation and proteolytic treatment, simultaneous XY-FISH and post-hybridization immunofluorescence staining could be performed to visualize LC homeostasis *in situ* (Fig 1A–B). A first round of staining for Langerin and Ki-67 followed by antibody stripping and secondary FISH/ immunofluorescence demonstrated that persisting host LCs were proliferating (Fig 1C).

Donor epidermal LC chimerism was significantly lower after NA conditioning compared with MA conditioning (day 28: 7% *vs.* 95%, p=0.001; day 56: 34% *vs.* 100%, p=0.05; day 84: 94% *vs.* 97%, p=0.48) (Fig 2A). Donor LC chimerism after NA conditioning (Fig 2A) was also significantly lower than the donor DC chimerism in peripheral blood or bone marrow (day 28: 7% vs. 100%, p=0.001; day 56: 34% vs. 100%; p=0.05) (Fig 2B–C) or the total $CD33^+$ myeloid compartment of blood (Fig 2D). $CD3^+$ T cells in the blood also showed lower chimerism after NA conditioning compared with MA conditioning (day 28: 78% *vs.* 100; p=0.02) (Fig 2E), in keeping with the delayed onset of GVHD after NA conditioning (11). Based on presence or absence of acute GVHD (≥ grade 2) at day 28, median CD3 cell chimerism was 78% among recipients without GVHD (n=17), and 100% among those with GVHD (n=7) (p=0.07).

Although persistence of host LCs after allo-HCT has been implicated in GVHD, we did not find a correlation between the highly variable degrees of LC chimerism on day 28 after NA conditioning and clinical or histopathological acute and chronic skin GVHD. Levels of day-28 LC-chimerism (<50% *vs* >50%) were not significantly associated with clinical skin stage 2–4 acute GVHD (45% *vs* 44%, p=0.95), histo-pathological stage 1–4 acute skin GVHD (43% *vs* 67%, p=0.14), or the rate of chronic GVHD meeting NIH criteria (32% *vs* 45% at 2 years, HR=1.2, p=0.77). Among patients with chronic GVHD, the types of chronic skin GVHD were similar between groups defined by day-28 LC chimerism. Day-28 LC-chimerism was also not associated with presence or absence of acute GVHD at this time (data not shown).

Since clinical GVHD reflects a late effector response and is likely to play a role in promoting donor LC engraftment, we also examined skin biopsies for early inflammatory changes (dermal cell infiltration and epidermal thickening) that were not classified as skin GVHD by our pathologist. We found that increased skin inflammation scores were strongly associated with earlier donor LC chimerism (p=0.009; Fig 2F).

DISCUSSION

These results show that recipient LCs proliferate *in situ* after allo-HCT and that, compared with MA conditioning, minimally toxic transplant NA conditioning consisting of low-dose

TBI (2–3 Gy) and fludarabine is associated with a two-month delay in donor LC-engraftment. Direct histological analysis is consistent with the findings in mice that inflammation is required to attract donor-derived LC-precursors to the epidermis.

Delayed LC-engraftment was reported after conditioning with a reduced-intensity regimen of fludarabine (150 mg/m^2) and melphalan (140mg/m^2) compared with conventional high-dose regimens (10). With the NA conditioning regimen examined in the present study, we observed an even greater delay in donor LC-engraftment, resulting in only 7% donor signal at day 28 and 34% at day 56, compared with 37% on day 40 that was previously reported after reduced-intensity conditioning. In contrast, the kinetics of donor engraftment of peripheral blood and bone marrow DCs was rapid and independent of conditioning intensity, in keeping with previous reports (10,12,13). Unlike previous reports we were able to measure LC-chimerism *in situ* thus avoiding potential bias introduced by the isolation of LCs through *in vitro* culture (10,13).

The role of LCs in GVHD remains controversial. Although some studies have shown that UVB-irradiation during allo-HCT decreases the risk of acute skin GVHD, the findings were based on relatively small sample sizes and may also be explained by mechanisms unrelated to UVB-mediated depletion of LCs (14). Targeted depletion of host LCs in animal models of allo-HCT does not protect from GVHD, presumably because other host DCs are able to prime allo-reactive donor T cells (15). LCs are sufficient to induce cutaneous GVHD, however, when they are the only remaining host antigen-presenting cell in recipients of MHC-mismatched (2) and MHC-matched grafts (7), and when mismatched donor lymphocytes and a local inflammatory stimulus are given to mixed chimeras (8). Complete loss of DCs but preservation of LCs and macrophages occurs in humans with DC, monocyte, B and NK lymphoid (DCML) deficiency / monocytopenia with *Mycobacterium avium* complex (monoMAC) due to GATA2-mutation. A potential role of LCs in human GVHD is suggested by the observation that these patients still experience GVHD when given hematopoietic allografts, although the contribution of macrophages and other non-professional antigen-presenting cells to donor T cell activation cannot be excluded (16,17).

Although the presence of skin inflammation correlated with donor LC-engraftment in our study, many potential explanations can account for the lack of relationship between histological or clinical GVHD and LC-chimerism. First, recipient LCs constitute an afferent pathway of donor T cell activation, while GVHD is a temporally separated effector phenomenon. Second, although persistent recipient LCs may promote GVHD, skin inflammation rapidly mobilizes them (18) increasing the proportion of donor LCs in animal models (2). Thus recipient LCs are more likely to survive in the absence of GVHD. Finally, the overall grading of clinical GVHD is retrospective and, hence, not easily recorded as a time-dependent variable. For these reasons, clinically useful predictions of GVHD are unlikely to be derived from the measurement of LC-chimerism.

PATIENTS AND METHODS

Patients

From November 2006 to February 2009, punch biopsies were obtained from the skin at the posterior iliac crest of recipients who had routine marrow biopsies under local anesthesia prospectively on days 28, 56 and 84 days after allo-HCT for treatment of hematologic malignancies. Informed consent was documented with the use of forms approved by the Institutional Review Board at the Fred Hutchinson Cancer Research Center. Patient characteristics are summarized in Table 1. All grafts were G-CSF-mobilized peripheral blood mononuclear cells. A pathologist graded skin biopsies for GVHD in a blinded fashion, according to standard criteria (19). Skin inflammation was also scored according to the degree of dermal infiltration and epidermal thickening and categorized as none (0); mild (1); moderate (2); or severe (3).

Combined immunofluorescence and FISH

Four millimeter punch biopsies of skin were fixed in 10% buffered formalin overnight and embedded in paraffin. Six micrometer sections were subjected to antigen retrieval using antigen unmasking solution (Vector Laboratories, Burlingame, CA). Dual FISH and immunofluorescence were performed as previously described (20) using the following antibodies and probes: Langerin Clone DCGM4 (Beckmann Coulter, Miamai, FL) with Cy2 anti–mouse IgG or biotinylated anti-mouse IgG and strepatividin Cy5 (Jackson Immunoresearch Inc., West Grove, PA); Ki-67 rabbit polyclonal (Vector Laboratories) with biotinylated anti–rabbit IgG, followed by streptavidin-Cy3 (Jackson Immunoresearch Inc.); SpectrumOrange X and SpectrumGreen Y chromosome enumeration probes (Vysis Inc., Des Plains, IL). FISH was performed before Langerin staining, except when Ki-67 was analyzed. In this case, Langerin and Ki-67 were stained before FISH and then Langerin was re-stained.

Leukocyte chimerism

$CD11c^+$ DCs, $CD3^+$ T cells and $CD33^+$ myeloid cells in peripheral blood, and $CD11c^+$ DCs from the bone marrow were sorted and subjected to VNTR-based chimerism analysis.

Statistical methods

Comparisons of chimerism values between groups were performed using a 2-sample Wilcoxon test. Comparisons of proportions of patients between groups defined by LC-chimerism were performed using Chi-squared tests. Linear regression was used to evaluate correlation between skin GVHD stage, skin inflammation and LC-chimerism.

Acknowledgments

We thank Helen Crawford and Bonnie Larson for assistance with manuscript preparation. We are also grateful to the physicians, nurses, physician assistants, nurse practitioners, pharmacists, and support staff caring for our patients, and to the patients who participated in this study.

Funding Support Information: The authors are grateful for research funding from the Dana Foundation, Human Immunology Grant Program (M. Mielcarek and M. Merad), and from the National Institutes of Health, Bethesda, MD grants HL108307 (M. Mielcarek), CA018029, CA078902 and CA015704. The funders had no role in study

design, data collection and analysis, decision to publish, or preparation of the manuscript. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health nor its subsidiary Institutes and Centers.

ABBREVIATIONS

Allo-HCT	Allogeneic hematopoietic cell transplantation
DC	Dendritic cell
G-CSF	Granulocyte colony-stimulating factor
GVHD	Graft-versus-host disease
LC	Langerhans cell
MA	Myeloablative
NA	Non-myeloablative
TBI	Total body irradiation
VNTR	Variable-number tandem repeats
FISH	Fluorescence in situ hybridization

REFERENCES

1. Merad M, Manz MG, Karsunky H, et al. Langerhans cells renew in the skin throughout life under steady-state conditions. Nat Immunol. 2002; 3:1135. [Erratum appears in Nat Immunol 2003 Jan; 4(1):92]. [PubMed: 12415265]
2. Merad M, Hoffmann P, Ranheim E, et al. Depletion of host Langerhans cells before transplantation of donor alloreactive T cells prevents skin graft-versus-host disease. Nat Med. 2004; 10:510. [erratum appears in Nat Med. 2004 Jun;10(6):649]. [PubMed: 15098028]
3. Czernielewski JM, Demarchez M. Further evidence for the self-reproducing capacity of Langerhans cells in human skin. J Invest Dermatol. 1987; 88:17. [PubMed: 3540136]
4. Kanitakis J, Morelon E, Petruzzo P, Badet L, Dubernard JM. Self-renewal capacity of human epidermal Langerhans cells: observations made on a composite tissue allograft. Exp Dermatol. 2011; 20:145. [PubMed: 20707812]
5. Perreault C, Pelletier M, Landry D, Gyger M. Study of Langerhans cells after allogeneic bone marrow transplantation. Blood. 1984; 63:807. [PubMed: 6367851]
6. Perreault C, Pelletier M, Belanger R, et al. Persistence of host Langerhans cells following allogeneic bone marrow transplantation: possible relationship with acute graft-versus-host disease. Br J Haematol. 1985; 60:253. [PubMed: 3890932]
7. Durakovic N, Bezak KB, Skarica M, et al. Host-derived Langerhans cells persist after MHC-matched allografting independent of donor T cells and critically influence the alloresponses mediated by donor lymphocyte infusions. J Immunol. 2006; 177:4414. [PubMed: 16982876]
8. Bennett CL, Fallah-Arani F, Conlan T, et al. Langerhans cells regulate cutaneous injury by licensing CD8 effector cells recruited to the skin. Blood. 2011; 117:7063. [PubMed: 21566096]
9. Ginhoux F, Tacke F, Angeli V, et al. Langerhans cells arise from monocytes in vivo. Nat Immunol. 2006; 7:265. [PubMed: 16444257]
10. Collin MP, Hart DN, Jackson GH, et al. The fate of human Langerhans cells in hematopoietic stem cell transplantation. J Exp Med. 2006; 203:27. [PubMed: 16390938]
11. Mielcarek M, Martin PJ, Leisenring W, et al. Graft-versus-host disease after nonmyeloablative versus conventional hematopoietic stem cell transplantation. Blood. 2003; 102:756. [PubMed: 12663454]

12. Auffermann-Gretzinger S, Lossos IS, Vayntrub TA, et al. Rapid establishment of dendritic cell chimerism in allogeneic hematopoietic cell transplant recipients. Blood. 2002; 99:1442. [PubMed: 11830498]

13. Auffermann-Gretzinger S, Eger L, Bornhauser M, et al. Fast appearance of donor dendritic cells in human skin: dynamics of skin and blood dendritic cells after allogeneic hematopoietic cell transplantation. Transplantation. 2006; 81:866. [PubMed: 16570010]

14. Kreutz M, Karrer S, Hoffmann P, et al. Whole-body UVB irradiation during allogeneic hematopoietic cell transplantation is safe and decreases acute graft-versus-host disease. J Invest Dermatol. 2012; 132:179. [PubMed: 21850024]

15. Li H, Kaplan DH, Matte-Martone C, et al. Langerhans cells are not required for graft-versus-host disease. Blood. 2011; 117:697. [PubMed: 20944073]

16. Bigley V, Haniffa M, Doulatov S, et al. The human syndrome of dendritic cell, monocyte, B and NK lymphoid deficiency. J Exp Med. 2011; 208:227. [PubMed: 21242295]

17. Cuellar-Rodriguez J, Gea-Banacloche J, Freeman AF, et al. Successful allogeneic hematopoietic stem cell transplantation for GATA2 deficiency. Blood. 2011; 118:3715. [PubMed: 21816832]

18. Kaplan G, Nusrat A, Witmer MD, Nath I, Cohn ZA. Distribution and turnover of Langerhans cells during delayed immune responses in human skin. J Exp Med. 1987; 165:763. [PubMed: 3546582]

19. Lerner KG, Kao GF, Storb R, Buckner CD, Clift RA, Thomas ED. Histopathology of graft-vs.-host reaction (GvHR) in human recipients of marrow from HL-A-matched sibling donors. Transplant Proc. 1974; 6:367. [PubMed: 4155153]

20. Bogunovic M, Ginhoux F, Wagers A, et al. Identification of a radio-resistant and cycling dermal dendritic cell population in mice and men. J Exp Med. 2006; 203:2627. [PubMed: 17116734]

21. Storb R, Deeg HJ, Whitehead J, et al. Methotrexate and cyclosporine compared with cyclosporine alone for prophylaxis of acute graft versus host disease after marrow transplantation for leukemia. N Engl J Med. 1986; 314:729. [PubMed: 3513012]

22. Nash RA, Etzioni R, Storb R, et al. Tacrolimus (FK506) alone or in combination with methotrexate or methylprednisolone for the prevention of acute graft-versus-host disease after marrow transplantation from HLA-matched siblings: A single-center study. Blood. 1995; 85:3746. [PubMed: 7540071]

23. McSweeney PA, Niederwieser D, Shizuru JA, et al. Hematopoietic cell transplantation in older patients with hematologic malignancies: replacing high-dose cytotoxic therapy with graft-versus-tumor effects. Blood. 2001; 97:3390. [PubMed: 11369628]

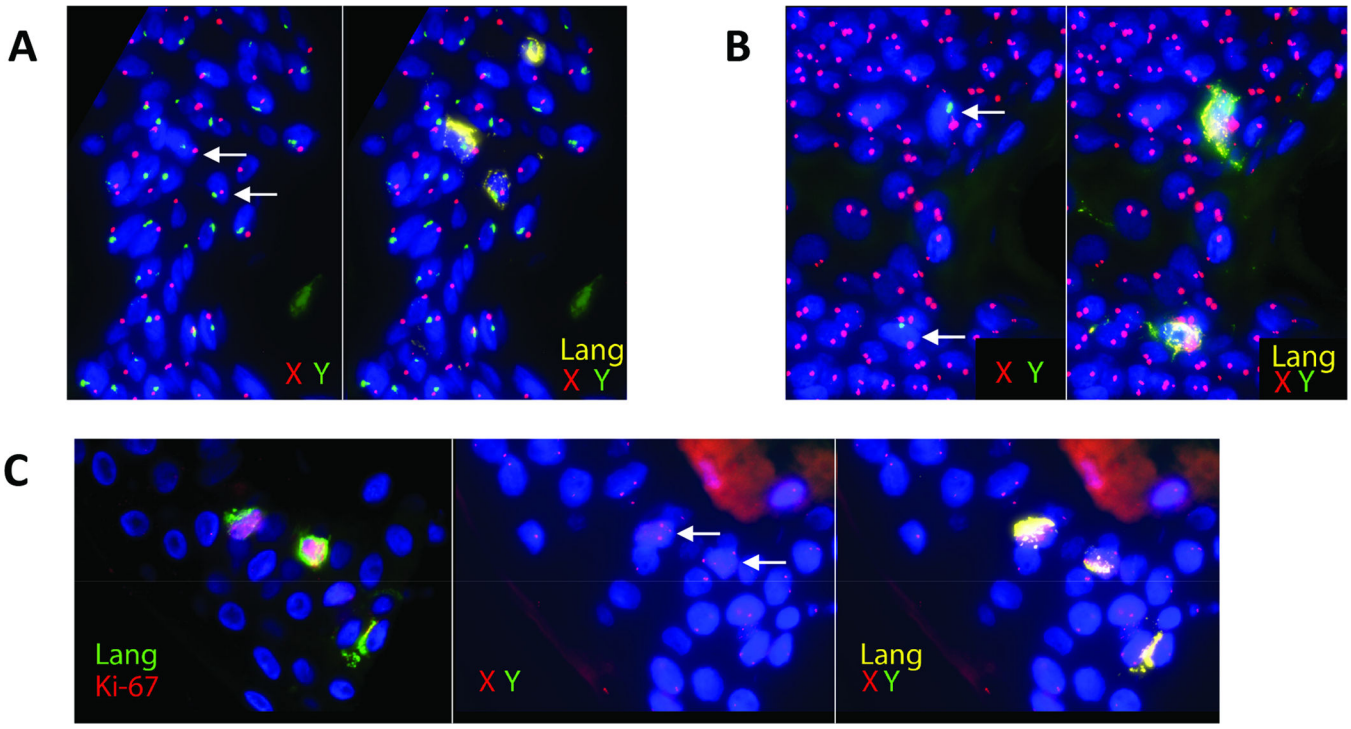

Figure 1. Assessment of Langerhans cell chimerism and proliferation after allogeneic hematopoietic cell transplantation

A) Day 28 after nonmyeloablative conditioning and transplantation with a female donor and male recipient. XY-FISH (left panel) combined with anti-Langerin antibody (right panel) demonstrate persistent male recipient Langerhans cells in the epidermis (white arrows).

B) Day 84 after nonmyeloablative conditioning and transplantation with a male donor and female recipient. XY-FISH (left panel) combined with anti-Langerin antibody (right panel) demonstrate male donor Langerhans cells in the epidermis (white arrows).

C) Day 28 after nonmyeloablative conditioning and transplantation with a male donor and female recipient. Pairs of recently divided Ki-67-positive Langerhans cells were identified by immunofluorescence, the positions noted and images recorded (left panel). Sections were then subjected to FISH and restaining with anti-Langerin as shown in (A) and (B). The same fields were then located to determine whether the proliferating cells were derived from the donor or recipient. In total, 23 Ki-67^{+} pairs of cells were identified at day 28 from 3 different patients and 23/23 (100%) were recipient-derived.

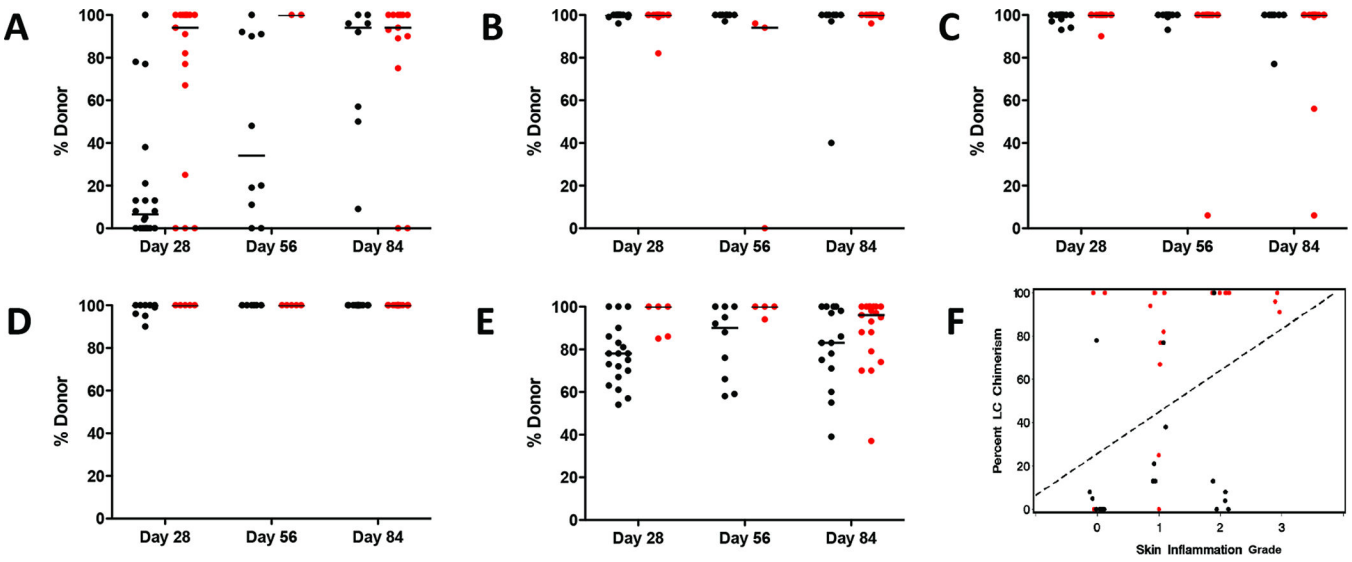

Figure 2. Langerhans cell chimerism and hematopoietic cell chimerism after allogeneic hematopoietic cell transplantation

A) The kinetics of donor Langerhans cells chimers after allogeneic HCT were assessed prospectively by combined XY-FISH and Langerin-immunohistochemistry as illustrated in Figure 1A and B. Patients were prepared with nonmyeloablative (black dots) or myeloablative (red dots) regimens. For each sample, at least two sections were examined and at least 20 cells with two sex chromosomes were counted. The median donor epidermal Langerhans cells chimerism was significantly lower after nonmyeloablative conditioning than after myeloablative conditioning (day 28: 7% *vs.* 95%, p=0.001; day 56: 34% *vs.* 100%, p=0.05; day 84: 94% vs. 97%, p=0.48). Horizontal lines indicate median values.

B–E) The chimerism of different cell populations was assessed prospectively by sorting cells from peripheral blood or aspirated marrow and subjecting them to VNTR-based chimerism analysis. Patients were prepared with nonmyeloablative (NA; black dots) or myeloablative (MA; red dots) regimens. (B) peripheral blood CD11c+ myeloid DCs; (C) bone marrow CD11c+ myeloid DCs; (D) peripheral blood CD33+ cells; (E) peripheral blood CD3+ T cells. Horizontal lines indicate median values. Except for median levels of CD3 T cell chimerism on day-28 (78% *vs.* 100%, p=0.02), conditioning intensity was not associated with statistically significant differences in levels of chimerism for these populations.

F) Correlation between skin inflammation score and degree of donor Langerhans cell chimerism on day 28 after transplantation. The degree of skin inflammation was assessed as described in *Patients and Methods* and correlated with percentages of donor Langerhans cell chimerism (p=0.009). Data were derived from patients prepared with nonmyeloablative (black dots) or myeloablative (red dots) regimens.

Table 1

Recipient and donor characteristics, GVHD and skin pathology of study subjects

		Conditioning Intensity	
	All Patients	**Nonmyeloablative***	**Myeloablative†**
N	48	23	25
Median age (range), years	51 (22–72)	57 (37–72)	44 (22–63)
Median follow up (range), days	461 (73–1113)	518 (73–1113)	370 (88–1108)
Female/Male Recipient, n	21/27	8/15	13/12
Diagnosis, n			
Acute myeloid leukemia	20	5	15
Myelodysplastic syndrome	8	3	5
Chronic myeloid leukemia	3	-	3
Acute lymphoblastic leukemia	3	1	2
Lymphoma	10	10	-
Multiple myeloma	4	4	-
Donor Type, n			
HLA-identical sibling	17	9	8
HLA-mismatched related	4	1	3
HLA-matched unrelated	21	9	12
HLA-mismatched unrelated	6	4	2
Immunosuppression, n			
Calcineurin-inhibitor +MTX‡	25	-	25
Calcineurin-inhibitor + MMF§	23	23	-
Acute GVHD			
Grade II–IV, n	36	19	17
Grade III–IV, n	4	4	-
Skin involvement, n	24	15	9
Skin grade >2, (%)	56	68	41
Median onset (range), days	31 (6–74)	34 (15–74)	27 (6–74)
Chronic GVHD			
Requiring systemic treatment, n	19	7	12
Skin involvement, n	16	6	10
Median onset (range), days	183 (91–367)	207 (91–367)	183 (126–323)
Day 28 LC chimerism			
% donor, median (range)	23 (0–100)	7 (0–100)	95 (0–100)
% of patients with <20% donor LCs	50	80	20
Day 28 skin pathology			
Median acute GVHD grade (range)	1 (0–2)	0 (0–2)	1 (0–2)

	All Patients	Conditioning Intensity	
		Nonmyeloablative*	Myeloablative†
% patients with grade >0	55	45	65
Median inflammation grade (range)	1 (0–3)	1 (0–2)	1 (0–3)
% patients with grade >0	65	58	75

*Nonmyeloablative conditioning (n=23): TBI 2 Gy, fludarabine 90 mg/m^2 (n=15); TBI 3–4 Gy, fludarabine 90 mg/m^2 (n=3); TBI 2 Gy only (n=2); TBI 2 Gy, fludarabine, 90 mg/m^2, ^{90}Y-ibritumomab, 0.4 mCi/Kg (n=3).

†Myeloablative conditioning (n=25): Busulfan, ≥ BU 8.0 mg/kg PO or ≥ BU 6.4 mg/kg IV, with cyclophosphamide, 120 mg/kg (n=11); TBI 10–14.4 Gy with cyclophosphamide, 120 mg/kg (n=7); TBI 2 Gy; fludarabine, 120 mg/m^2 with ^{131}I-conjugated-anti-CD45 and cyclophosphamide 69 mg/kg (n=2); fludarabine, 120 mg/m^2 with treosulfan, 42 g/m^2 (n=5).

‡Cyclosporine or tacrolimus twice daily PO or IV (whole blood target levels, 120–360 ng/mL and 5–15 ng/mL, respectively) from day −1 until day +50. In the absence of GVHD, calicineurin-inhibitors were tapered from day +50 through day +180; methotrexate IV at a dose of 15 mg/m^2 on day +1 and at 10 mg/m^2 on days +3, +6 and +11 (21,22).

§Cyclosporine or tacrolimus twice daily PO or IV (whole blood target levels, 120–360 ng/mL and 5–20 ng/mL, respectively) from day −3 until day +180. In the absence of GVHD, calcineurin-inhibitors were tapered from day +56 through day +180; mycophenolate mofetil (MMF), 15mg/kg PO twice (related donors) or thrice (unrelated donors) daily, from day 0 to day +27. For recipients of unrelated grafts, MMF prophylaxis was typically extended until 40 to 180 days after HCT, according to specific protocols (23).