Resistance to Cutaneous Graft-vs.-Host Disease Is Not Induced in T Cell Receptor δ Gene–mutant Mice

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Summary

The function of murine dendritic epidermal cells (dEC) remains largely speculative, probably because of the lack of a suitable in vivo model, although previous studies suggest that γ/δ+ dEC may have originally evolved to serve as a self-protection mechanism(s). Our previous study demonstrated that the epidermis of mice that had spontaneously recovered from cutaneous graft-vs.-host disease (GVHD) induced by local injection of CD4+ autoreactive T cells contained unexpectedly large numbers of dEC and became resistant to subsequent attempts to induce GVHD in a site-restricted manner, suggesting that the resistance is mediated by dEC. However, because α/β+ dEC as well as γ/δ+ dEC were greatly increased in number in the epidermis, it was unclear whether γ/δ+ dEC are indeed responsible for this protection. The availability of this murine model and mice selectively lacking γ/δ T cells as a result of disruption of the T cell receptor Ca gene segment allowed us to investigate the role of γ/δ+ dEC. In the epidermis of γ/δ T cell–deficient mice (δ−/−), a congenital lack of γ/δ+ dEC was substituted for by α/β+ dEC of either a CD4−8+ or a CD4−8− phenotype. After intradermal injection of the autoreactive T cells, δ−/− mice developed significantly enhanced delayed-type hypersensitivity responses and cutaneous GVHD, which persisted longer than in heterozygous littermate controls (δ+/-). Surprisingly, resistance to the cutaneous GVHD was not induced in the epidermis of δ−/− mice after spontaneous recovery from the GVHD, whereas the “susceptible” epidermis of δ−/− mice contained large numbers of α/β+ dEC comparable to those in the “resistant” epidermis of δ+/− mice. Injection of day 16 fetal thymocytes from wild-type mice into δ−/− mice resulted in the appearance of donor-type γ/δ+ dEC in the epidermis, and reconstitution with γ/δ+ dEC restored the protective immune response of the epidermis against the GVHD to nearly normal levels. These results indicate that γ/δ+ dEC are responsible for the site-restricted protection against cutaneous GVHD.

Although considerable information on murine dendritic epidermal cells (dEC) expressing monomorphic TCR-γ/δ heterodimers has accumulated during the last decade (1, 2), the physiological function of dEC remains largely speculative. Previous studies, including our own (3–5), suggest that these γ/δ+ dEC residing in the epidermis may have originally evolved to serve as self-protection mechanism(s). The availability of mice congenitally lacking TCR-γ/δ–expressing cells by gene targeting has enabled us to investigate the functional role of γ/δ+ dEC in vivo: if γ/δ+ dEC are indeed specialized for a self-protection mechanism in the epidermis by recognizing damaged self-antigens as suggested (5), then it might be anticipated that mice lacking γ/δ+ T cells would develop a severe inflammatory skin disease evoked by an unregulated T cell–mediated attack on the epidermis. However, contrary to our expectation, prior studies using mice congenitally lacking γ/δ+ T cells have never documented spontaneous development of inflammatory skin diseases. This finding was not surprising when we considered the presumptive redundancy of self-protective mechanisms: under conditions of physiologic antigen stimulation, alternative self-protection mechanisms would be expected to mobilize and mask a congenital lack of γ/δ+ dEC. In light of such functionally redundant pathways of self-protection mechanisms, we reasoned that γ/δ+ dEC–mediated self-protection mechanisms would become evi-

Abbreviations used in this paper: B6 mice, C57Bl/6 mice; dEC, dendritic epidermal cells; δ−/−, δ+/−, δ+/+, homozygous, heterozygous, or wild type for the δ gene disruption, respectively.
dent only when excessive self-inflicted immune responses against the epidermis are induced in mice congenitally lacking γ/δ T cells in such a site-restricted manner that it cannot afford to be substituted for by other subtle self-protection mechanisms.

In this regard, we have previously established a murine model of cutaneous GVHD best suited for elucidation of the potential role of γ/δ TEC as part of the self-protection mechanisms: cutaneous GVHD characterized by selective destruction of epidermal structures associated with massive T cell infiltrates can be induced in normal mice by intradermal inoculation of CD4 T autoreactive T cells into the footpad (6); this experimentally induced cutaneous GVHD was a self-limiting event, and the epidermis spontaneously recovered from the destruction becomes resistant to subsequent attempts to induce cutaneous GVHD (4); and the long-term, local resistance to cutaneous GVHD is likely to depend on the expansion of γ/δ + TEC within the epidermis (4, 7). However, no direct evidence to indicate that the resistance is indeed mediated by γ/δ + TEC has been demonstrated in these studies. The use of our murine model for cutaneous GVHD and mice congenitally lacking γ/δ T T cells has provided the opportunity to delineate, in the absence of other confounding issues, the role of γ/δ + TEC as the tissue-selective, self-protection mechanisms.

Materials and Methods

Mice. Mice homozygous (δ−/−) and heterozygous (δ+/−) for the δ gene disruption on the (C57BL/6 × 129)F1 background were generated, as described in detail previously (8). These mice were backcrossed at least three times to C57B1/6 (B6) mice. PCR analysis of tail DNA confirmed the absence of the normal δ gene. B10.Thy-1.1 mice were bred and maintained in our animal facilities, and B6 mice were obtained from Charles River Japan, Inc. (Atsugi, Kanagawa, Japan). These mice were maintained under pathogen-free conditions in our animal facility.

GVHD-inducing Autoreactive T Cells. The derivation and maintenance of the cloned TCR-α/β CD4 T cell line BB5 have been described in detail previously (9); BB5 is an autoreactive T cell clone derived from B6 mice and is specific for I-A b. BB5 is capable of migrating into the epidermis upon its intradermal inoculation into the footpads of syngeneic mice and causes the destruction of the epidermis, histologic changes identical to those seen in chronic cutaneous GVHD (6, 10).

Assay for DTH Responses. As described previously (6, 10), the autoreactive T cells were harvested from culture 7–10 d after antigenic stimulation. Before the T cells were used in experiments, dead cells in the preparation were removed on a lymphocyte separation media (Sigma Chemical Co., St. Louis, MO) density gradient. After being washed with HBSS three times, remaining cells with >97% viability were injected intradermally in a volume of 25 μl into hind footpads of recipient mice (2 × 106 cells/footpad). The resultant swelling was measured with a dial thickness gauge at several time points after injection of the T cells.

Assay for Cutaneous GVHD. The cutaneous GVHD induced by the intradermal injection of the autoreactive T cells was evaluated both semiquantitatively by the intensity of epidermal invasion of T cells and the severity of epidermal cell damage, as previously described (4, 6, 10). The results were expressed as the mean number of lymphoid cells ± SD per linear millimeter of epidermis that was calculated from that within the total epidermal length surveyed. The severity of the epidermal cell damage was scored according to the grading system described by Lerner et al. (11) for cutaneous GVHD.

Immunofluorescence Staining. Epidermal sheets from the footpads were stained by the two-color immunofluorescence method, as previously described (7). The following mAbs were used: FITC-labeled anti-Thy-1.1 (mouse IgG2b; Meiji Laboratories, Tokyo, Japan); FITC-labeled anti-Thy-1.2 (clone 30–H12, rat IgG2b; Becton Dickinson & Co., Mountain View, CA); PE-conjugated anti-Thy-1.2 (clone 53–2.1, rat IgG2a; Pharmingen, San Diego, CA), FITC- or PE-conjugated CD3ε (clone 145–2C11, hamster IgG; Pharmingen); FITC- or PE-conjugated anti-CD4 (clone RM4–5, rat IgG2a, k; Pharmingen); FITC- or PE-conjugated anti-CD8α (clone YTS 169.4, rat IgG2b; CALTAG Laboratories, South San Francisco, CA); PE-conjugated anti-CD8β (clone 53–6.8, rat IgG1; Pharmingen); FITC- or PE-conjugated anti-TCR-α/β (clone H57–597, hamster IgG; Pharmingen); FITC- or PE-conjugated anti-TCR-γ/δ (clone GL3, hamster IgG; Pharmingen); and FITC- or PE-conjugated anti-Vγ5 (clone F536, hamster IgG; Pharmingen). The densities of TEC were assessed by counting 10 random fields of 0.125 mm2 per ear or footpad specimen with the aid of an ocular grid, as described previously (4, 7).

Reconstitution of δ−/− Mice with γ/δ+ TEC Precursors. For the reconstitution of δ−/− mice with γ/δ + TEC precursors, day 14, 15, or 16 fetal thymocytes obtained from B10.Thy-1.1 wild-type mice (δ+/+) were injected (~1.2–1.5 × 10⁷ cells/mouse) into the tail vein of δ−/− mice (Thy1.2). In some experiments, these fetal thymocytes were divided into two samples. The first sample was left unfracated, and Vγ5 + T cells were purified from the second by positive selection by flow cytometry. Our preliminary experiments demonstrated that intravenous injection of the day 16 fetal thymocytes (unfractated population) resulted in the appearance of donor-type Thy-1.1 TEC in the footpad epidermis of δ−/− mice 2 mo after cell transfer, as previously described by Payer et al. (12).

Results and Discussion

Mice homozygous for the mutant TCR-δ gene (δ−/−) were outwardly indistinguishable from heterozygous (δ+/−) or wild-type (δ+/+) littermates on gross physical inspection. As predicted by previous work (8) using epidermal sheets prepared from ear skin of δ−/− and δ+/+ mice, γ/δ + TEC and δ−/− mice, γ/δ + TEC expressing identical TCR encoded by Vγ5/Vδ1 genes were completely absent from the epidermis of δ−/− mice, and the vast majority of TEC (density of Thy1+ TEC: 152 ± 59/mm2, n = 13) in δ−/− mice were found to react with anti-CD3ε (92%) and anti-TCR-α/β mAbs (80%); these α/β + TEC expressing identical TCR encoded by Vγ5/Vδ1 genes were completely absent from the epidermis of δ−/− mice, and the vast majority of TEC (density of Thy1+ TEC: 152 ± 59/mm2, n = 13) in δ−/− mice were found to react with anti-CD3ε (92%) and anti-TCR-α/β mAbs (80%); these α/β + TEC expressing identical TCR encoded by Vγ5/Vδ1 genes were only sparsely populated. The morphologic appearance and distribution of α/β + TEC found in δ−/− mice were different from γ/δ + TEC in δ−/− mice: in the ear of δ−/− mice, γ/δ + TEC were diffusely distributed in a regular, dense network throughout the epidermis as well as those in δ−/− littermates, whereas α/β + TEC in δ−/− mice were unevenly distributed in many areas; and
most of $\alpha/\beta$ dEC, unlike $\gamma/\delta$ dEC showing dendritic shape, were angular and less dendritic. 53% of Thy1+ dEC identified in the epidermis of $\delta^{-/-}$ mice expressed CD8 molecules composed of both $\alpha$ and $\beta$ chains, although the remaining dEC were negative for CD4 and CD8, the same phenotype as typical $\gamma/\delta^+$ dEC. These results, taken together with our previous observation that bone marrow-derived dEC expressing TCR-$\alpha/\beta$ CD3 and CD8 preferentially migrate to the areas devoid of the regular network of the original $\gamma/\delta^+$ dEC (13), suggest that the CD8+ subset has a tendency to localize within the epidermis. The complete absence of $\gamma/\delta^+$ dEC and the compensatory appearance of CD8+ $\alpha/\beta^+$ dEC were also confirmed in other sites, such as the footpad epidermis of $\delta^{-/-}$ mice. We additionally demonstrated that there was no significant difference in the number of epidermal Langerhans cells between $\delta^{-/-}$ and $\delta^{+/-}$ mice (data not shown).

Experiments were conducted to determine whether $\delta^{-/-}$ mice could mount delayed-type hypersensitivity (DTH) responses comparable to those in normal or $\delta^{+/-}$ mice after intradermal injection with GVHD-inducing autoreactive T cells. As shown in Fig. 1, the magnitude and duration of footpad swelling that was elicited in $\delta^{-/-}$ mice were significantly higher than those in $\delta^{+/-}$ mice: whereas the DTH responses in $\delta^{+/-}$ mice had diminished by day 5 and completely subsided by day 8, in $\delta^{-/-}$ mice a significant delay in the resolution of the DTH responses was observed, and it took almost 16–18 d to completely resolve the response. These findings suggest that $\gamma/\delta^+$ dEC may serve a regulatory function in limiting epidermal damage induced by a specific population of $\alpha/\beta^+$ T cells.

On the basis of the altered DTH responses in $\delta^{-/-}$ mice, our next series of experiments focused on the potential role of $\gamma/\delta^+$ in the self-protective mechanism against cutaneous GVHD. To evaluate the influence of complete absence of $\gamma/\delta^+$ dEC in the epidermis on the cutaneous GVHD, autoreactive T cells were injected intradermally into the footpads of $\delta^{-/-}$ and $\delta^{+/-}$ mice, and at various times after injection, foodpad specimens were taken for histologic assessment of cutaneous GVHD. As in the DTH responses, $\delta^{-/-}$ mice developed significantly exaggerated cutaneous GVHD, which persisted longer than that in $\delta^{+/-}$ mice (data not shown). This delay in the resolution of DTH and GVHD observed in $\delta^{-/-}$ mice may be partly explained by
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Data shown represent a typical result from three independent experiments.

*On day -21, 2 × 10⁶ BB5 cells were injected into the footpads of δ⁺/⁻ or δ⁻/⁻ mice to induce resistance to cutaneous GVHD.

*On day 0, 2 × 10⁶ BB5 cells were again injected into the same footpad sites.

Recent suggestions that intraepithelial γ/δ⁺ T cells function in repair of damaged epithelial tissues by secreting keratinocyte growth factor (14). We next asked whether δ⁻/⁻ and δ⁺/⁻ mice spontaneously recovered from epidermal destruction in cutaneous GVHD could become resistant to a second induction of GVHD, as demonstrated previously in normal B6 mice (4). Surprisingly, as shown in Table 1 and Fig. 2, the epidermis of δ⁻/⁻ mice recovered from destruction remained susceptible to the second induction of cutaneous GVHD: resistance to cutaneous GVHD was not induced in the epidermis of δ⁻/⁻ mice; in contrast, the epidermis of δ⁺/⁻ mice was protected against the second induction of the GVHD.

Immunohistochemical examination of epidermal sheets obtained from the footpad after spontaneous recovery from GVHD showed that the "susceptible" epidermis of δ⁻/⁻ mice contained no γ/δ⁺ dEC but a large number of α/β⁺ dEC comparable to those in the "resistant" epidermis of δ⁺/⁻ mice (Fig. 3), indicating that the inability of δ⁻/⁻ mice to induce resistance could be due to complete deletion of γ/δ⁺ dEC from the epidermis. However, the possibility remained to be ruled out that the inability of δ⁻/⁻

![Figure 3](image-url)

Table 1. Resistance to Cutaneous GVHD Is Not Induced in TCR-δ-mutant Mice

| Recipient | T cell clone injected on day -21 to cause GVHD resistance* | Injection of T cell clone on day 0 | Number of lymphoid cells invading the epidermis on day 5 (grade) |
|-----------|----------------------------------------------------------|----------------------------------|---------------------------------------------------------------|
| δ⁺/⁻      | None                                                     | BB5                              | 124 ± 25 0 0 1 3 1                                           |
|           | BB5                                                      | BB5                              | 13 ± 115 4 1 0 0 0                                           |
| δ⁻/⁻      | None                                                     | BB5                              | 136 ± 27 0 0 1 2 2                                          |
|           | BB5                                                      | BB5                              | 119 ± 43 0 0 0 3 1                                           |

Note: *P < 0.005 compared with control mice without pretreatment on day -21.
mice to induce resistance resulted from other alterations necessarily unrelated to the complete absence of γ/δ+ dEC in the δ−/− mice. We therefore tested whether the reconstitution of δ−/− mice with γ/δ+ dEC could restore the capacity to induce resistance to cutaneous GVHD. Day 16 fetal thymocytes obtained from δ+/+ B10.Thy1.1 mice were used for the source of the dEC precursors. As reported by Payer et al. (12), intravenous injection of day 16 fetal thymocytes into δ−/− mice resulted in the appearance of donor-type γ/δ+ dEC 3 mo after injection (Fig. 4 A), although the density of the donor-type γ/δ+ dEC present in the footpad epidermis of the δ−/− recipient was lower than that in δ+/+ mice: most of the γ/δ+ were found to express Vγ5. To address whether these fetal thymocyte-derived γ/δ+ dEC could participate in the protective immune responses as do γ/δ+ dEC originally residing in the epidermis of δ+/− mice.

Table 2. Reconstitution of δ−/− Mice with δ+/+-derived Fetal Thymocytes Restores GVHD Resistance

| Recipient | T cell clone injected on day -21 to cause GVHD resistance* | Injection of T cell clone on day 0* | Number of lymphoid cells invading the epidermis on day 5 | Number of mice with cutaneous GVHD lesions on day 5 (Grade) |
|-----------|----------------------------------------------------------|----------------------------------|--------------------------------|------------------|
| δ−/−      | None                                                     | BB5                              | 117 ± 37                        | 0 0 1 2 1         |
|           | BB5                                                      | BB5                              | 138 ± 26                        | 0 0 1 3 0         |
| δ−/− + δ+/+ | None                                                    | BB5                              | 127 ± 20                        | 0 0 0 3 1         |
|           | BB5                                                      | BB5                              | 43 ± 32                        | 2 1 1 0           |

Data are cumulative from two independent experiments.
*On day -21, 2 × 10⁶ BB5 cells were injected to induce resistance, and on day 0, 2 × 10⁶ BB5 cells were again injected as described in Table 1. δ−/− mice that had been intravenously injected with fetal thymocytes obtained from δ+/+ B10.Thy1.1 mice 2 mo before were used for the γ/δ+ dEC–reconstituted δ−/− mice. At the time of killing, the reconstitution of δ−/− mice with γ/δ+ dEC was confirmed by immunofluorescence staining of epidermal sheets obtained from the ear, and only the data in those mice in which the reconstitution with γ/δ+ dEC was confirmed are given. δ P < 0.05 compared with control mice without pretreatment on day -21.
and δ+/− mice, the γ/δ+ dEC–reconstituted δ−/− mice were tested for their ability to induce resistance to cutaneous GVHD. As shown in Fig. 4 B, the parallel expansion of α/β+ dEC and fetal thymocyte–derived γ/δ+ dEC was observed in the epidermis of the γ/δ+ dEC–reconstituted δ−/− mice that had recovered spontaneously from the first induction of GVHD; and the capacity of the mice to protect the epidermis against cutaneous GVHD was subsequently restored toward levels close to that of δ−/− mice (Table 2); no significant difference was detected in the density and phenotype of α/β+ dEC between the “resistant” epidermis of the γ/δ+ dEC–reconstituted δ−/− mice and the “susceptible” epidermis of δ−/− mice that had not received day 16 fetal thymocytes (973 ± 167 vs. 985 ± 82/mm2; n = 4). These results indicate that fetal thymocyte–derived γ/δ+ dEC expressing TCR Vy5 are essential for resistance to the induction of cutaneous GVHD under conditions in which the epidermis is severely damaged in a site-restricted manner, although they are not absolutely required for the expansion or recruitment of α/β+ dEC precursors into the regenerating epidermis. However, we were unable to determine conclusively whether a particular subset of γ/δ+ dEC–bearing TCR Vy5 could be the only cell type responsible for the resistance because, for unknown reasons, the reconstitution of δ−/− mice with γ/δ+ dEC was possible only when unfractionated fetal thymocytes, but not the Vy5+ population purified from the former, were used.

There are a number of different ways in which activated γ/δ+ dEC could mediate the site-restricted resistance to a second induction of GVHD. The most straightforward interpretation of our results might be that the resistance is directly mediated by γ/δ+ dEC that eliminate or suppress the autoreactive T cells to cause the GVHD. However, recent studies (15, 16) have suggested an immunoregulatory, rather than an effector (cytotoxic), function of γ/δ+ T cells that can control the activation and differentiation of α/β+ T cells in large numbers: this appears to be a particularly effective means of maintaining immunological homeostasis in the epidermis by relatively small numbers of γ/δ+ dEC. Thus, direct lysis or suppression of the autoreactive T cells by γ/δ+ dEC may not be the mechanism responsible for the resistance in our system. Instead, we favored the second possibility that α/β+ dEC would also be necessary to act synergistically with γ/δ+ dEC in protecting the epidermis against the GVHD because our results demonstrate that the expansion of α/β+ dEC was excessive relative to that of γ/δ+ dEC in the “resistant” epidermis, and our previous studies with lethally irradiated bone marrow chimeras (8) showed that the expansion of adult thymus–derived Thy1+ dEC expressing TCR-α/β is crucial to the induction of the resistance. Consistent with this possibility, our recent preliminary studies with the use of α/β T cell–deficient mice (β−/−) have shown that, like δ−/− mice, significant delay in the spontaneous recovery from GVHD was observed and that, despite an expansion of γ/δ+ dEC, the resistance to a second induction of GVHD after spontaneous recovery was incomplete in these mice (Shiohara, T., unpublished data). An important role for activated γ/δ+ dEC in the resistance to GVHD might be to activate more numerous secondary effector cells, such as α/β+ dEC, via secretion of specific combinations of cytokines. Thus, our experimental model provides important paradigms to unravel the complex nature of the cross-talk among γ/δ+ dEC and α/β+ dEC.

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