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Depletion of host Langerhans cells before transplantation of donor alloreactive T cells prevents skin graft-versus-host disease

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Abstract

Skin is the most commonly affected organ in graft-versus-host disease (GVHD). To explore the role of Langerhans cells in GVHD, the principal dendritic cells of the skin, we studied the fate of these cells in mice transplanted with allogeneic bone marrow. In contrast to other dendritic cells, host Langerhans cells were replaced by donor Langerhans cells only when donor T cells were administered along with bone marrow, and the extent of Langerhans cell chimerism correlated with the dose of donor T cells injected. Donor T cells depleted host Langerhans cells through a Fas-dependent pathway and induced the production in skin of CCL20, which was required for the recruitment of donor Langerhans cells. Administration of donor T cells to bone marrow–chimeric mice with persistent host Langerhans cells, but not to mice whose Langerhans cells had been replaced, resulted in marked skin GVHD. These findings indicate a crucial role for donor T cells in host Langerhans cell replacement, and show that host dendritic cells can persist in nonlymphoid tissue for the duration of an animal's life and can trigger GVHD despite complete blood chimerism.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Allogeneic bone marrow or mobilized blood stem cell transplantation is the treatment of choice for patients with certain malignancies and other life-threatening conditions. Extensive use of this approach has been restricted, however, by the occurrence of GVHD, a frequent and serious complication of bone marrow transplantation^{1–3}. Although the cellular and molecular mechanisms that underlie GVHD have not been completely elucidated, alloreactive donor T lymphocytes are known to be essential effector cells^{1,2,4}, whereas host cells have been postulated to be the key stimulators of donor T cells^{5,6}.

Skin, gut and liver are the organs targeted in GVHD, with the skin being the most frequently affected². One cell type that may have an important role in skin GVHD is the Langerhans cell⁷, the major antigen-presenting cell in the skin^{7,8}. Like other dendritic cells, Langerhans cells take up and process antigens in their environment, then travel through lymphatic vessels to the draining lymph nodes, where they present antigenic determinants to T lymphocytes^{9,10}. We recently discovered that Langerhans cells self-renew in the skin throughout life, and are replaced by bone marrow–derived precursors only during inflammatory injury¹¹. The remarkable stability of Langerhans cells in the skin contrasts with dendritic cell populations in other organs, which undergo constant replacement by circulating bone marrow–derived precursors.

Because replacement of host Langerhans cells by donor-derived cells may have a profound effect on the outcome of GVHD in the skin, we decided to explore the fate of host Langerhans cells in mice transplanted with allogeneic bone marrow or purified hematopoietic stem cells (HSCs). We found that Langerhans cells remain of host origin for at least 18 months after transplantation of a purified population of allogeneic HSCs or T-cell-derived (TCD) bone marrow, but are replaced by donor-derived Langerhans cells if donor T cells are administered together with bone marrow. By contrast, dendritic cells in the blood, liver and lymphoid organs were replaced by 4 weeks, whether or not donor T cells were added to the graft. Most importantly, in mice with persistent host Langerhans cells after transplantation with TCD bone marrow, but not in mice lacking such cells, injection of donor T cells induced marked skin GVHD.

RESULTS

Langerhans cell chimerism

To analyze Langerhans cell chimerism in the skin of mice transplanted with fully major histocompatibility complex (MHC)-mismatched allogeneic bone marrow, we reconstituted lethally irradiated BALB/c mice with either whole bone marrow, purified HSCs, TCD bone marrow or TCD bone marrow in combination with peripheral blood T cells (all obtained from C57BL/6 mice). Langerhans cells remained of host origin for at least 12 months after transplantation of a purified population of allogeneic stem cells or TCD bone marrow (Fig. 1a). By contrast, host Langerhans cells were largely replaced by donor-derived Langerhans cells when we added donor T cells to the TCD bone marrow graft or when we transplanted whole bone marrow, which contains ~2% mature T cells¹² (Fig. 1a). These results suggest that alloreactive T cells are required for the recruitment of circulating donor Langerhans cell precursors to the skin. Other DC populations, however, were replaced by donor cells by 4

weeks after transplantation, whether or not the graft contained T cells (Fig. 1b). Allogeneic T cells obtained from bone marrow, spleen or peripheral blood were equally able to induce Langerhans cell chimerism (Fig. 1c), and they did so in a dose-dependent manner (Fig. 1d,e). Consistent with other reports⁴, we observed skin GVHD only in mice that received donor T cells together with their grafts (data not shown).

Role of cytotoxic T cells in Langerhans cell chimerism

To explore whether cytotoxic T cells are required for Langerhans cell chimerism, we transplanted BALB/c mice with C57BL/6 TCD bone marrow supplemented with peripheral blood wild-type T cells, T cells deficient for the perforin cytotoxic-dependent pathway (*Prf1*^{-/-}) or Fas ligand-defective T cells isolated from mice homozygous for the generalized lymphoproliferative disease (*gld*) mutation. *Prf1*^{-/-} T cells, like wild-type T cells, induced complete depletion of host Langerhans cells by week 5 after transplantation, followed by strong recruitment of donor-derived Langerhans cells (Fig. 2a,b). By contrast, *gld* T cells were substantially less efficient at inducing host Langerhans cell depletion; 40–60% of host Langerhans cells were still present in the skin 5 weeks after transplantation (Fig. 2a,b). The extent of Langerhans cell chimerism induced by *gld* T cells was lower than that induced by wild-type or *Prf1*^{-/-} T cells (Fig. 2a,b). All T-cell populations, including *gld* T cells, were able to infiltrate the skin of BALB/c recipient mice (Fig. 2c), but mice that received *gld* T cells experienced less skin GVHD compared with those that received *Prf1*^{-/-} or wild-type T cells (Fig. 2d–g).

Lymph node DC chimerism after allogeneic transplantation

To assess whether the persistence of host Langerhans cells in the skin leads to the persistence of dendritic cells in the draining lymph nodes, we analyzed the proportion of remaining host dendritic cells in skin and non-skin-draining lymph nodes of recipient mice. Four months after transplantation, mice with no Langerhans cell chimerism (TCD bone marrow recipients) or partial Langerhans cell chimerism (TCD bone marrow + *gld* T-cell recipients) had a higher proportion of host dendritic cells in skin-draining (inguinal) lymph nodes (20% and 3%, respectively, of the total inguinal DC population), compared with mesenteric lymph nodes from the same mice or skin-draining lymph nodes from mice that received wild-type (0.4%) or *Prf1*^{-/-} T cells (0.3%; Fig. 2h). These differences persisted 8 months after transplantation (Fig. 2h).

Skin chemokine profile

We have shown that the proinflammatory chemokines CCL2 (monocyte chemoattractant protein-1; MCP-1) and CCL7 (MCP-3) have a major role in the rapid trafficking of circulating Langerhans cell precursors to injured skin¹¹. CCL20 (macrophage inflammatory protein-3α) is another chemokine that attracts human CD34⁺ cell-derived Langerhans cells *in vitro*¹³, but its role *in vivo* has not been clarified. One week after transplantation, CCL20, CCL2 and CCL7 transcript levels were 10, 17 and 13 times higher, respectively, in the skin of mice that received donor T cells compared with those that did not receive T cells (Fig. 3a). CCL20 transcripts remained at similar levels 3 weeks after transplantation, whereas CCL2 and CCL7 decreased around 2 weeks after transplantation (data not shown).

CCR2 chemokine ligands facilitate Langerhans cell chimerism

Because alloreactive T cells induced the secretion of CCR2 ligands, including CCL2 and CCL7, we wanted to know whether these chemokines also had a role in the recruitment of donor-derived Langerhans cell precursors to the skin after allogeneic bone marrow transplantation. Mice reconstituted with 100% *Ccr2*^{-/-} bone marrow had Langerhans cell chimerism similar to that of mice reconstituted with *Ccr2*^{+/+} bone marrow (Fig. 3b). However, when mice were injected with a 1:1 mixture of *Ccr2*^{-/-} and *Ccr2*^{+/+} TCD bone marrow and wild-type peripheral blood T cells, *Ccr2*^{-/-} Langerhans cells were undetectable in the skin for at least 12 weeks after bone marrow transplantation (Fig. 3c), suggesting that CCR2 ligands facilitate but are not required for Langerhans cell recruitment to the skin after allogeneic bone marrow transplantation.

CCR6 ligands are required for Langerhans cell chimerism

CCR6 is the only known receptor for CCL20 (ref. 13). To analyze the role of CCL20 in Langerhans cell chimerism after allogeneic transplantation, we injected lethally irradiated BALB/c mice with *Ccr6*^{-/-} TCD bone marrow cells that are unable to respond to CCL20 chemokine gradients *in vivo*, together with wild-type peripheral blood T cells. Notably, 6 weeks after transplantation, mice reconstituted with *Ccr6*^{+/+} TCD bone marrow had sixfold more donor Langerhans cells compared with mice reconstituted with *Ccr6*^{-/-} TCD bone marrow (Fig. 3d). The difference in Langerhans cell chimerism between the two groups persisted 12 weeks after transplantation (Fig. 3d). The chimerism defect associated with *Ccr6*^{-/-} or *Ccr2*^{-/-} bone marrow was limited to the Langerhans cell population, as both *Ccr2*^{-/-} and *Ccr6*^{-/-} bone marrow contributed to splenic dendritic cells, circulating monocytes, T cells (Fig. 3e,f) and B cells (data not shown) with the same efficiency as did wild-type bone marrow cells.

Although skin-homing T cells express high levels of CCR6 and respond to CCL20 *in vitro*¹⁴, our results indicate that *Ccr6*^{-/-} and *Ccr6*^{+/+} T cells were similarly able to infiltrate the skin of recipient mice and induce complete depletion of host Langerhans cells and recruitment of donor Langerhans cells (Supplementary Fig. 1 online). Both *Ccr6*^{-/-} and *Ccr6*^{+/+} T cells were also able to induce skin GVHD (Supplementary Fig. 1). These results suggest that the defect of Langerhans cell chimerism observed in mice transplanted with *Ccr6*^{-/-} bone marrow is caused by defective Langerhans cell precursors, and that a similar defect in peripheral blood T cells does not interfere with the replacement of host Langerhans cells by donor Langerhans cells.

Role of host Langerhans cells in induction of skin GVHD

To explore the roles of host and donor Langerhans cells in skin GVHD, we reconstituted BALB/c mice with either TCD bone marrow or whole bone marrow from C57BL/6 mice. Although both groups had complete chimerism of white cells in the blood, as described above, Langerhans cell chimerism was achieved only in mice that received whole bone marrow. Eight weeks later, both groups were lethally irradiated again and injected with TCD bone marrow, together with peripheral blood T cells isolated from C57BL/6 mice. After this procedure, donor T cells progressively and selectively accumulated in the skin of mice that had persistent host Langerhans cells (Fig. 4a). Five weeks after the second transplant, the

percentage of T cells in the skin of those mice had increased by more than 15-fold. By contrast, in mice without host Langerhans cells, the percentage of T cells in skin declined to less than one-third of pretransplant levels. In addition, in mice with persistent host Langerhans cells, delayed administration of donor T cells resulted in the complete depletion of host Langerhans cells and induced the recruitment of donor Langerhans cells (Fig. 4b). Most importantly, mice that had persistent host Langerhans cells before the second transplant developed marked (grade 4) skin GVHD (Fig. 4c). By contrast, mice that had only donor Langerhans and dendritic cells in skin-draining lymph nodes did not develop any skin lesions (Fig. 4d).

To determine whether these results could be therapeutically exploited to protect against skin GVHD in patients, we exposed mice that were previously reconstituted with TCD bone marrow to ultraviolet light¹¹. Forty-five days later, the mice underwent a second transplant with allogeneic TCD bone marrow and alloreactive T cells (Fig. 5a). In mice previously reconstituted with TCD bone marrow and exposed to ultraviolet light, host Langerhans cells were almost completely depleted (0.15% epidermal cells) compared with mice not exposed to ultraviolet light (3.15% epidermal cells; Fig. 5b and Table 1). In mice that had not been reconstituted with TCD bone marrow, ultraviolet light induced the depletion of epidermal Langerhans cells (data not shown), followed by their replacement with endogenous bone marrow-derived Langerhans cells, which remained in the epidermis at the time of the second transplant (Fig. 5b and data not shown). Notably, mice reconstituted with TCD bone marrow and not subsequently exposed to ultraviolet light, as well as ultraviolet-treated BALB/c mice that were not reconstituted with TCD bone marrow, developed severe skin GVHD (Fig. 5c,d and Table 1). By contrast, mice that were reconstituted with TCD bone marrow and then exposed to ultraviolet light developed little or no skin GVHD (Fig. 5e and Table 1).

DISCUSSION

The failure of circulating hematopoietic precursors to replace host Langerhans cells in recipients of allogeneic bone marrow transplants was demonstrated in two different experimental models. First, Langerhans cells remained of host origin for at least 12 months after transplantation of a purified population of allogeneic HSCs. Second, more than 18 months after transplantation with allogeneic TCD bone marrow, Langerhans cells were not replaced by donor precursors, whereas all other DC populations, including liver, blood and dendritic cells in lymphoid organs, were of donor origin within 3 to 4 weeks. The difference between our results and those of earlier studies, which showed that Langerhans cells are rapidly replaced by donor-derived Langerhans cells after allogeneic bone marrow transplantation^{15,16}, seems to be related to the presence or absence of donor T cells in the bone marrow graft. When mice were reconstituted with purified stem cells or TCD bone marrow, host Langerhans cells were never replaced by donor precursors. By contrast, Langerhans cells were always replaced by donor precursors when whole bone marrow (which contains 2% mature T cells) was transplanted or when allogeneic T cells were added to TCD bone marrow grafts. Because whole allogeneic bone marrow was used in these earlier studies^{15,16}, the Langerhans cell chimerism that ensued was probably caused by the presence of donor T cells in the bone marrow allograft. Consistent with this interpretation,

we found that the level of Langerhans cell chimerism increased with the dose of coadministered allogeneic T cells.

Studies of Langerhans cell chimerism after allogeneic bone marrow transplantation are difficult in humans because of the lack of easily identifiable markers that distinguish donor from recipient Langerhans cells. Nonetheless, an elegant study analyzed Langerhans cell chimerism in eight children with MHC class II deficiency (bare lymphocyte syndrome) transplanted with allogeneic bone marrow, and searched for donor MHC II⁺ Langerhans cells in the skin after transplantation¹⁷. Consistent with our findings, donor Langerhans cells were not detected in the two children reconstituted with TCD allogeneic bone marrow, despite unequivocal engraftment of donor cells in the peripheral blood and dermis, whereas donor Langerhans cells were found in children who received whole allogeneic bone marrow grafts. Although these results must be confirmed in a larger number of patients, they suggest that in humans as well as mice Langerhans cell chimerism after allogeneic bone marrow transplantation is induced only in the presence of alloreactive T cells.

We previously reported that the recruitment of circulating Langerhans cell precursors depends not only on available Langerhans cell sites in the epidermis, but also on the secretion of proinflammatory chemokines capable of attracting bone marrow–derived Langerhans cell precursors from the blood to the skin¹¹. As shown in the current study, allogeneic T cells not only create available Langerhans cell sites by depleting host Langerhans cells, but also induce the secretion in the skin of proinflammatory chemokines including MCP and CCL20. Our results reveal for the first time an important role for CCL20 in the recruitment of host Langerhans cells *in vivo*. Low levels of donor Langerhans cells persisted for at least 12 weeks after transplantation of *Ccr6*^{−/−} TCD bone marrow, suggesting that the role of CCL20 in the trafficking of donor-derived Langerhans cell precursors to the skin is not redundant. CCL20 induces the migration of human CD34⁺ progenitor–derived Langerhans cells *in vitro*¹³, and CCL20 is increased in inflamed human skin¹⁴. A role for CCL20 in the recruitment of Langerhans cells would seem to be at odds with the finding that *Ccr6*^{−/−} mice have normal levels of Langerhans cells in their skin¹⁸. However, our observation that CCL20 is important for Langerhans cell recruitment to inflamed, but not to normal, skin seems to reconcile these findings. Although skin-homing T cells express high levels of CCR6 and respond to CCL20 *in vitro*¹⁴, we found that T cells, in contrast to Langerhans cell precursors, do not require CCR6 to infiltrate the skin.

Consistent with our study of ultraviolet light–induced skin injury after congenic bone marrow transplantation¹¹, *Ccr2*^{−/−} TCD bone marrow transplanted alone gave rise to wild-type levels of Langerhans cells. By contrast, when *Ccr2*^{−/−} TCD bone marrow was transplanted with an equal amount of *Ccr2*^{+/+} TCD bone marrow, *Ccr2*^{−/−} progenitors consistently failed to give rise to Langerhans cells, indicating that *Ccr2*^{−/−} Langerhans cell precursors cannot compete with *Ccr2*^{+/+} Langerhans cell precursors to occupy the limited number of Langerhans cell sites in skin. These results suggest that after allogeneic transplantation, the recruitment of donor Langerhans cells to recipient skin requires CCL20 chemokine induction in the skin and is facilitated by MCP chemokines. Cooperation of chemokines in the recruitment of leukocytes has previously been shown to be necessary for

B cells to enter lymph nodes¹⁹. Entry of Langerhans cell precursors into inflamed skin seems to be another example of this phenomenon.

Several studies have shown that dendritic cells in different tissues can migrate to draining lymph nodes in the absence of inflammatory signals^{20–24}. Langerhans cells are also thought to constitutively migrate to draining lymph nodes to give rise to lymph node dendritic cells^{25–27}. This suggests that in the absence of Langerhans cell chimerism, host dendritic cells should be detectable in skin-draining lymph nodes but not in other lymphoid tissues. Accordingly, 8 months after transplantation of TCD bone marrow alone, the frequency of host-derived dendritic cells in skin-draining lymph nodes was as high as 20%, which is consistent with the reported frequency of dendritic cell–derived Langerhans cells in human lymph nodes, whereas host dendritic cells were absent in the other lymphoid tissues examined. In addition, no host dendritic cells were detected in the skin-draining lymph nodes of mice with complete Langerhans cell chimerism. Together, these results provide further evidence for the constitutive migration of Langerhans cells to draining lymph nodes.

The observation that high levels of host Langerhans cells in the skin and high levels of host dendritic cells in skin-draining lymph nodes persist in mice after TCD bone marrow transplantation has important implications for the development of GVHD in patients who undergo this procedure. The results of the present study show that after allogeneic transplantation, dendritic cell chimerism in the blood does not correlate with dendritic cell chimerism in skin, suggesting that host dendritic cells may persist in patients after allogeneic bone marrow transplantation even if they are found to be fully chimeric in the blood. To test whether host tissue dendritic cells that persist in complete bone marrow and blood chimeric mice can have a role in GVHD, we administered donor T cells together with TCD bone marrow to mice with only host or donor Langerhans cells in the skin. Notably, the donor T cells induced skin GVHD in mice with persistent host Langerhans cells, but not in mice in which host Langerhans cells had already been replaced by donor Langerhans cells. Although these results provide the first direct evidence that host Langerhans cells induce GVHD, previous studies have shown that host dendritic cells are essential for both the activation^{5,6,28} and effector phases of acute GVHD²⁹. In the latter study, donor T cells induced GVHD only if host hematopoietic cells were present in target tissues²⁹. In this regard, host Langerhans cells persisting in skin may be responsible for the continued activation of donor T cells and for the production of cytokines that are crucial for the tissue damage observed in GVHD²⁹. We also found that in mice that had predominantly host Langerhans cells after reconstitution with allogeneic TCD bone marrow, exposure to ultraviolet light induced the depletion of host Langerhans cells and rendered the mice resistant to skin GVHD. By contrast, control mice in which the majority of Langerhans cells were of host origin developed severe GVHD. Protection against skin GVHD was not related to a nonspecific effect of ultraviolet light, as nontransplanted BALB/c mice developed severe skin GVHD after injection of allogeneic T cells despite their exposure to ultraviolet light.

Chronic GVHD with involvement of the skin is a common complication of allogeneic bone marrow transplantation³⁰. Because we found that Langerhans cell replacement is dependent on the number of donor T cells present in the graft, varying degrees of Langerhans cell

chimerism can be expected in bone marrow–transplanted patients. Indeed, host Langerhans cells have been found in the skin of patients more than 1 year after transplantation³¹. Phototherapy using ultraviolet light improves cutaneous lesions but not other lesions of chronic GVHD³⁰. It will thus be interesting to determine whether the improvement of skin GVHD observed in phototherapy-treated patients is related to the induction of Langerhans cell chimerism in the skin.

Our results reveal new information about the cellular and molecular mechanisms leading to Langerhans cell chimerism after allogeneic bone marrow transplantation. Alloreactive T cells induce Langerhans cell chimerism through two successive checkpoints: Fas ligand–dependent depletion of host Langerhans cells, and induction in the skin of CCL20 and MCP inflammatory chemokines that cooperate in the recruitment of circulating donor Langerhans cells. Our demonstration that depletion of host Langerhans cells by ultraviolet light before injection of alloreactive T cells protects against skin GVHD establishes the basis for clinical trials testing the beneficial effect of ultraviolet light in patients who are candidates for allogeneic bone marrow transplants.

METHODS

Mice

We purchased 5- to 7-week-old male C57BL/6 (CD45.2⁺H-2^b), C57BL/6 (CD45.1⁺H-2^b), C57BL/6 Fas ligand–defective (*gld*) mice³² and C57BL/6 perforin-deficient (*Prf1*^{−/−})³³ mice from The Jackson Laboratory. Seven- to eight-week-old male BALB/c (H-2^d) mice were obtained from the Stanford University Department of Comparative Medicine. C57BL/6 *Ccr6*^{−/−} and C57BL/6 *Ccr2*^{−/−} mice were generated as described (refs. 18,34, respectively). The Stanford University Committee on Animal Welfare approved all mouse protocols.

Cytokines and antibodies

We purchased recombinant mouse granulocyte-macrophage colony-stimulating factor and tumor necrosis factor- α from PeproTech. We purchased all monoclonal antibodies from PharMingen, except the monoclonal antibody to Thy1.2, which we purchased from Caltag.

Allogeneic stem cell and bone marrow transplantation

We lethally irradiated BALB/c recipient mice with 800 rad using an X-ray source, then injected them intravenously with 1×10^3 purified HSCs, or 2×10^6 or 25×10^6 TCD bone marrow cells isolated from C57BL/6 mice. We purified HSCs using a protocol described elsewhere³⁵. We obtained TCD bone marrow as described previously³⁶. Where indicated, we lethally irradiated BALB/c mice (800 rad) and injected then with 25×10^6 whole bone marrow cells isolated from C57BL/6 mice.

Isolation of T cells

We purified TCR- $\alpha\beta$ CD8⁺CD4⁺ triple-positive and propidium iodide (PI)-negative cells from peripheral blood or spleen using a FACS Vantage flow cytometer (Becton Dickinson). To prepare bone marrow T cells, we purified TCR- $\alpha\beta$ ⁺ cells by FACS from an enriched Thy1.2⁺ bone marrow cell population. The purity of each T-cell preparation was >98%, as

assessed by flow cytometry. We used a low dose of donor T cells (1.5×10^5) in most of the transplantation experiments to avoid early death due to acute GVHD, and to study the Langerhans cell chimerism that occurs mainly between 5 and 9 weeks after transplantation.

Isolation and characterization of skin cells

We isolated skin cells as described elsewhere¹¹. We determined the percentage of epidermal cells that were host-derived (I–A^d) or donor-derived (I–A^b) Langerhans cells by flow cytometry, gating on CD45⁺PI[–] epidermal cells. We determined the percentage of donor TCRαβ⁺ skin-infiltrating T cells by gating on PI[–] skin cells.

Isolation of spleen, liver and lymph node dendritic cells

We harvested spleen, liver and lymph nodes and injected them with collagenase D (1 mg/ml; Boehringer Mannheim) for 45 min (spleen and lymph nodes) or 90 min (liver) at 37 °C. We resuspended digested organs in medium and layered the cells above a Nycoprep solution (Nycomed). We collected the enriched dendritic cells at the interface and determined the proportion of host and donor dendritic cells by flow cytometry, gating on CD11c⁺ cells.

Histopathologic examination of tissues

We embedded formalin-fixed tissues in paraffin, cut them into 4-μm sections and stained them with H&E. We blindly coded all histopathology slides, and a pathologist graded the GVHD lesions as described³⁷.

Quantitative analysis of chemokine RNA expression in the skin

We extracted total RNA from bulk homogenized skin cells. We quantitatively analyzed chemokines and cyclophilin transcripts by the fluorogenic SYBR green PCR assay, using an MX4000 Multiplex Quantitative PCR System (Stratagene). We normalized relative RNA levels to cyclophilin and expressed the results as a ratio of chemokine to cyclophilin, which allowed us to determine the fold increase of relative RNA levels between transplanted and nontransplanted BALB/c mice.

Transplantation of *Ccr6*^{–/–} or *Ccr2*^{–/–} cells

We reconstituted lethally irradiated BALB/c mice with TCD bone marrow cells that were *Ccr6*^{–/–}, *Ccr6*^{+/+}, *Ccr2*^{–/–} or *Ccr2*^{+/+}, or a 1:1 mixture of *Ccr2*^{–/–} CD45.2⁺ and *Ccr2*^{+/+} CD45.1⁺ or *Ccr2*^{+/+} CD45.2⁺ and *Ccr2*^{+/+} CD45.1⁺. We administered 2×10^6 TCD bone marrow cells either alone or in combination with wild-type peripheral blood T cells (all isolated from C57BL/6 mice). Where indicated, we injected BALB/c mice intravenously with 2×10^6 TCD bone marrow cells isolated from wild-type CD45.1⁺ C57BL/6 mice, alone or in combination with peripheral blood T cells isolated from *Ccr6*^{–/–} or *Ccr6*^{+/+} CD45.2⁺ C57BL/6 mice.

Repeat transplantation

We reconstituted lethally irradiated BALB/c mice (ten mice per group) with 2×10^6 TCD bone marrow cells or 25×10^6 whole bone marrow isolated from C57BL/6 mice. Eight weeks later, we lethally irradiated the mice again (800 rad) and injected them intravenously

with 2×10^6 TCD bone marrow cells together with 2.5×10^5 spleen T cells isolated from C57BL/6 mice.

Induction of Langerhans cell chimerism before injection of allogeneic T cells

In two separate experiments, we reconstituted groups of three or five BALB/c mice with TCD bone marrow or whole bone marrow. Two weeks after transplantation, we either left the transplanted mice untreated, or exposed transplanted and nontransplanted BALB/c mice to ultraviolet light (wavelength 254 nm, voltage 8 W, ultraviolet source 38 cm from the target) for 30 min. Three weeks later, we analyzed Langerhans cell chimerism in the epidermis by flow cytometry. Eight weeks after the first transplant (that is, 6 weeks after exposure to ultraviolet light), we lethally irradiated the mice (800 rad) again and reconstituted them with 5×10^6 TCD bone marrow cells together with 2×10^5 spleen T cells isolated from C57BL/6 mice.

Statistical analysis

We compared GVHD scores between the treatment groups and tested the differences for statistical significance using the Student *t* test. We set the level of significance at $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Host Langerhans cells (LCs) persist in the skin after allogeneic stem cell transplantation. **(a)** BALB/c mice were reconstituted with low or high dose of TCD bone marrow (BM), purified HSCs, whole bone marrow or TCD bone marrow, together with peripheral blood (PB) T cells. Contour plots show percent of host and donor Langerhans cells in CD45⁺-gated epidermal cells after transplantation (Tx). **(b)** Percent of donor dendritic cells (DCs) and circulating monocytes (Mo) 4 weeks after transplantation. Mean of four separate experiments is shown. **(c)** Percent of host- and donor-derived Langerhans cells, 6–9 weeks after transplantation of TCD bone marrow alone or together with T cells purified from bone marrow, peripheral blood or spleen. **(d,e)** Epidermal cells were isolated 9 weeks after transplantation of 2×10^6 TCD bone marrow together with either 3×10^4 or 2×10^5 peripheral blood T cells. Contour plots **(d)** and bar graph **(e)** show percent of host- and donor-derived Langerhans cells. Results represent mean of two different experiments.

Figure 2.

Allogeneic T cells deplete host Langerhans cells (LCs) through a Fas ligand-dependent pathway. BALB/c mice were injected with TCD bone marrow (BM), alone or with *gld*, *Prf1*^{-/-} or wild-type (WT) peripheral blood T cells. (a) Proportion of donor and host Langerhans cells in epidermis 5 weeks after transplantation. (b,c) Percent host and donor Langerhans cells in epidermis (b), and donor T cells in skin (c) of recipient mice. Mean of three experiments is shown. (d-g) Histological examination 4 weeks after transplantation reveals normal skin in mice transplanted with TCD bone marrow alone (d) or together with *gld* T cells (e). Skin GVHD with necrotic keratinocytes and focal inflammatory infiltrate is seen in mice injected with *Prf1*^{-/-} T cells (f), and severe GVHD with numerous necrotic keratinocytes and predominantly lymphoid inflammatory infiltrate is seen in mice injected

with wild-type T cells (**g**). Magnification, $\times 200$. (**h**) Dot plots show percentage of host dendritic cells (DCs) among gated CD11c⁺ cells in mesenteric lymph nodes (MLNs) and peripheral skin-draining (inguinal) lymph nodes (PLNs) at 4 and 8 months after transplantation. One of two experiments is shown.

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Figure 3.

Expression of CCR2 and CCR6 on donor bone marrow (BM) cells is required for Langerhans cell (LC) chimerism. **(a)** Fold increase of relative levels of chemokine transcripts in skin of treated mice compared with nontransplanted BALB/c control mice. Data represent mean of two different experiments. **(b–d)** Mice were injected with either *Ccr2*^{-/-} or *Ccr2*^{+/+} **(b)**, a 1:1 mixture of CD45.2⁺ *Ccr2*^{-/-} and CD45.1⁺ *Ccr2*^{+/+} or CD45.2⁺ *Ccr2*^{+/+} and CD45.1⁺ *Ccr2*^{+/+} cells **(c)**, or *Ccr6*^{-/-} or *Ccr6*^{+/+} TCD bone marrow **(d)**, alone or in addition to wild-type (WT) T cells. Results shown are mean of three separate

experiments. **(e,f)** Mice were transplanted as described in **c** and **d**. Source of spleen dendritic cells (DCs), T cells and monocytes (Mo) was determined 5 weeks later.

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Figure 4.

Host Langerhans cells (LCs) that persist in chimeric mice induce severe skin GVHD.

BALB/c mice were reconstituted with whole C57BL/6 bone marrow (BM) or TCD bone marrow, lethally irradiated 8 weeks later, and injected again with TCD bone marrow and T cells from C57BL/6 mice. Plots show percentage of infiltrating donor T cells in skin (**a**) and percentage of host and donor LCs in CD45⁺-gated epidermal cells (**b**), before and 3 and 5 weeks after second transplantation (Tx). (**c,d**) Histological examination 4 weeks after second transplant reveals severe (grade 4) GVHD, characterized by extensive apoptosis of basal keratinocytes, vacuolization and infiltration of inflammatory cells, in mice that had only host Langerhans cells at time of second transplantation (**c**). No skin GVHD was observed in mice that had full Langerhans cell chimerism at time of the second transplant (**d**). Results shown are representative of two separate mice. Magnification, $\times 200$.

Figure 5.

Depletion of host Langerhans cells (LCs) prevents skin GVHD. **(a)** BALB/c mice were reconstituted with C57BL/6 TCD bone marrow (BM) and either left untreated or exposed to ultraviolet (UV) light. Six weeks after ultraviolet exposure, all mice were reconstituted with TCD bone marrow and T cells from C57BL/6 mice. **(b)** Contour plots show percentage of host and donor Langerhans cells in gated PI⁻ epidermal cells 3 weeks after exposure to ultraviolet light. **(c–e)** Skin sections obtained 3 weeks after second transplant and stained with H&E are shown at $\times 200$ magnification. Mice not previously reconstituted with TCD bone marrow but exposed to ultraviolet light **(c)** and mice previously reconstituted with TCD bone marrow, but not exposed to ultraviolet light **(d)**, show extensive mononuclear infiltrates in superficial dermis that focally infiltrate epidermis, associated with necrotic keratinocytes and vacuolization of basal keratinocytes (grade 2 GVHD). Mice previously reconstituted with TCD bone marrow and exposed to ultraviolet light **(e)** lack infiltrates in skin, and show only rare necrotic keratinocytes (grade 0.5 GVHD).

Table 1

Attenuation of skin GVHD with ultraviolet light

Groups	No. of mice	Donor cell engraftment	% Langerhans cell chimerism	Skin GVHD score
T cells + UV	8	1.0 ± 0.5	0.14 ± 0.11	2.6 ± 0.4
T cells + TCD BM	6	96 ± 0.4	1.22 ± 0.6	2.4 ± 0.48
T cells + TCD	6	95 ± 0.5	75 ± 5.0	0.5 ± 0.3*
BM + UV				

In two separate experiments, BALB/c mice were treated as described in Figure 5a. Donor cell engraftment was analyzed 2 weeks after the first transplant with TCD bone marrow (BM), and measured as percentage of H-2K^b cells in nucleated peripheral blood cells. Percentage of Langerhans cell chimerism was analyzed 3 weeks after ultraviolet exposure and measured as percentage of epidermal CD45⁺CD11c⁺I-Ab⁺ cells. GVHD in skin was scored 3 weeks after injection of donor T cells, as described in Methods. Data are presented as mean ± s.e.m.

*, $P < 0.01$ for skin GVHD scores in mice that received TCD BM + UV before second transplant, compared with control groups.