An inflammatory checkpoint regulates recruitment of graft–versus–host reactive T cells to peripheral tissues

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Transfer of T cells to freshly irradiated allogeneic recipients leads to their rapid recruitment to nonlymphoid tissues, where they induce graft–versus–host disease (GVHD). In contrast, when donor T cells are transferred to established mixed chimeras (MCs), GVHD is not induced despite a robust graft–versus–host (GVH) reaction that eliminates normal and malignant host hematopoietic cells. We demonstrate here that donor GVH–reactive T cells transferred to MCs or freshly irradiated mice undergo similar expansion and activation, with similar up-regulation of homing molecules required for entry to nonlymphoid tissues. Using dynamic two-photon in vivo microscopy, we show that these activated T cells do not enter GVHD target tissues in established MCs, contrary to the dogma that activated T cells inevitably traffic to nonlymphoid tissues. Instead, we show that the presence of inflammation within a nonlymphoid tissue is a prerequisite for the trafficking of activated T cells to that site. Our studies help to explain the paradox whereby GVH–reactive T cells can mediate graft–versus–leukemia responses without inducing GVHD in established MCs.

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Abbreviations used: BMT, BM transplantation; DLI, donor leukocyte infusion; GVH, graft-versus-host; GVHD, graft-versus-host disease; GVL, graft-versus-leukemia; MC, mixed chimera; TCD, T cell–depleted; TLR, Toll-like receptor.

Allogeneic BM transplantation (BMT) is an important treatment for hematological malignancies. The therapeutic potential of BMT relies on a graft–versus–leukemia (GVL) effect in which donor T cells eradicate tumor cells expressing host or tumor-associated antigens. Unfortunately, the GVL response is frequently associated with the development of graft-versus-host disease (GVHD). An understanding of the mechanisms that lead to GVHD is critical for optimizing this therapeutic modality.

The induction of GVHD requires the recruitment of effector T cells to peripheral tissues (1). After their transfer to freshly irradiated allogeneic recipients, naive donor T cells are initially retained within secondary lymphoid tissues (2). Here, they are activated by host APCs and undergo a rapid burst of proliferation (3, 4). After an interval of 3–4 d, large numbers of lymphoblasts enter the peripheral circulation and then exit to nonlymphoid tissues such as the skin and gut (2, 5, 6), where

they induce the profound tissue injury that characterizes GVHD.

It is well established that T cell activation leads to increased tropism for nonlymphoid tissues (7, 8) and changes in trafficking potential are likely to be an important prerequisite for the recruitment of graft–versus–host (GVH–reactive) T cells to peripheral tissues (1). However, it is not known whether such changes are sufficient for the induction of GVHD. Indeed, T cell reactivity against antigens expressed by the host does not automatically lead to tissue injury. For example, a state of "adaptive tolerance" in which rapid T cell proliferation is followed by an eventual state of unresponsiveness is observed in several model systems after transfer of transgenic T cells to hosts that ubiquitously express antigens recognized by the relevant TCR (for review see reference 9). In some cases, there is a lack of tissue destruction despite the marked infiltration of target organs by activated T cells (10, 11).

Importantly, T cell reactivity against host antigens may also be dissociated from damage

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to peripheral tissues under circumstances in which T cells are not tolerant. For example, although central, deletional tolerance to donor and recipient antigens is induced in established mixed chimeras (MCs; in which hematopoietic elements of both the donor and allogeneic recipient coexist) (12, 13), this state can readily be broken after the delayed transfer of naive, nontolerant donor T cells (14). In this situation, donor T cells induce a GVH reaction that eradicates normal and malignant host hematopoietic cells, but do so without inducing GVHD (14–16). In clinical studies, delayed transfer of T cells to allogeneic chimeras is also associated with a low risk of GVHD (17). The precise mechanisms underlying the lack of GVHD are not known but may relate to the resolution of the early inflammatory cascade induced by the conditioning protocol (18, 19). In this regard, nonmyeloablative protocols that generate low levels of tissue injury are associated with a reduced incidence of GVHD as compared with that observed after myeloablative conditioning (20).

To determine the role of inflammation in the development of GVHD, we examined the behavior of T cells after transfer to allogeneic recipients under various conditions. We first demonstrate that diminished alloreactivity does not explain the failure of donor leukocyte infusion (DLI)-derived T cells to induce GVHD in established MCs. Instead, there is marked clonal expansion of GVH-reactive T cells, which despite their presence within the peripheral circulation, fail to accumulate in skin or gut. This failure is not an intrinsic, absolute deficiency in skin or gut tropism because the cells induce severe GVHD after transfer to secondary, freshly irradiated recipients. Thus, recruitment of activated T cells to nonlymphoid organs is subject to tight regulation by T cell–extrinsic factors within the host. Finally, we demonstrate here a critical role for local inflammation in permitting entry of activated T cells to a GVHD target tissue.

RESULTS
Recruitment of alloreactive T cells to peripheral tissues depends upon the host environment
Transfer of small numbers of T cells on day 0 to freshly irradiated allogeneic recipients leads to severe, lethal GVHD. In contrast, when large numbers of T cells are transferred as delayed DLIs to established MCs, a GVH reaction occurs that eradicates host hematopoietic elements but does not lead to significant GVHD (14, 16). We first considered the possibility that intrinsic properties of alloreactive T cells are different in the two situations. To compare the donor T cell response elicited in these two contexts, we transferred 10^6 purified CD8$^+$ T cells from 2C transgenic mice together with 10^7 B6 CD45.1 donor splenocytes to established B6 CD45.2 + BALB/c→BALB/c MCs or to freshly irradiated BALB/c mice. The latter group of mice also received T cell–depleted (TCD) B6 BM to prevent death from marrow aplasia. 2C mice are transgenic for a high-affinity TCR that recognizes the L^d MHC class I alloantigen (21) expressed by the recipient strain. B6 splenocytes containing additional polyclonal B6 T cells were cotransferred to both groups to provide CD4

help, which is required for full expansion and/or survival of the transgenic CD8$^+$ T cells (22, 23) and to provide polyclonal CD8$^+$ T cells. Thus, 2C CD8$^+$ T cells were used as a known GVH-reactive marker population in the context of a "normal" GVH reaction.

As expected, lethally irradiated mice receiving allogeneic T cells on day 0 developed severe, lethal GVHD characterized by histological evidence of severe colitis (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20060376/DC1). In contrast, when these T cells were administered to established MCs, mice did not develop clinical GVHD and no histological evidence of colitis was observed at day 10 or thereafter. Skin of freshly irradiated mice also showed evidence of GVHD, whereas that from MCs did not (not depicted). MCs did, however, show sustained increases in donor chimerism, consistent with a donor T cell–mediated lymphohematopoietic GVH reaction (Fig. S1). Irradiated, syngeneic control mice, as expected, did not develop GVHD (not depicted).

We next examined T cell proliferation and tissue accumulation in these models. In both freshly irradiated mice and established MCs, we observed rapid proliferation (as evaluated by CFSE dilution) of the transferred 2C CD8$^+$ T cells within the recipient spleens (Fig. 1 A) and lymph nodes (not depicted). By day 6 after transfer to established MCs or freshly irradiated recipients, the proportion of the splenic 2C CD8$^+$ T cell population that had undergone seven or more divisions was 90.6 ± 1.9% and 99.4 ± 0.2%, respectively. In contrast, by day 6, only 7–8% of 2C CD8$^+$ T cells had undergone seven or more divisions after transfer to freshly irradiated syngeneic recipients (Fig. 1 A). We evaluated tissue accumulation by measuring absolute numbers of 2C CD8$^+$ T cells in the spleen, lymph nodes, blood, BM, and the intra-epithelial compartment of the small and large intestine at timed intervals after transfer to the two cohorts of allogeneic recipient mice (Fig. 1, B–E, and not depicted). In the freshly irradiated mice, increased numbers of 2C CD8$^+$ T cells were observed in the spleen, lymph nodes, BM, peripheral circulation, and the intra-epithelial compartment of the intestine, peaking at ∼3–6 d after transfer (Fig. 1, B–E, and not depicted). In the MCs, there was a delayed but dramatic increase in the numbers of 2C CD8$^+$ T cells in the spleen, peaking at ∼12 d after transfer (Fig. 1 B), with less expansion evident within the lymph nodes and BM (Fig. 1 C and not depicted). However, despite the presence of similar peak numbers within the peripheral circulation in these two groups (Fig. 1 D), 2C CD8$^+$ T cells failed to accumulate within the intra-epithelial compartment of the gut in MCs (Fig. 1 E). Similarly, polyclonal donor CD4$^+$ and CD8$^+$ T cells failed to accumulate in the intestines of established MCs, whereas large numbers of these cells were present in the intestines of freshly irradiated mice (not depicted). Only limited accumulations of 2C CD8$^+$ T cells occurred in the spleens and lymph nodes of freshly irradiated syngeneic control mice, but no significant accumulation was observed in the gut (Fig. 1, B, C, and E).
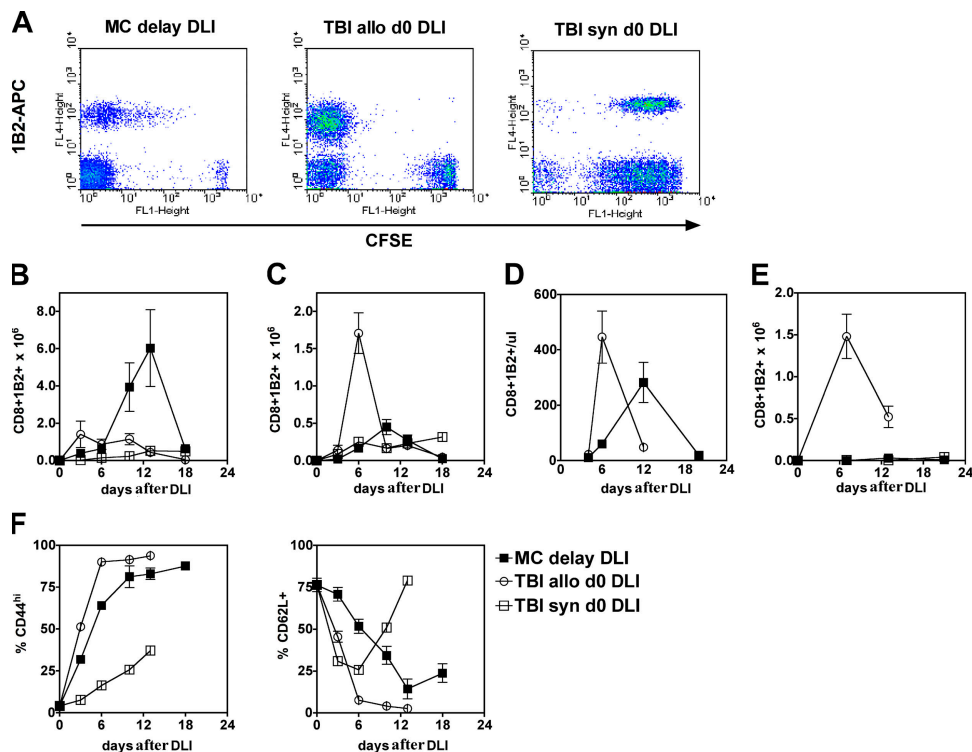
Figure 1. Distribution of antihost CD8+ CTLs between lymphoid and nonlymphoid tissues differs according to host environment. (A) Proliferation of transferred 2C CD8+ T cells as evaluated by CFSE dilution. Dot plots showing CFSE staining (x axis) versus staining with the clonotypic marker 1B2 (y axis) in gated CD8+ T cells derived from recipient spleen on day +6 after transfer to established MCs (MC), freshly irradiated allogeneic recipients (TBI allo), or syngeneic recipients (TBI syn). Note that the CFSE+1B2− population represents polyclonal CFSE-labeled B6 CD8+ T cells that were cotransferred. (B–E) Absolute numbers expressed as mean ± SEM (n = 3 mice per group, except syngeneic mice, which comprised 1–2 mice per group) of antihost 2C trans- genic CD8+ CTLs in the spleen (B), lymph nodes (C), peripheral blood (D), and intra-epithelial compartment of the intestine (E) after DLIs on day 0 to freshly irradiated allogeneic (○) or syngeneic mice (□), or to established MCs (■). Data shown in A–E are one of three independent experiments performed, which had similar results. Peripheral blood enu- meration was not performed in syngeneic control mice. (F) Mean per- centage (±SEM) of antihost 2C transgenic CD8+ CTLs in the spleen that were CD44high and CD62L+ after DLIs to MCs (■) and freshly irradiated allogeneic (○) or syngeneic mice (□). Data are representative of one out of three independent experiments.

The failure of GVH-reactive T cells to accumulate within GVHD target organs of established MC recipients could reflect either a reduced capacity of stimulated T cells to traffic to these sites or, alternatively, a failure of these cells to survive, proliferate, or be retained in these locations. To evaluate the role of trafficking, we first measured the expression of homing markers on splenic 2C CD8+ T cells from each group. Consistent with their activation in vivo, 2C CD8+ T cells derived from the spleens of mice in the two groups expressed a broadly similar CD44highCD62L− CD45RBlow CD27high phenotype by day 12, although there were differ- ences in the kinetics with which this phenotype was acquired (Fig. 1 F and not depicted). In contrast, after transfer to syn- geneic controls, 2C CD8+ T cells showed only moderate up- regulation of CD44 and transient, partial down-regulation of CD62L, consistent with the effects of lymphopenia-induced proliferation (24, 25). At the transcriptional level, we observed equivalent reductions in CCR7 expression in 2C CD8+ T cells after transfer to freshly irradiated mice and established

MCs as compared with naive cells (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20060376/DC1). Furthermore, although there were minor differences between the two groups, 2C CD8+ T cell expression of the effector cell–associated chemokine receptor CXCR3 increased sub- stantially in both groups compared with naive cells (Fig. S2). To examine whether "imprinting" mediated by intestinal DCs (26–28) was different between the two groups, we com- pared expression of the small intestinal homing receptor $\alpha_4\beta_7$ on 2C CD8+ T cells within meseneteric lymph nodes after transfer. The levels of $\alpha_4\beta_7$ expression were similar in 2C CD8+ T cells derived from mesenteric lymph nodes in both groups, and these levels were higher than those observed in naive T cells (Fig. S2). Thus, activated GVH-reactive T cells in both groups modified their expression of homing receptors to a similar extent.

We obtained very similar findings in another recipi- ent (BDF1) and donor (B6) strain combination. Thus, 2C CD8+ T cells underwent similar activation and expansion

in the spleens of B6 + BDF1→BDF1 MCs and freshly irradiated BDF1 mice (Fig. S3, which is available at http://www.jem.org/cgi/content/full/jem.20060376/DC1, and not depicted). In both groups, 2C CD8$^+$ T cells expressed a broadly similar CD44^{high} CD45RB^{low} CD62L$^-$ phenotype (not depicted). As for the BALB/c recipient and B6 donor combination, there was a complete absence of donor T cell accumulation within the gut of MCs and these recipients did not develop colitis (Fig. S3 and not depicted). Furthermore, similar expansions of donor T cells after DLIs were observed in MCs prepared with nonmyeloablative conditioning and those generated after lethal irradiation and reconstitution with donor and recipient TCD BM (not depicted).

To examine the trafficking of donor T cells to GVHD target tissues directly, we used intravital microscopy of the skin. In MCs, T cell transfer does not induce GVHD within the skin, whereas it does so in freshly irradiated mice (not depicted). To examine trafficking, we transferred 4×10^6 purified T cells derived from transgenic B6 GFP mice to freshly irradiated BDF1 mice or to established B6 + BDF1→BDF1 MCs. On days 5 and 12 after transfer, the ear pinnae of surviving recipient mice were imaged using two-photon microscopy. In freshly irradiated recipients, we observed marked accumulation of GFP$^+$ T cells, particularly in relation to hair follicles (Fig. 2, A and C). In contrast, when these cells were transferred to established B6 + BDF1→BDF1 MCs, the numbers of GFP$^+$ T cells accumulating within the skin were minimal (Fig. 2, B and D). To examine the behavior of T cells in real time, we used second harmonics to identify vessels that corresponded in size to postcapillary venules and observed the movement of GFP$^+$ T cells within them. In freshly irradiated recipients, large numbers of GFP$^+$ T cells demonstrated sticking or rolling to postcapillary venule walls on day 5 (Fig. 2 E and Video S1, which is available at http://www.jem.org/cgi/content/full/jem.20060376/DC1). In contrast, when identical numbers of cells were transferred to established B6 + BDF1→BDF1 MCs, the numbers of GFP$^+$ T cells rolling or sticking to the vessel walls were minimal on days 5 and 12 (Fig. 2 F and Video S2), despite the presence of similar numbers of these cells in the peripheral circulation. Thus, after transfer to MCs, alloantigen-activated T cells undergo marked clonal expansion and modify their expression of homing receptors but fail to enter nonlymphoid organs and induce GVHD.

### T cells primed after transfer to established MCs induce GVHD in freshly irradiated recipients

The behavior of activated T cell populations depends on both intrinsic characteristics that develop during initial priming and on extrinsic factors within the postpriming environment (29). One possible explanation for the failure of GVH–reactive T cells to enter the peripheral tissues of established MCs is that these cells have an intrinsic defect in tissue homing and/or function. To test this possibility, we transferred donor T cells activated in established MCs to secondary, lethally irradiated recipients (Fig. 3 A). Thus, 2.5×10^7
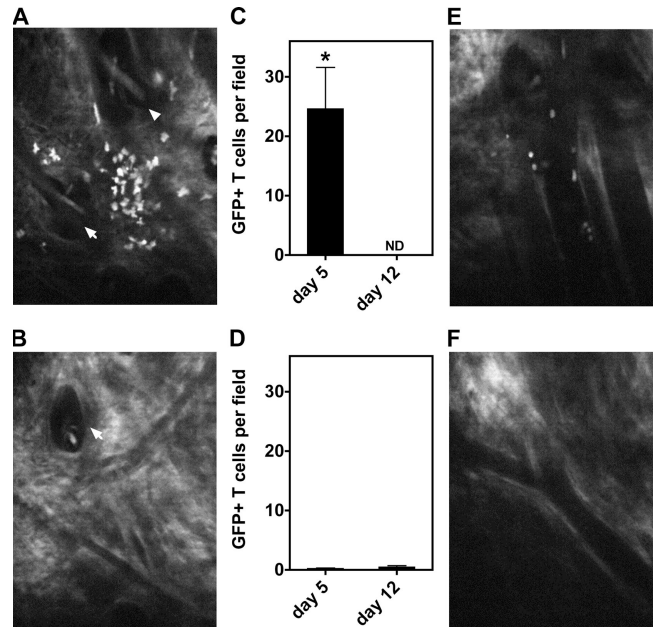
Figure 2. Absence of donor T cells in skin after transfer to established MCs. Representative images of skin (A and B, day 5) and mean $\pm$ SEM ($n = 3$ per group) number of GFP$^+$ T cells per field (C and D) derived from two-photon imaging of recipient ear pinnae after transfer to freshly irradiated recipients (A and C) and MCs (B and D) *, P < 0.05 day 5 freshly irradiated mice versus MC. White arrows indicate hair follicles. Representative images of postcapillary venules imaged on day 5 after GFP$^+$ T cell transfer to freshly irradiated recipients (E) or MCs (F). Freshly irradiated mice died before day 12 and therefore could not be assessed at this time point. ND, not done.

B6 CD45.1 splenocytes were administered to established B6 CD45.2 + BDF1→BDF1 MCs. By day 28 after transfer, recipient mice demonstrated conversion to full donor chimerism, although none developed GVHD (Fig. 3 B). On day 12 after transfer, we killed some of the recipient mice and sorted CD45.1$^+$ T cells from the spleen by immunomagnetic selection (day 12 T cells). Day 12 was chosen because this time point corresponded to the peak of polyclonal donor CD4$^+$ and CD8$^+$ T cell expansion in this model (not depicted). We then transferred $2.5–4 \times 10^6$ purified day 12 T cells together with TCD B6 BM or TCD BM alone on day 0 to lethally irradiated secondary BDF1 recipients. In two independent experiments, we observed that day 12 T cells induced severe clinical (Fig. 3 C) and histological (not depicted) GVHD upon transfer to lethally irradiated recipients. To exclude the possibility that small numbers of naive T cells present within the day 12 T cell population were responsible for inducing GVHD in secondary recipients, we compared the effect of transferring 2.5×10^6 similarly selected CD45.1$^+$ naive T cells with that of 2.5×10^6 day 12 T cells. As expected, recipients of naive T cells developed clinical and histological evidence of GVHD (not depicted), but the median survival was >100 d versus only 31 d in recipients of day 12 T cells. Furthermore, because donor CD4$^+$ T cells increase the severity of GVHD
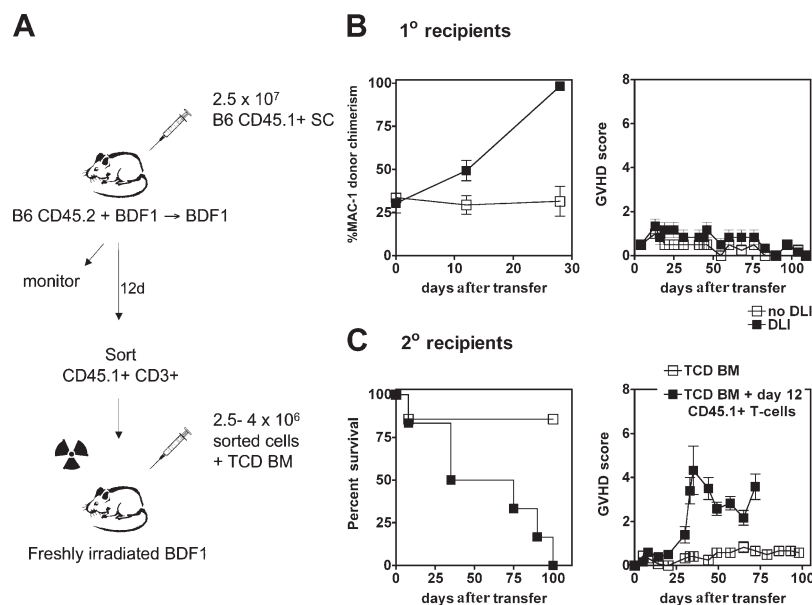
Figure 3.   T cells derived from MCs induce GVHD upon transfer to freshly irradiated recipients. (A) 2.5×10^7 naive B6 CD45.1 splenocytes were transferred to established B6 + BDF1→BDF1 MCs. On day 12, CD45.1$^+$CD3$^+$ T cells were sorted from recipient spleens and 2.5–4 × 10^6 cells were transferred to secondary irradiated recipients. (B) Transfer of naive donor splenocytes (■) to MCs induces conversion to full donor chimerism (left) but not GVHD (right) as compared with untreated MCs (□). (C) Survival (left) and clinical GVHD scores (right) after transfer of 4 × 10^6 day 12 CD45.1$^+$ T cells plus TCD donor BM (■; n = 6) or TCD BM alone (□; n = 7) to freshly irradiated secondary recipients. Data shows one of two independent experiments with similar results (survival comparison, P = 0.004 day 12 CD45.1$^+$ T cells plus TCD donor BM vs. TCD BM alone).

in this model (30), it is noteworthy that day 12 T cells induced accelerated GVHD despite reduced numbers of donor CD4$^+$ T cells being transferred compared with the naive inoculum (ratio CD4/CD8, 1:1 in day 12 T cells and 2:1 in naive T cells). These findings indicate that there is no absolute intrinsic homing or functional defect in T cells activated after transfer to established MCs.

T cells from mice with GVHD fail to induce GVHD in established MCs

The data described above suggested that T cell–extrinsic factors in the host are crucial in regulating the capacity of GVH-reactive T cells to induce GVHD. To further test this possibility, we performed the converse experiment in which T cells from freshly irradiated mice were transferred to established MCs (Fig. 4 A). First, 10^7 B6 CD45.2 splenocytes and TCD B6 BM were transferred on day 0 to lethally irradiated B6DF1 recipient mice. This dose of splenocytes is sufficient to induce severe, lethal GVHD (not depicted). On day 4 after transfer, mice were killed and T cells from recipient spleens were purified by immunomagnetic selection. 2.5 × 10^6 day 4 T cells (CD4/CD8 ratio, 3:1, reflecting their ratio in recipient spleens) were then transferred either to freshly irradiated BDF1 mice or to established B6 CD45.2 + BDF1→BDF1 MCs (Fig. 4 A). The former group of mice also received TCD B6 BM to prevent death from marrow aplasia. In three independent experiments, transfer of T cells from mice developing GVHD induced rapid lethality in irradiated recipi-

ents. In contrast, when these cells were transferred to established MCs, they caused no death, no clinical or histological GVHD, and no increases in the levels of donor chimerism (Fig. 4, B and C, and not depicted). Strikingly, donor T cells failed to engraft after transfer to secondary MC recipients as evaluated by flow cytometry of peripheral blood, suggesting that they failed to survive in this host environment. In parallel experiments in which B6 GFP$^+$ T cells obtained from primary, irradiated BDF1 recipients were transferred to MCs, no GFP$^+$ T cells were evident in the circulation by day 5 after transfer (not depicted). In contrast, GFP$^+$ T cells were present in large numbers in the circulation of secondary, freshly irradiated recipients and showed evidence of rolling/tethering in the postcapillary venules of recipient ear pinnae.

Localized Toll–like receptor (TLR) agonist triggers local GVHD following transfer of T cells to established MC

Collectively, the data in Figs. 3 and 4 indicate that T cell–extrinsic factors regulate the capacity of activated T cells to induce GVHD. However, the failure of day 4 effector T cells to engraft in secondary MC recipients prevented a direct evaluation of the role of extrinsic factors on the homing of GVH-reactive T cells originally activated in the presence of inflammation. Thus, we adopted an alternative strategy in which we altered the extrinsic environment in MCs and evaluated its influence upon the behavior of donor T cells. We first considered the possibility that inflammation such as that induced by innate immune activation would be required
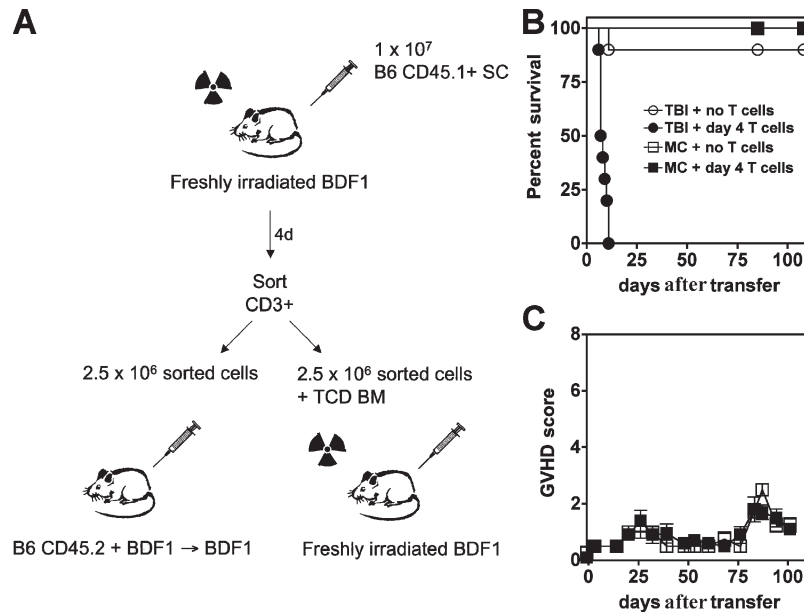
Figure 4. T cells derived from mice developing GVHD are unable to induce GVHD upon transfer to MCs. (A) 10^7 naive B6 CD45.1 spleno-cytes plus TCD BM were transferred to freshly irradiated BDF1 recipients. On day 4, $CD3^+$ T cells were sorted from recipient spleens and 2.5×10^6 cells were transferred to secondary irradiated BDF1 mice or to established B6 + BDF1→BDF1 MCs. (B) Survival and (C) clinical GVHD scores after transfer of 2.5×10^6 day 4 T cells plus TCD donor BM (●; $n = 10$) or TCD BM alone (○; $n = 11$) to freshly irradiated secondary recipients, or after transfer of day 4 T cells (■; $n = 11$) or nil (□; $n = 8$) to established MCs. Pooled data are shown from two independent experiments of the same design ($P < 0.0001$ survival comparison day 4 T cells plus TCD donor BM vs. each of three other groups).

to permit tissue entry of activated T cells. The early phase after lethal irradiation is characterized by substantial activation of the innate immune system and systemic inflammation (31, 32). Therefore, we reasoned that induction of innate immune activation via TLR ligation at the time of T cell transfer to established MCs would generate conditions permissive for the induction of GVHD. To model the effects of TLR triggering, we used a synthetic TLR7 agonist (an imidazoquinoline derivative, R–848), which induces substantial innate immune activation in vivo (33). R–848 was administered to established MCs that either did or did not receive 3×10^7 B6 splenocytes (Fig. 5 A). MCs receiving R–848 alone or 3×10^7 donor splenocytes alone developed no signs of GVHD. In sharp contrast, MCs receiving both R–848 agonist and donor splenocytes developed significant weight loss (Fig. 5 A), with histological evidence of GVHD at day +50 affecting the lung, liver, gut, and skin (not depicted). Thus, systemic exposure to a TLR agonist is sufficient to permit access of activated T cells to peripheral tissues and the induction of GVHD.

Systemic activation of a TLR could promote GVHD in MCs by inducing inflammation in GVHD target tissues that promotes T cell recruitment or, alternatively, by altering the intrinsic character of T cells in the DLI, perhaps in part by overcoming the activity of regulatory T cells (34, 35). To distinguish among these possibilities, we next asked whether local activation of the same TLR would induce local versus systemic GVHD. For this purpose, we applied imiquimod, an

imidazoquinoline derivative that acts as a TLR7 agonist (33, 36), to localized skin areas of MCs receiving DLI. Imiquimod has previously been shown not to induce significant T cell recruitment in otherwise untreated mice (36). In this experiment, the ear pinnae of established MCs were painted with imiquimod at the time of transfer of 9×10^6 GFP^+ donor T cells. The extent of GFP^+ T cell recruitment to the skin was then examined. As expected, only very minor infiltration of T cells was observed in imiquimod-treated controls given syngeneic T cells (Fig. 5, B and D). Likewise, MCs that were given donor T cells without imiquimod showed very little skin infiltration (Fig. 5, B and D). In contrast, massive skin infiltration of donor T cells was observed in imiquimod-treated MCs, peaking on day 12 after transfer (Fig. 5, B and D). We used in vivo flow cytometry as a means of evaluating the number of GFP^+ T cells in the input arteriolar circulation. As shown in Fig. 5 C, no differences were observed in the numbers of GFP^+ cells in MCs treated or not treated with imiquimod, indicating that the level of donor T cell expansion and circulation was similar in both groups. In additional experiments (Fig. 5 E), we confirmed that local inflammation induced only local GVHD in imiquimod-treated MCs by treating the left flank of recipient mice with agonist on days 0 and 5 after T cell transfer and leaving the right flank untreated. The mice developed unilateral GVHD in the treated skin and not in the skin of the untreated flank. No GVHD was observed in similarly treated mice that did not receive donor T cells (Fig. 5 E). Thus, local inflammation was sufficient
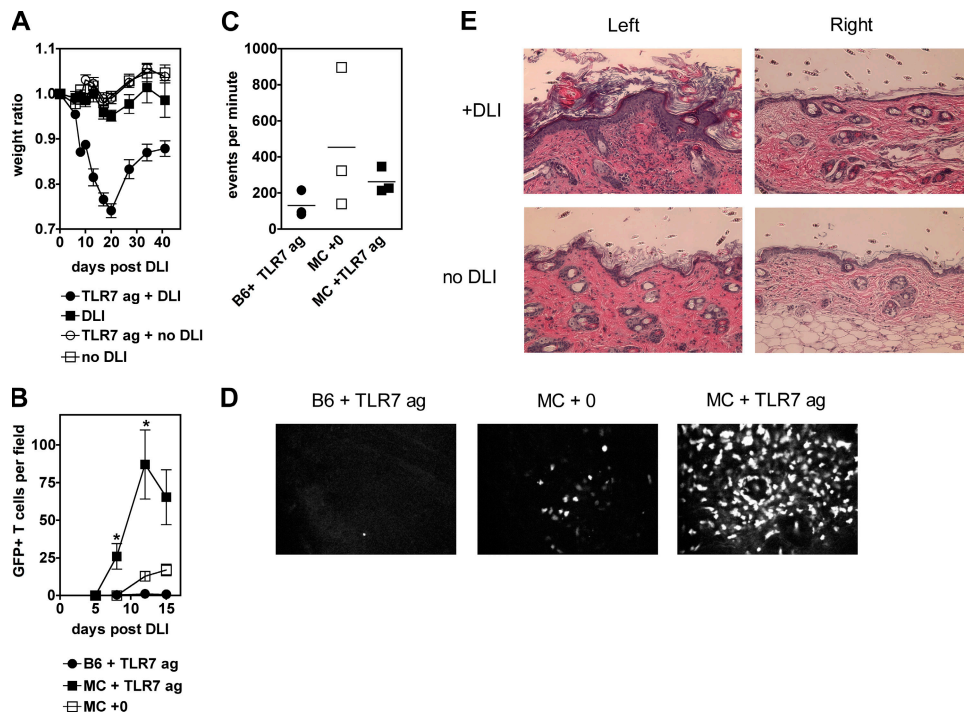
Figure 5. Localized TLR–induced inflammation permits access to skin for activated T cells. (A) Mean ± SEM weight ratio of untreated MCs (□; $n = 3$) or at timed intervals in MCs after transfer of 3×10^7 splenocytes alone (■; $n = 7$), 3×10^7 splenocytes plus 50 μg R-848 (given as 12.5 μg by i.p. injections at 3-d intervals from day 0; ●; $n = 7$), or 50 μg R-848 alone (○; $n = 4$). (B) Mean ± SEM ($n = 3$ per group) number of GFP+ T cells per field at time intervals after transfer of 9×10^6 GFP+ T cells to syngeneic control mice treated topically with imiquimod (●), to untreated MCs (□), or to imiquimod-treated MCs (■). *, $P < 0.05$ days 8 and 12 treated versus untreated MCs. (C) In vivo flow cytometry was used to examine the frequency of GFP+ T cells in the arteriolar circulation of ear pinnae on day 12 after transfer of 9×10^6 GFP+ T cells to the above groups. (D) Representative images derived from two-photon imaging of recipient ear pinnae on day 12 after transfer of 9×10^6 GFP+ donor T cells to B6 mice treated topically with imiquimod (left), to untreated MCs (middle), or to imiquimod-treated MCs (right). (E) The left flank was treated with imiquimod, and the right flank was left untreated in MCs receiving (top) or not receiving (bottom) 3×10^7 donor splenocytes. Representative images are shown of histology on day 14 after transfer.

and necessary to permit local donor T cell recruitment and GVHD. Collectively, our data indicate that the presence of peripheral tissue inflammation through activation of the innate immune response is a checkpoint of overriding importance in the recruitment of activated T cells.

DISCUSSION

After activation in secondary lymphoid tissues, T cells acquire the ability to traffic to nonlymphoid organs (37, 38). Our studies have shown that there is an additional requirement for tissue inflammation to permit trafficking to peripheral tissues. Activation by host APCs in MCs (16, 39) leads to marked expansion of GVH-reactive T cells to an extent similar to that observed in freshly irradiated recipients. However, in established MCs, this response is confined to the lympho-hematopoietic system. Significantly, we have demonstrated that such GVH-reactive T cells are fully capable of causing GVHD when placed into an inflamed host environment. Moreover, if localized skin inflammation is generated in MCs at the time of T cell transfer, localized GVHD is induced. Although tissue inflammation has been shown to support T cell accumulation in other models (40–42), to our knowledge

this is the first demonstration that tissue inflammation controls the development of GVHD at the local level. This is also the first direct demonstration that tissue inflammation regulates T cell accumulation in a parenchymal tissue by controlling T cell access to that tissue.

Although no intrinsic, absolute homing deficiency of T cells activated in established MCs was demonstrated, we have not excluded an incomplete intrinsic functional defect. In this context, we noted delayed kinetics of CD62L downregulation in the GVH-reactive CD8+ T cells after transfer to established MCs compared with freshly irradiated mice, a finding that may reflect differences in the initial levels of donor T cell activation induced upon transfer to the two groups. However, preferential recirculation of activated T cells to secondary lymphoid organs is unlikely to be a major explanation for the lack of GVHD in MCs because peak circulating numbers of GVH-reactive T cells were similar in freshly irradiated mice and established MCs receiving DLIs. Moreover, at the peak of the response, only a minor fraction of GVH-reactive CD8+ T cells expressed CD62L in either group. "Reprogramming" of T cells by activated DCs (28) might change the recirculatory patterns of these cells upon transfer

to freshly irradiated recipients or in the presence of local tissue inflammation and explain their new capacity to induce GVHD under such conditions. However, the local nature of the GVHD induced in the presence of local inflammation indicates that, even after such complete activation by fully activated APCs, the T cells do not gain access to noninflamed skin. Thus, imprinting by tissue-specific APCs in MCs seems unlikely to be the primary explanation for the failure of GVH-reactive T cells to access peripheral tissues.

Major differences between the environments in freshly irradiated recipients and established MCs include stimuli driving lymphopenia-induced proliferation, the extent of suppression mediated by regulatory cell populations, and the degree of direct tissue injury or tissue inflammation induced by irradiation. For example, the transfer of T cells to lymphopenic hosts is associated with enhanced proliferation and the acquisition of some effector functions (24, 25, 43, 44). However, this phenomenon does not appear to influence trafficking because transfer of T cells to irradiated, lymphopenic syngeneic hosts did not lead to accumulation of these cells within the gut. Furthermore, we did not observe severe GVHD after transfer of polyclonal T cells to nonirradiated allogeneic RAG knockout mice, indicating that lymphopenia-associated proliferation is insufficient to confer marked GVHD-inducing capacity of alloreactive T cells in the absence of tissue inflammation (unpublished data). Another possibility is that regulatory cell populations in MCs are important in preventing the access of activated T cells to GVHD target organs. This contrasts with the situation in freshly irradiated recipients, in which the integrity of host regulatory mechanisms may be severely compromised (34, 35, 45). However, because localized TLR-induced skin inflammation led to localized T cell recruitment and tissue injury, we conclude that the presence of tissue inflammation is sufficient to override any regulatory influences that may be present in established MCs.

Our data demonstrate that local tissue factors control the recruitment of and the development of tissue damage by GVH-reactive T cells activated under identical conditions. Conflicting data have been obtained in less clinically relevant models regarding the role of local inflammation in causing T cell entry into peripheral tissues. Several recent studies involving a model host antigen have shown that inflammation can convert autoreactivity into overt autoimmune disease (10, 46–49). However, memory T cells were shown to readily infiltrate noninflamed tissue in some of these studies (10, 11). Our data are consistent with an important role for innate immune activation and inflammation through exposure to microbial products that act as TLR ligands in inducing T cell recruitment to nonlymphoid tissues. Importantly, we have demonstrated here that localized inflammation induced by a TLR agonist induces localized T cell accumulation and localized T cell–induced injury, suggesting that this inflammatory checkpoint exists at the level of individual tissues. The precise mechanisms through which local TLR activation leads to the local accumulation of activated T cells within peripheral

tissues remain to be determined. TLR7 activation can modulate endothelial–T cell interactions (50), T cell proliferation and cytokine production (51), and APC functions (33). Thus, a local TLR-mediated inflammatory response could potentially modulate the recruitment of activated T cells by influencing their initial access or their subsequent in situ proliferation, retention, or survival.

Comparison of the behavior of T cells after transfer to freshly irradiated mice and established MCs indicates that one mechanism by which host conditioning influences recruitment of activated T cells to nonlymphoid tissues is at the level of access. Initial rolling and subsequent arrest of T cells upon the vascular endothelium of nonlymphoid tissues requires sequential and cooperative interactions between endothelial selectins, chemokines, integrins, and their respective ligands or receptors expressed by T cells (for review see reference 52). In this context, irradiation has been demonstrated to induce up-regulation of E-selectin and intercellular adhesion molecule 1 on endothelial cells in vitro (53). Similarly, experiments involving the transfer of syngeneic T cells to freshly irradiated recipients indicate that irradiation can induce the early and transient up-regulation of certain inflammatory chemokines and adhesion molecules (32, 54, 55) in nonlymphoid tissues. In contrast, up-regulated expression of these molecules by peripheral tissues is greater and sustained for longer periods after the transfer of allogeneic T cells to freshly irradiated recipients (32, 54, 55). These data suggest a model wherein inflammation induced by conditioning plays an important role in the initial recruitment of T cells, which induce an inflammatory cascade promoting further recruitment of T cells to GVHD target organs. According to this concept, endothelial cell recovery from the initial effects of conditioning may be an important determinant of the failure of activated T cells to roll or arrest on the vascular endothelium of established MCs.

In summary, we have demonstrated that activation and clonal expansion of GVH-reactive T cells can be dissociated almost completely from their ability to access gut or skin. This dissociation reflects tight control by local tissue-dependent factors rather than an absolute, intrinsic homing defect. Most significantly, the presence of inflammation within nonlymphoid tissues is a prerequisite for trafficking of GVH-reactive T cells to those specific sites and the induction of GVHD. Thus, reducing the degree of tissue inflammation induced by conditioning protocols may be crucial in the design of strategies to prevent GVHD. The restriction of the GVH reaction to the spleen, lymph nodes, and BM after delayed T cell transfer to MCs helps to explain the development of protective immunity against leukemia and lymphoma cells that reside at the same location after DLI (15, 16, 39). It will be of interest to determine whether a local inflammatory response to tumor infiltration at these sites is a requirement for full development of a GVL effect. A related issue is whether therapeutic induction of local inflammation might permit the induction of graft–versus–tumor effects outside the lymphoid and hematopoietic systems of the host.

MATERIALS AND METHODS

Animals. Animals were used under a protocol approved by our institutional Subcommittee on Research Animal Care, and experiments were performed in accordance with National Institutes of Health guidelines. Female BALB/cJ, C57BL/6, and B6.SJL (CD45.1) mice were purchased from the Frederick Cancer Research Facility. Female recipient B6 × DBA/2 (BDF1) mice were purchased from Charles River Laboratories. Female donor C57BL/6-transgenic (UBC-GFP)30Scha/J mice, which express GFP under the control of the human ubiqutin C promoter, were purchased from The Jackson Laboratory. 2C T cell receptor transgenic mice (H2^b on C57BL/6 background) were provided by D. Loh (Washington University, St. Louis, MO). Donors were aged 6–13 wk and recipients were aged 11–12 wk at the time of BMT.

BMT, donor leukocyte infusion, and purification of T cells. MCs were established by reconstitution of lethally irradiated recipients with a mixture of TCD allogeneic (15 × 10^6) and host-type (5 × 10^6) BM cells or TCD allogeneic (10–15 × 10^6) BM cells alone. Recipient mice were lethally irradiated (B6 and B6DF1 10.25 Gy, BALB/c 8 Gy, ^{137}Cs source, 0.8 Gy/min), and TCD BM cells were injected i.v. 4–8 h later. TCD was performed using anti-CD4/anti-CD8 microbeads (Miltenyi Biotec). In additional experiments, B6 + BDF1→BDF1 MCs were generated using a nonmyeloablative protocol as described previously (56). MCs were used in experiments between 2–3 mo after initial BMT.

Splenocyte suspensions were used for DLIs and were prepared as described previously (57). In experiments that involved transfer of activated donor T cells derived from freshly irradiated mice, recipients of BMT and donor splenocytes were killed on day 4 and splenocytes were harvested. Day 4 T cells were purified by passage through nylon wool to remove dead cells and then by negative selection to remove non–T cells (pan T isolation kit; Miltenyi Biotec). In experiments that involved transfer of activated T cells derived from established MCs, DLI recipients were killed on day 12 and splenocytes were harvested. Day 12 CD45.1$^+$ T cells were purified by negative selection to remove non–T cells (pan T isolation kit; Miltenyi Biotec) and then incubated with biotinylated anti–CD45.1 antibody followed by positive selection with anti-biotin–conjugated microbeads (Miltenyi Biotec). Where required, naive donor 2C CD8$^+$ T cells were isolated (purity >93%) by using anti-CD8 microbeads (Miltenyi Biotec) or after activation in vivo by negative selection of untouched T cells and positive selection using IgG1 1B2 clonotypic antibody and anti–mouse IgG1 microbeads (>95% purity). In experiments to examine proliferation of transferred donor T cells, polyclonal B6 splenocytes and 2C CD8$^+$ T cells were labeled with 1 μM CFSE (Invitrogen) as described previously (24). In experiments designed to evaluate the effect of systemic TLR activation, 12.5 μg of the TLR7 agonist R-848 was administered by i.p. injection every 72 h for four doses from the day of splenocyte transfer to established MCs. In additional experiments, 1.25 mg imiquimod (3M Pharmaceuticals) was applied to the ear pinnae or flank of MCs on days 0 and 5 after splenocyte transfer to MCs.

Assessment of GVHD. Clinical GVHD was assessed by scoring five parameters, including hunching, ruffled fur, diarrhea, periorbital edema, and activity. Histopathologic analysis of GVHD target organs was performed single blind by scoring changes in skin (dermal/epidermal lymphocyte infiltration, dyskeratotic epidermal keratinocytes, and epidermal thickening) and colon (crypt regeneration, apoptosis in crypt epithelial cells, crypt loss, surface colonocyte attenuation, inflammatory cell infiltration in lamina propria, mucosal ulceration, and thickening of mucosa). A severity scale from 0 to 4 was used: 0, normal; 0.5, focal and rare; 1, focal and mild; 2, diffuse and mild; 3, diffuse and moderate; 4, diffuse and severe.

Isolation of lymphocytes from intestines. Mice were killed and perfused via the heart with 40 ml PBS containing 10 U/ml heparin. The gut, cut longitudinally, was rinsed in cold HBSS, sectioned into 5-mm pieces, and shaken at 120 rpm at 37°C for 30 min in RPMI 1640/5% FCS. The suspension was then passed serially through a steel mesh, nylon mesh, and nylon wool column before enrichment for lymphocytes by density centrifugation (400 g for 30 min) over a Ficoll-Histopaque gradient (Sigma-Aldrich).

Flow cytometry. The following antibodies were used: anti–LPAM-1 (α4β7, DATK32), anti-H2-D^d-biotin (34-2-12), anti–CD4-PE (RM4-5), anti–CD8β-PE (H35-17.2), anti–CD11b-PE (M1/70), anti–CD44-FITC (IM7), anti–CD45.1-biotin (A20), anti–CD45.2-FITC (104), anti–CD62L-FITC (MEL-14), anti–rat IgG2a-biotin (RG7/1.30), and all appropriate isotype controls (all purchased from BD Biosciences). Detection of biotinylated antibodies was performed using either PE or APC linked to streptavidin (BD Biosciences). CD8$^+$ T cells bearing the 2C TCR were identified via the clonotype-specific mAb, 1B2, and anti–mouse IgG1-APC (X56; BD Biosciences). Enumeration of CD8$^+$1B2$^+$ T cells in peripheral blood was performed using TRUCOUNT (BD Biosciences) beads according to the manufacturer's instructions.

Quantitative real-time PCR. Methods for RNA extraction, RT, primers/conditions for quantitative PCR, and analysis of quantity values for gene expression have been described previously (58). Calculated values for the gene of interest were normalized to the housekeeping gene GAPDH.

Intravital two-photon microscopy and flow cytometry. Trafficking of GFP$^+$ T cells to the skin was imaged noninvasively using a custom-built two-photon fluorescence microscope designed specifically for in vivo imaging. In brief, output of a femtosecond titanium sapphire laser (890 nm; Coherent Mira) was injected into a video rate laser scanning platform consisting of a spinning polygon and a galvanometer that steer the laser beam in the fast (x) and slow (y) axes, respectively. The laser beam was focused onto the sample (mouse ear skin) using a 60×, 1.2NA water immersion objective lens (Olympus). Two-photon excited fluorescence was collected by the same objective lens, separated from the excitation beam by a dichroic filter, and detected with a photomultiplier tube (HC-124-2; Hamamamtsu) through a band pass filter (500–600 nm; Edmund Optics). Images were acquired at 30 frames per second and either stored to a digital video recorder or to a computer hard drive. In the case of static images, a 1-s frame average was performed to improve the signal/noise ratio. Circulating T cells were measured with an in vivo flow cytometer as described previously (59).

Statistical analysis. Survival data were analyzed using the log-rank test. Otherwise, statistical analyses were performed using the Student's t test or the Mann-Whitney test for nonparametric data. A p-value of <0.05 was considered to be significant.

Online supplemental material. Fig. S1 shows survival, clinical, and histological GVHD scores and chimerism after transfer of DLIs to allogeneic recipients. Fig. S2 shows expression of CCR7, CXCR3, and α$_4$β$_7$ upon CD8$^+$ CTL after transfer to allogeneic recipients. Fig. S3 shows the distribution of 2C CD8$^+$ T cells after transfer to freshly irradiated BDF1 mice or to B6 + BDF1→BDF1 MCs. Video S1 shows trafficking of GFP$^+$ donor T cells in the ear pinnae of freshly irradiated mice on day 5 after transfer. Video S2 shows trafficking of GFP$^+$ donor T cells in the ear pinnae of MCs on day 5 after transfer.

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