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The CD100 Receptor Interacts with Its Plexin B2 Ligand to Regulate Epidermal γδ T Cell Function

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SUMMARY

γδ T cells respond rapidly to keratinocyte damage, providing essential contributions to the skin wound healing process. The molecular interactions regulating their response are unknown. Here, we identify a role for interaction of plexin B2 with the CD100 receptor in epithelial repair. In vitro blocking of plexin B2 or CD100 inhibited γδ T cell activation. Furthermore, CD100 deficiency in vivo resulted in delayed repair of cutaneous wounds due to a disrupted γδ T cell response to keratinocyte damage. Ligation of CD100 in γδ T cells induced cellular rounding via signals through ERK kinase and cofilin. Defects in this rounding process were evident in the absence of CD100-mediated signals, thereby providing a mechanistic explanation for the defective wound healing in CD100-deficient animals. The discovery of immune functions for plexin B2 and CD100 provides insight into the complex cell-cell interactions between epithelial resident γδ T cells and the neighboring cells they support.

INTRODUCTION

Wound repair in the skin is a complex process involving numerous cell types and cooperation of these cell types is crucial to complete healing. Epithelial γδ T cells are the exclusive T cell population in mouse epidermis and play a fundamental role in the wound healing process. These Thy1$^+$ dendritic epidermal T cells (DETCs) provide a first line of defense against environmental assault. They express a monoclonal Vγ3Vδ1 T cell receptor (TCR) that recognizes a poorly characterized antigen on damaged or diseased keratinocytes (Girardi et al., 2001; Jameson et al., 2002; Jameson et al., 2004; Strid et al., 2008). DETCs have a characteristic dendritic morphology enabling multiple contacts with neighboring keratinocytes and Langerhans cells. DETCs extend their dendrites to monitor keratinocytes for signs of damage or disease and retract them in response to keratinocyte damage, which allows for proliferation and migration of both DETCs and keratinocytes, crucial to the wound healing process (Chodaczek et al., 2012; Grose et al., 2002; Jameson et al., 2002). The molecular interactions regulating the DETC response to keratinocytes are poorly defined. It has been proposed that DETCs recognize a stress- or damage-induced keratinocyte self-antigen through their canonical TCR (Havran et al., 1991; Jameson et al., 2004; Komori et al., 2012). There is, however, no requirement for antigen presentation by MHC class I or class II molecules (Havran et al., 1991), although DETCs do appear to be selected by a molecule expressed by thymic stroma (Barbee et al., 2011; Boyden et al., 2008; Lewis et al., 2006). In addition, DETCs do not express many of the usual coreceptors that are important for αβ T cell activation, such as CD4 or CD8 or the costimulatory molecule CD28 (Hayday, 2000). However, the nature of the DETC-keratinocyte interaction suggests that molecules, in addition to the TCR, likely play a crucial role in the DETC response. This notion is supported by the recent identification of *j*unctional *a*dhesion *m*olecule-*l*ike molecule (JAML) and *c*oxsackie and *a*denovirus *r*eceptor (CAR) as a crucial receptor ligand pair for costimulation of epithelial γδ T cells (Witherden et al., 2010).

The nervous system, like the immune system, relies on multiple cell-cell contacts for activation, proliferation, and migration. A growing body of evidence indicates many parallels between the nervous and immune systems and highlights a number of shared features (Khan et al., 2001; Tordjman et al., 2002). One family of molecules, the plexins, was first described as playing a role in cell adhesion (Ohta et al., 1995) and has since been shown to play a fundamental role in the nervous system (Waimey and Cheng, 2006). Plexins are large transmembrane proteins containing a sema domain and a highly conserved cytoplasmic domain (Tamagnone et al., 1999). They are highly

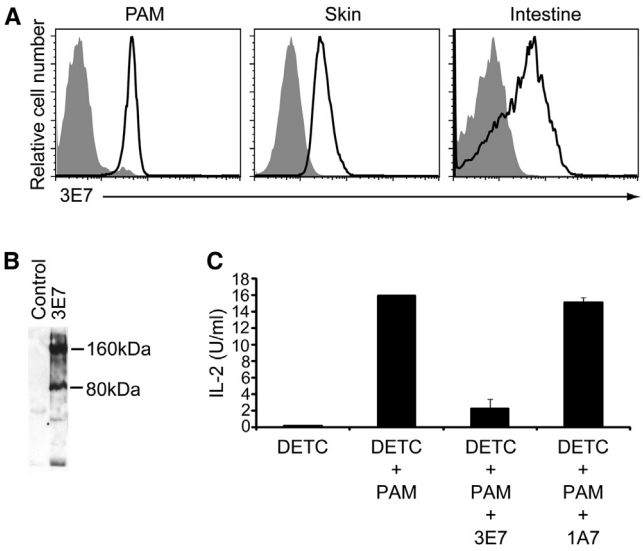

Figure 1. mAb 3E7 Recognizes Plexin B2 Expressed on Skin and Intestinal Epithelial Cells and Blocks Keratinocyte-Induced IL-2 Production by DETCs

(A) Cell surface staining of PAM 2-12 keratinocytes, freshly isolated skin and intestinal epithelial cells with mAb 3E7.

(B) Immunoprecipitation from PAM 2-12 lysates with control mAb or mAb 3E7.

(C) 7-17 DETC were cultured either alone or cocultured with irradiated PAM 2-12 cells for 24 hr in the presence of media alone (PAM+DETC), mAb 3E7, or control mAb 1A7. Culture supernatants were collected and the amount of IL-2 produced was measured with the IL-2-dependent CTLL cell line and an MTT assay. Histograms represent mean ± SD. Data are representative of three or more experiments. See also Figure S1.

expressed in neurons (Tamagnone et al., 1999; Worzfeld et al., 2004) and mediate axon guidance cues (Halloran and Wolman, 2006). In the developing nervous system, plexins control axon guidance by acting as functional receptors for semaphorins (Kruger et al., 2005; Tamagnone et al., 1999).

Semaphorins are a large family of membrane-bound and soluble proteins that deliver directional cues through their interaction with plexins (Fiore and Püschel, 2003). When bound by semaphorins, plexins modify the cytoskeleton through regulation of small GTP-bound proteins (Driessens et al., 2001). A number of studies have demonstrated an important role for semaphorins in the immune system (Kruger et al., 2005; Moretti et al., 2006) through interaction with both plexins (Chabbert-de Ponnat et al., 2005; Walzer et al., 2005; Wong et al., 2003) and nonplexin ligands (Kikutani et al., 2007). CD100, also known as Sema4D, is one of the most-well-characterized semaphorins on T cells (Kumanogoh et al., 2000; Shi et al., 2000). CD100 is highly expressed on all T cells and more weakly on B cells and APCs. CD100 is upregulated upon activation and is expressed as a 150 kDa transmembrane or 120 kDa soluble protein. CD100 binds with high affinity to plexin B1 in the central nervous system (Tamagnone et al., 1999) and with lower affinity to CD72 on B cells in the immune system (Kumanogoh et al., 2000). Analysis of CD100-deficient animals has demonstrated a crucial role for CD100 in both humoral and cellular immunity (Shi et al., 2000). It is unknown, however, what intracellular signals are generated through CD100.

We hypothesized that other cell surface proteins, in addition to the TCR, would play a fundamental role in DETC-keratinocyte interactions. To test this hypothesis, we generated monoclonal antibodies to keratinocyte cell surface proteins and screened for antibodies that impacted the DETC response to keratinocytes in vitro. We identified a plexin family member, plexin B2, on keratinocytes as crucial to activation of DETCs. Plexin B2 was found to exert its effect on DETCs through interaction with the semaphorin ligand CD100. CD100 mediates γδ T cell morphology changes both in vitro and in vivo and plays a fundamental role in wound repair. Mice deficient in CD100 showed a defective γδ T cell response to keratinocyte damage resulting in delayed healing of cutaneous wounds. Our results therefore demonstrate a receptor-ligand pair involved in γδ T cell responses in the skin. Their interaction was crucial for epidermal γδ T cell activation and probably functions in other epithelial tissues, such as the intestine, where γδ T cells also play a central role in tissue maintenance.

RESULTS

The DETC Response to Stressed Keratinocytes Requires Interaction with Plexin B2

To address the requirement for other receptors, in addition to the TCR, that are involved in antigen-mediated DETC responses, we generated monoclonal antibodies (mAbs) to cell surface molecules expressed by the keratinocyte cell line PAM 2-12. Antibodies were initially screened for their ability to block or augment the activation of γδ T cells by stressed keratinocytes. One mAb, 3E7, was identified as recognizing a highly expressed keratinocyte cell surface protein (Figure 1A). In addition to its expression on freshly isolated keratinocytes and the keratinocyte cell line PAM 2-12, this protein was also expressed on epithelial cells isolated from mouse intestine (Figure 1A), another tissue in which γδ T cells play an important role in homeostasis and repair (Chen et al., 2002).

Immunoprecipitation of the protein recognized by mAb 3E7 from PAM 2-12 cells revealed two major bands of apparent molecular sizes of 80 and 160 kDa (Figure 1B). The identity of these proteins was determined by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) and N-terminal protein sequencing. All peptides sequenced matched those of mouse plexin B2 (Figure S1A available online), a member of the plexin family of transmembrane proteins. The N-terminal sequence of the 80kDa protein begins immediately after a consensus sequence for the proprotein convertase family (Figure S1A) that is characteristic of plexin family members (Artigiani et al., 2003). Thus, the 160 kDa protein is probably the posttranslationally cleaved α subunit of plexin B2 and the 80kDa protein is the β subunit.

Although an immunological function has been described for some plexin family members (Chabbert-de Ponnat et al., 2005; Holl et al., 2011; Walzer et al., 2005; Wong et al., 2003), a role for the plexin B subfamily in T cell responses has not been described. Plexin B2 shows a wide cell and tissue distribution outside of the nervous system (Su et al., 2002; Zielonka et al., 2010) and, interestingly, is expressed in many tissues where γδ T cells localize, such as intestine (Figure 1A) and other epithelia (Su et al., 2002; Zielonka et al., 2010). To assess the requirement

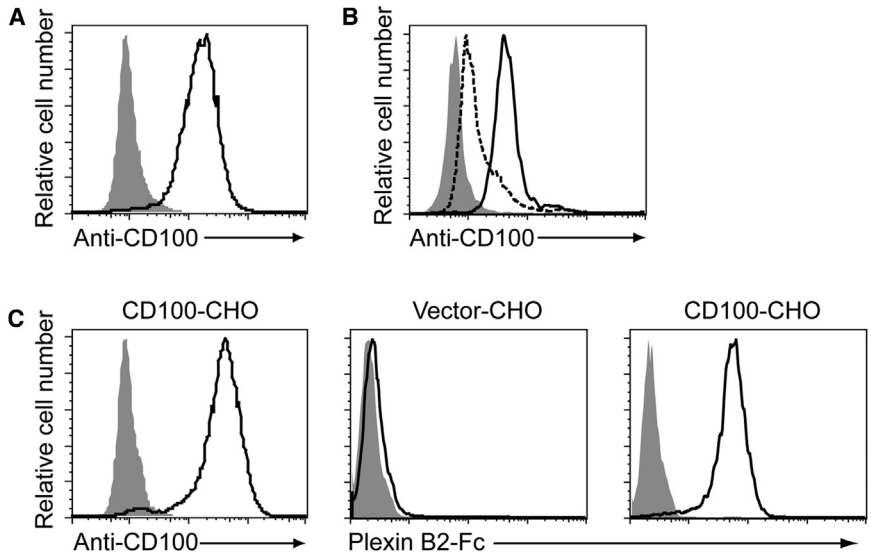

Figure 2. CD100 Is Expressed by DETCs and Is a Ligand for Plexin B2

(A) Surface expression of CD100 on 7-17 DETCs. Shaded area represents control staining.

(B) CD100 expression on resting and activated DETCs isolated from the skin of C57BL/6 mice. Cells were either unstimulated (dashed line) or stimulated (solid line) for 16 hr with 5 μg/ml Concanavalin A then stained for CD100 expression. Shaded area represents control staining.

(C) CHO cells were transfected with the expression vector pcDNA3 containing mouse CD100 cDNA. CD100-transfected or vector-transfected cells were stained with anti-CD100 or plexin B2-Fc, subsequently stained with fluorescent-labeled secondary reagents, and analyzed by flow cytometry (solid line). Shaded areas represent staining with secondary reagents alone. Data are representative of more than three independent experiments.

for plexin B2 in DETC-keratinocyte interactions, we set up in vitro cocultures in the presence or absence of the plexin B2 mAb 3E7. Irradiated PAM 2-12 keratinocytes were treated with mAb 3E7 or a control mAb, 1A7, then the 7-17 DETC line was added. Analysis of culture supernatants for the presence of IL-2 24 hr later, as an indicator of DETC activation, revealed a block in DETC activation upon addition of the plexin B2 mAb (Figure 1C). In contrast, media alone or the control mAb were unable to affect the DETC response to keratinocytes (Figure 1C). This result suggests that DETC responses to stressed keratinocytes depend on interactions with plexin B2.

The specificity of this effect for γδ T cell activation was assessed with the K^d-restricted CD8 T cell, clone 4, specific for a peptide (HA) derived from the hemagglutinin protein of influenza virus (Redmond et al., 2008). PAM 2-12 keratinocytes were able to present HA peptide to clone 4 T cells (Figure S1B). The activation of clone 4 cells was unaffected by preincubation of PAM 2-12 keratinocytes with mAb 3E7 or a control mAb (Figure S1B). Thus, it appears that plexin B2 is required for epidermal γδ T cell activation, but is dispensable for the activation of αβ T cells.

CD100 Is the DETC Ligand for Plexin B2

The strong homology in the extracellular domain between plexin B1 and plexin B2 raises the possibility that these two family members could interact with the same semaphorin, CD100, a notion supported by the promiscuous binding of other plexins and semaphorins (Kruger et al., 2005; Kumanogoh and Kikutani, 2010) and the colocalization of plexin B2 and CD100 in both the nervous system (Worzfeld et al., 2004) and pancreas (Zielonka et al., 2010). Indeed, CD100 is expressed on both the 7-17 DETC cell line and on freshly isolated DETCs (Figures 2A and 2B).

In order to test directly our hypothesis that CD100 is a ligand for plexin B2, we produced a soluble fusion protein of the extracellular domain of plexin B2 and the Fc portion of human IgG1. FACS analysis of CHO-K1 (CHO) cells expressing mouse CD100 (CD100-CHO) demonstrated that plexin B2-Fc can bind to CD100-CHO, but not to untransfected CHO cells (Figure 2C). Although these data showed that plexin B2 and CD100 could, indeed, interact, as had been suggested previously (Masuda et al., 2004), it did not demonstrate whether these two proteins form a receptor-ligand pair in vivo in the skin.

To address the interaction of Plexin B2 and CD100 ex vivo, we isolated epidermal cells from C57BL/6 wild-type and CD100-deficient (*Sema4d*$^{-/-}$; Shi et al., 2000) mice and established short-term DETC lines. FACS analysis of these DETCs revealed plexin B2-Fc binding to wild-type DETCs (Figure 3A). In contrast, plexin B2-Fc was unable to bind to CD100-deficient DETCs, supporting the notion that plexin B2 and CD100 do form a receptor-ligand pair in vivo. In addition, surface plasmon resonance (SPR) analysis of CD100 binding to immobilized plexin B2 (Figure 3B) demonstrated specific binding of CD100 to plexin B2 and no binding to immobilized control molecules (Figure 3B). In coculture assays with wild-type PAM 2-12 keratinocytes and CD100-deficient DETCs, a similar defect in activation, measured as IL-2 production, was seen (Figure 3C) as observed after plexin B2 blockade (Figure 1C). Taken together, these results support a fundamental role for CD100-plexin B2 interactions in DETC function in the skin.

CD100-Deficient Animals Show Delayed Wound Healing

Mice lacking CD100 have apparently normal numbers and phenotype of γδ T cells in the skin (Figure S2). Unlike mice lacking γδ T cells, CD100-deficient animals show normal epidermal homeostasis with no increased keratinocyte apoptosis or altered homeostatic numbers of DETCs or keratinocytes (Figure S2). However, analysis of αβ T cell responses in these animals has demonstrated a crucial role for CD100 in antigen-specific αβ T cell activation (Kumanogoh et al., 2002; Shi et al., 2000). This result, together with the defective DETC activation seen in vitro after plexin B2 blockade or CD100 deficiency, led us to hypothesize that CD100 and plexin B2 would play a role in DETC activation in the skin. This hypothesis was further supported by the observation that the expression of plexin B2 is modulated after epidermal wounding. Eighteen hours after full thickness punch biopsy wounds in the back of wild-type mice,

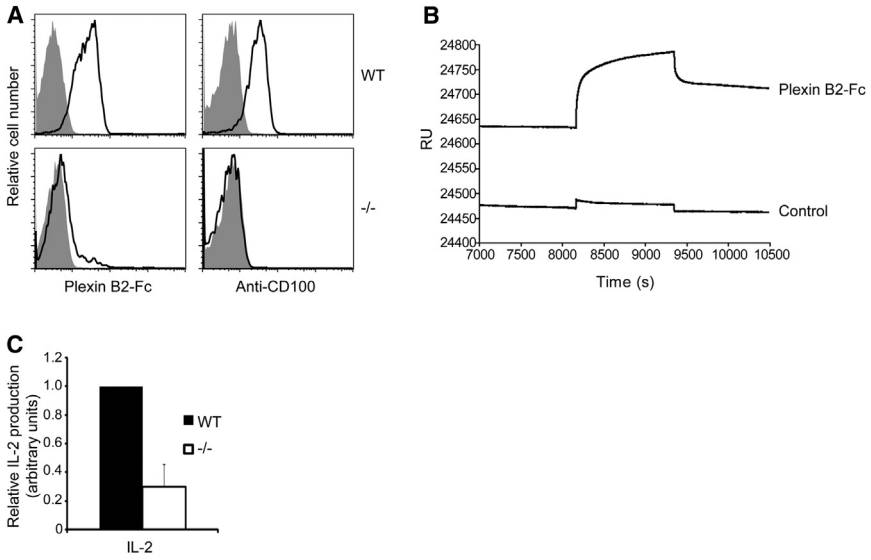

Figure 3. Plexin B2 Binding to CD100 Is Required for DETC Activation

(A) Epidermal cells were isolated from wild-type (WT) and CD100-deficient (−/−) mice and short-term DETC cultures set up. Thy1$^+$γδTCR$^+$ cells were stained for plexin B2 ligand or CD100 expression (solid lines). Shaded areas represent control staining.

(B) SPR sensorgrams of soluble CD100-Fc passed over immobilized plexin B2-Fc or HL4E10 IgG (control) as a negative control. The specific interaction of CD100-Fc with plexin B2-Fc is characterized by complex, biphasic binding and dissociation behavior due to the bivalent nature of the analyte.

(C) Reduced keratinocyte-induced IL-2 production by CD100-deficient DETC. Short-term wild-type (WT) and CD100-deficient (−/−) DETC lines were cocultured with irradiated PAM 2-12 cells for 24 hr. Culture supernatants were collected and the amount of IL-2 produced was measured by ELISA. Histograms represent mean ± SD. Data are representative of three independent experiments.

upregulation of plexin B2 was observed (Figure 4A). This upregulation was sustained for at least 48 hr.

DETCs are critical to the healing response to cutaneous wounds (Jameson et al., 2002). They are activated in response to damage whereupon they lose their characteristic dendritic morphology, assume a rounded shape, and begin secreting cytokines and growth factors that aid in the wound repair process (Jameson et al., 2002). To determine whether CD100 plays a role in the γδ T cell response to keratinocyte damage, we chose to analyze wound healing in CD100-deficient animals. A comparison between wild-type and CD100-deficient mice revealed that the lack of CD100-mediated interactions resulted in altered wound closure kinetics (Figure 4B). CD100-deficient animals showed a significant 1 to 2 day delay in wound closure as compared to their wild-type counterparts. This effect is strikingly similar to the delay seen in mice lacking γδ T cells (Jameson et al., 2002) and suggests a crucial role for CD100 in DETC function.

Although in vitro activation assays suggested that plexin B2-CD100 interactions do not play a role in αβ T cell activation (Figure S1B), αβ T cells are an important component of the wound healing response (Engelhardt et al., 1998; Schäffer and Barbul, 1998). Therefore, to assess whether the defective wound closure seen in CD100-deficient animals was due to the lack of CD100 on γδ T cells or CD100 on all T cells, we generated bone marrow chimeras after lethal irradiation of wild-type and CD100-deficient animals. DETCs are resistant to radiation and remain in normal numbers in the epidermis (Gray et al., 2011). Wild-type animals reconstituted with CD100-deficient bone marrow showed normal wound closure kinetics, as did control wild-type animals reconstituted with wild-type bone marrow (Figure 4C). In contrast, CD100-deficient animals reconstituted with either wild-type or CD100-deficient bone marrow (Figure 4C) showed the same delay in wound closure as unmanipulated CD100-deficient animals (Figure 4B). Thus, wound closure was only disrupted when DETCs were CD100 deficient. Conversely, wound closure was normal when DETCs were wild-type, regardless of the CD100 phenotype of other T cells. These data suggest that it is indeed the deficiency of CD100 on DETCs that is responsible for the wound healing defect seen in CD100-deficient animals.

CD100 Is Required for DETC Rounding in Response to Activation

Plexin-semaphorin interactions have been well documented to regulate morphology, migration and proliferation in several cell types (Kruger et al., 2005; Yazdani and Terman, 2006). One of the early hallmarks of DETC activation in vivo is the rapid rounding of these characteristically dendritic T cells (Jameson et al., 2002). This morphology change appears to be both an early consequence of activation as well as a requirement for the effector function of these cells (Havran and Jameson, 2010). In order to proliferate and migrate to the site of damage, as well as to facilitate the proliferation and migration of their neighboring keratinocytes, DETCs probably need to retract their dendrites and release contacts with nearby cells.

In order to assess the possible role of CD100 in this process, we examined DETC morphology in vivo in the ear of wild-type and CD100-deficient animals in response to punch biopsy wounds. At 1 hr postwounding, DETCs in wild-type animals began to round up in response to keratinocyte damage and by 2 hr, the vast majority of DETCs in the immediate vicinity of the wound had assumed a rounded morphology (Figures 4D and 4E). In contrast, very few of the DETCs surrounding the wound in CD100-deficient animals had started to lose their dendritic processes even 2 hr after wounding (Figures 4D and 4E). These data are quantified in Figures 4F and 4G. At 2 hr postwounding, <5% of DETCs at the wound edge of wild-type animals still showed a dendritic morphology and >70% had retracted all their dendrites and were completely round. In stark contrast, in CD100-deficient animals, only ~10% of DETCs had rounded by 2 hr and almost 70% remained dendritic (Figure 4F). These data thus implicated CD100 in the rounding response to DETC activation signals. Similarly, morphometric

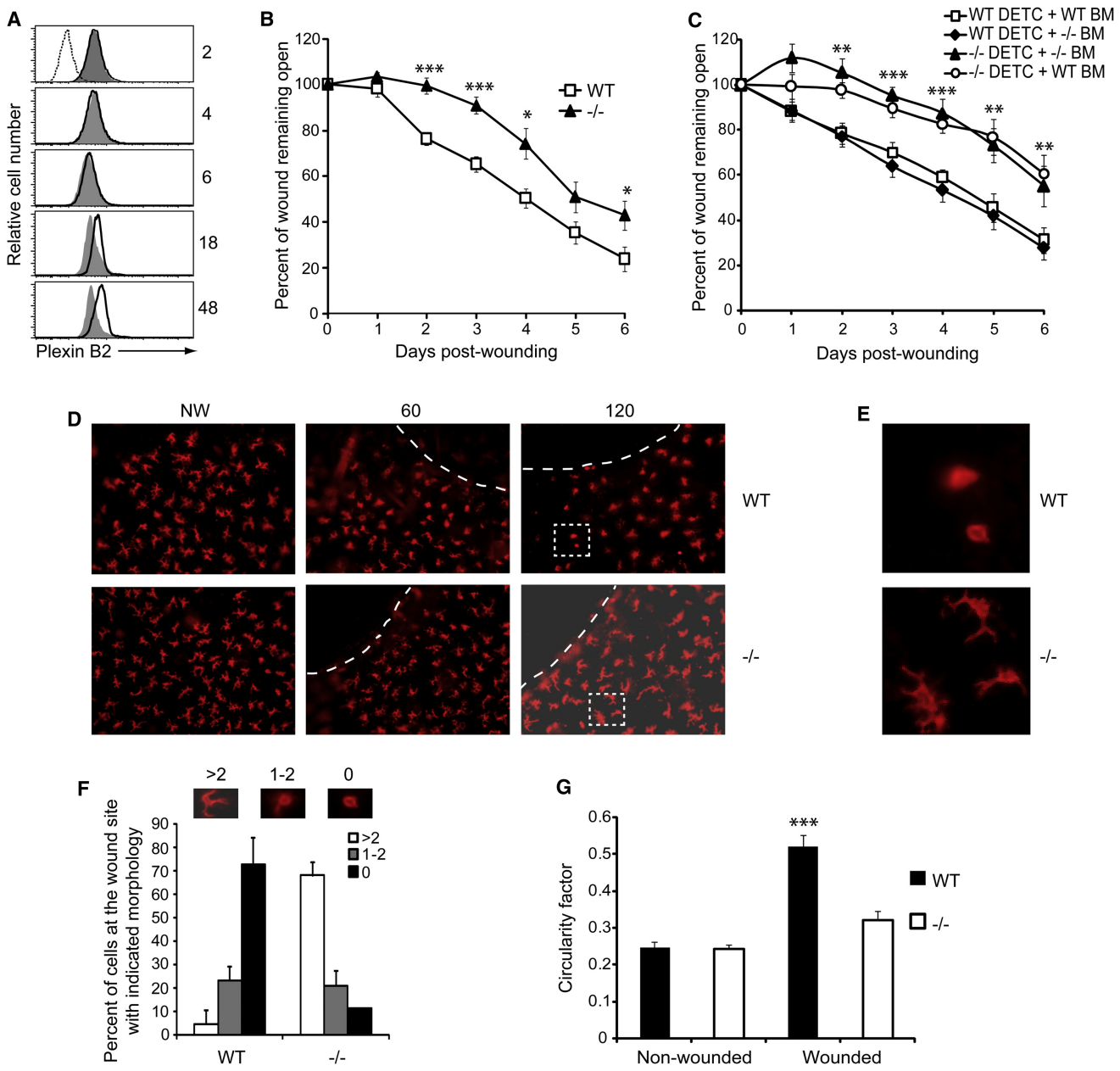

Figure 4. CD100 Deficiency Delays DETC Rounding and Wound Closure

(A) Expression of plexin B2 on keratinocytes isolated from the epidermis of nonwounded (shaded) and wounded (solid lines) wild-type animals at various times after wounding. Dotted line represents control staining.

(B) Analysis of wound closure kinetics in WT (open squares) and CD100-deficient (closed triangles) mice. Data represent mean ± SEM of at least ten wounds per mouse strain and are representative of more than three independent experiments.

(C) Wound closure kinetics in WT chimeric animals reconstituted with WT (open squares) or CD100-deficient (closed diamonds) bone marrow and CD100-deficient animals reconstituted with CD100-deficient (closed triangles) or WT (open circles) bone marrow. Data represent mean ± SEM of at least nine wounds per mouse strain and are representative of two independent experiments. For (B) and (C), ***$p < 0.001$, **$p < 0.01$ and *$p < 0.05$.

(D) Epidermal sheets from WT and CD100-deficient (−/−) mouse ears were stained for γδ TCR 0 min (NW), 60 min, and 120 min after wounding. Dashed lines represent the wound edge. Dotted squares represent areas shown in (E).

(E) Inset of representative cells from (D).

(F) The number of dendrites per cell at the wound edge was quantified 2 hr after wounding and is expressed as a percent of total cells with zero (0), one to two (1–2) or more than two (>2) dendrites. Histograms represent mean ± SD of three experiments. Individual examples of cells with 0, 1–2, or >2 dendrites are shown above the histograms.

(G) Circularity factor of DETCs in nonwounded tissue and adjacent to the wound edge in epidermal ear sheets from WT and CD100-deficient animals shown in (D). Histograms represent mean ± SD. ***$p < 0.0001$, compared to all other conditions. See also Figure S2.

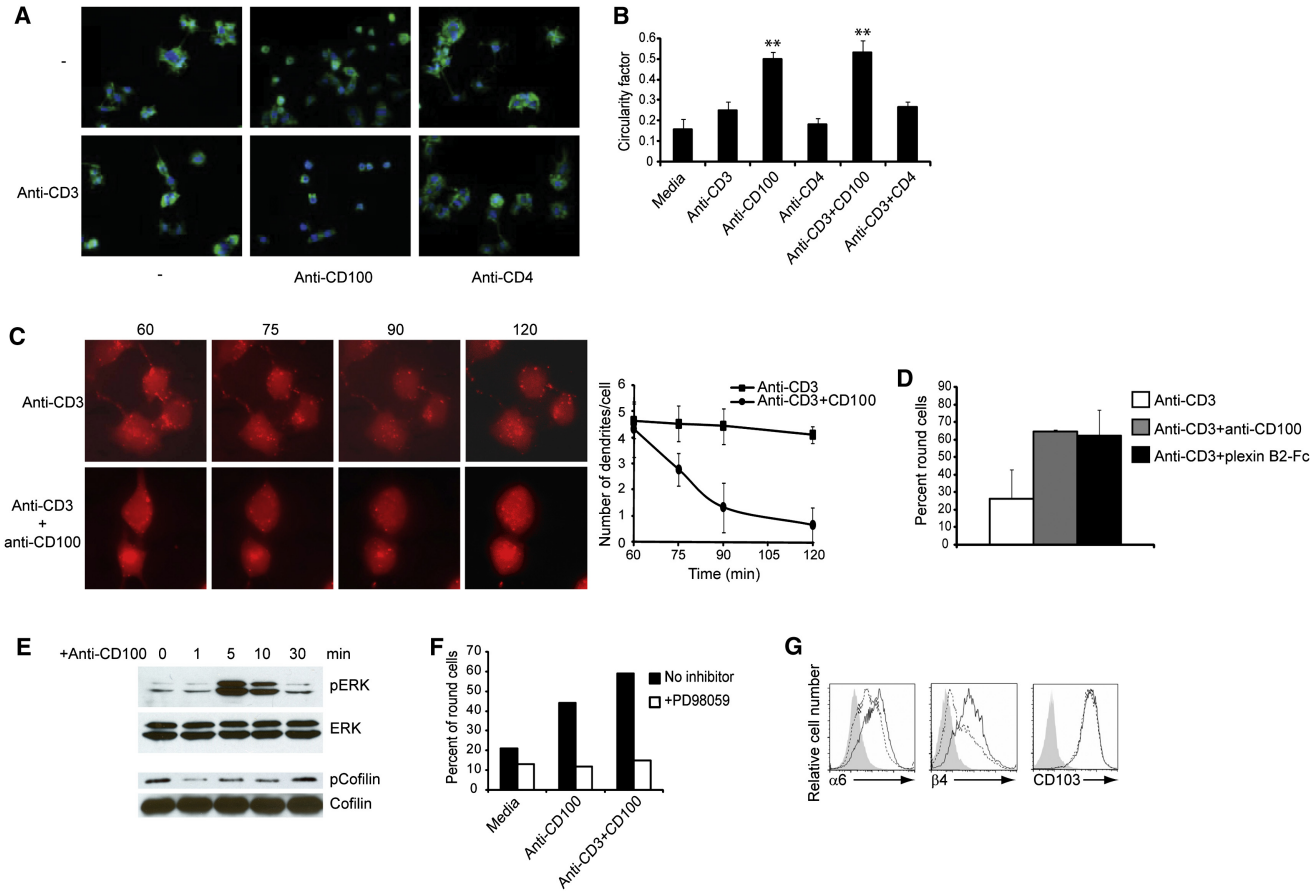

Figure 5. CD100 Ligation Induces DETC Rounding via Signals through ERK Kinase and Cofilin

(A) Analysis of morphology changes in 7-17 DETCs upon CD100 ligation. 7-17 cells were left untreated (left panels) or treated with anti-CD100 (middle panels) or anti-CD4 (right panels) either alone (top panels) or in combination with anti-CD3 (bottom panels) for 120 min and then fixed. Morphology was assessed by staining for actin and nuclei were stained with DAPI. More than 200 cells were analyzed per condition and data are representative of three experiments.

(B) Circularity factor of DETCs treated as in (A). Histograms represent mean ± SD. **$p < 0.005$ compared to all other treatments.

(C) Time course of DETC morphology changes. 7-17 cells labeled with cell-tracker CMTMR were treated with anti-CD3 (top panels) or anti-CD3 and anti-CD100 (bottom panels) for 120 min and analyzed by immunofluorescence microscopy at 1 min intervals during the last 60 min (left panels). Original magnification, 63×. Data are representative of five experiments. The number of dendrites per cell was quantified at various time points between 60 and 120 min and is plotted as mean ± SD of all cells analyzed (right panel).

(D) Morphology change in 7-17 DETCs upon plexin B2-CD100 interaction. Cells were treated with anti-CD3 alone or anti-CD3 in combination with either anti-CD100 or plexin B2-Fc for 120 min and then fixed. Cells were stained as in (A) and round cells quantified by counting more than 200 cells per condition. Histograms represent mean percentage of rounded cells ± SD and data are representative of three experiments.

(E) Time course of ERK phosphorylation and cofilin dephosphorylation in DETCs. Immunoblot analysis of ERK and cofilin phosphorylation in 7-17 lysates, after anti-CD100 treatment of cells for the indicated times, is shown. Total protein is shown as a control. Data are representative of three to four independent experiments.

(F) Effect of ERK inhibition on DETC rounding. Cells were treated as indicated in the presence (open histograms) or absence (solid histograms) of the ERK inhibitor PD98059. Histograms represent mean percentage of rounded cells. Data are representative of three experiments.

(G) Integrin upregulation upon CD100 ligation. FACS analysis of α6, β4 and CD103 expression on 7-17 DETC after stimulation with anti-CD100. Data are representative of three independent experiments. See also Movies S1, S2, S3, S4, S5, and S6 and Figure S3.

analysis of DETC circularity in these animals showed no difference in the overall cell shape circularity between wild-type and CD100-deficient animals in undamaged tissues, with a circularity factor value approaching 0.25 in both animals (Figure 4G). However, 2 hr after tissue damage, wild-type DETCs exhibited significantly greater circularity than CD100-deficient DETCs with a circularity factor value approaching 0.6 for wild-type DETCs and only 0.3 for CD100-deficient DETCs (Figure 4G).

In order to examine this process more directly, we set up an in vitro system to analyze the effect of CD100 signals on DETCs. As was seen in DETCs in vivo, 7-17 DETCs cultured in vitro with suboptimal concentrations of anti-CD3 in combination with anti-CD100 did in fact show disappearance of dendritic processes and cellular rounding (Figures 5A and 5B and Movie S1). This morphology change was rapid (Figure 5C and Movie S5) and not seen in control cultures (Figures 5A–5C and Movies S1, S2, S3, S4, and S6). The proportion of rounded cells increased

4-fold in the first hour of cultures with anti-CD3 and anti-CD100 and by 2 hr almost half the cells had assumed a rounded morphology (Figure S3A). In contrast, control cultures with anti-CD3 and anti-CD4 showed no significant increase in cellular rounding over the entire two hours of culture (Figure S3A). Interestingly, anti-CD100 alone was able to induce some cellular rounding with 39% of cells having a rounded morphology at 2 hr compared to 20% of cells, cultured with anti-CD3, and 48% of cells, cultured with a combination of anti-CD3 and anti-CD100 (Movie S3 and Figure S3A). This result was further supported by morphometric analysis of cell shape. Only cells treated with anti-CD100, either in the presence or absence of anti-CD3, resulted in significant morphological rounding of DETCs in the culture (Figure 5B). Treatment with anti-CD3 alone or control Abs did not cause an increase in circularity factor (Figure 5B). In addition, plexin B2-induced rounding of DETCs was comparable to that induced by anti-CD100 (Figure 5D).

Our data support the idea that interactions of CD100 with plexin B2 are responsible for the morphology change seen in DETCs in response to tissue damage. However, CD100 is known to have ligands in addition to plexin B2, namely plexin B1 and CD72. Although CD72 is not expressed in the epidermis, plexin B1 is expressed by keratinocytes. Therefore, to assess the role of plexin B1 in DETC morphology changes, we assessed rounding response to wounding in ear epidermal sheets in the presence or absence of antibodies specific for plexin B1 or plexin B2. Only ear sheets incubated with anti-plexin B2 showed a reduction in rounding of DETC in response to wounding (Figure S3B). No effect was seen with plexin B1 Abs (Figure S3B), again suggesting that it is indeed interactions of CD100 with plexin B2 that are important for the DETC morphology changes seen in response to tissue damage.

CD100 Signals through ERK and Cofilin and Modulates Integrin Expression

Much insight has been gained recently on the signals from semaphorins through plexins that regulate morphology changes. Integrins and the actin cytoskeleton are major targets of plexin signaling. In the case of CD100, signals through plexin B1 involve a number of signaling cascades, which include molecules such as R-Ras and RhoA, as well as PI3K, Akt, and ERK (Basile et al., 2007). These signals lead to a variety of outcomes including, modification of the cytoskeleton and subsequently changes in cell adhesion and migration. On the other hand, which signaling pathways are activated through CD100 in T cells is completely unknown. We hypothesized that as signals through CD100 result in morphology changes similar to those seen through plexins, pathways activated by CD100 ligation were probably also similar to those generated by plexin ligation. To address this question directly, we examined activation of ERK and cofilin in response to CD100 ligation. 7-17 DETCs were incubated at 37°C for various times with CD100 mAb. Ligation of CD100 led to rapid and transient phosphorylation of ERK and dephosphorylation of cofilin (Figure 5E). Activation of ERK was maximal at 5 min of stimulation and rapidly declined thereafter, and both ERK and cofilin activities had returned to resting levels by 30 min of stimulation (Figure 5E). Similar results were seen in CD100 transfected CHO cells (Figure S3C). Whether or not signaling through ERK was in fact required for the rounding response seen in DETCs after CD100 ligation was assessed by the DETC rounding response to CD100 signals in the presence or absence of the ERK inhibitor PD98059. DETCs preincubated with the ERK inhibitor prior to CD100 ligation showed a complete block in rounding (Figure 5F). This supports the notion that signals through the ERK pathway are required for the rounding of DETCs in response to CD100 ligation. Additional analysis of integrin expression revealed an increase in expression of both α6 and β4 integrins upon CD100 ligation (Figure 5G). In contrast, other integrins, such as CD103, showed no effect of CD100 ligation (Figure 5G). The α6β4 integrin has been well documented to be important for cell migration, particularly in the skin, and it is tempting to speculate that this change in expression may correlate with migration of DETCs to the wound site. Thus, these data provide a possible mechanism for the observed effect of CD100 on DETC morphology and provide insight as to how CD100 may also function on other T cell subsets.

DISCUSSION

Signals delivered between keratinocytes and DETCs are crucial for effective wound healing in the skin. Until now, the TCR (Jameson et al., 2004), NKG2D (Whang et al., 2009; Yoshida et al., 2012), and the recently described costimulatory molecule JAML (Witherden et al., 2010) were the only DETC molecules known to have a functional interaction with damaged keratinocytes in response to cutaneous wounds. Here, we describe a unique interaction between CD100 on DETCs and plexin B2 on keratinocytes and establish a fundamental role for this interaction in the DETC response to keratinocyte damage.

We found constitutive expression of plexin B2 on epithelial cells in both the skin and intestine. Although the functions ascribed to plexin B2 to date are associated with its expression in the nervous system, several reports have demonstrated a broad distribution outside of the nervous system (Perälä et al., 2011; Yu et al., 2008; Zielonka et al., 2010). In many tissues, however, expression of plexin B2 does not overlap with expression of a described ligand for plexin B2, Sema 4C (Zielonka et al., 2010), suggesting other semaphorins as ligands for plexin B2. CD100 (also known as Sema 4D) has also been suggested as a ligand for plexin B2, largely as a result of in vitro binding of CD100 to plexin B2 (Masuda et al., 2004). Here, we show that CD100 expressed on DETCs can, indeed, function as a ligand for plexin B2 in the epidermis.

DETCs are in constant contact with neighboring keratinocytes and act as sentinels surveying for signs of damage or disease. Upon keratinocyte damage, an as-yet-uncharacterized antigen is expressed on the surface of the keratinocytes and recognized by the canonical TCR expressed by DETCs (Komori et al., 2012). Recent identification of the costimulatory pair JAML and CAR (Verdino et al., 2010; Witherden et al., 2010) provided direct evidence that, similar to αβ T cells, complete activation of DETCs requires coordinated interaction of molecules in addition to the TCR. The first hint that the interaction between plexin B2 and CD100 was also important for DETC function came from the in vitro inhibition of γδ T cell activation in response to plexin B2 blockade on damaged keratinocytes. Similarly, DETCs isolated from CD100-deficient animals showed a reduced activation in response to keratinocyte damage. Together, these data

suggested a role for the interaction between plexin B2 on keratinocytes and CD100 on DETC for γδ T cell activation.

Complete activation of DETCs is crucial for effective wound healing in the skin (Jameson et al., 2002; Witherden et al., 2010). Initial in vivo analyses further supported a role for plexin B2 and CD100 in this wound healing response. Expression of plexin B2 was upregulated after in vivo wounding. Furthermore, mice deficient in CD100 showed a 2 day delay in wound closure compared with wild-type animals. This delay is strikingly similar to the observed delay in wound closure in mice lacking γδ T cells (Jameson et al., 2002) and suggested a crucial role for plexin B2 and CD100 in γδ T cell function in response to wounding.

In the nervous system, interactions between plexin B receptors and class 4 semaphorins have been shown to directly regulate the actin cytoskeleton and play a vital role in the migration of developing neurons (Deng et al., 2007; Friedel et al., 2007; Zhou et al., 2008). A more detailed analysis of the DETC defect in CD100-deficient animals revealed a striking correlation with the neuronal function for plexin-semaphorin interactions. Mice deficient in CD100 showed a delay in the DETC morphology change that typically occurs in response to activation by keratinocyte damage. In wild-type animals, DETCs are highly dendritic immobile cells under homeostatic conditions (Chodaczek et al., 2012; Gray et al., 2011). After disruption of this homeostasis through physical injury to the skin, a hallmark feature of the response of DETCs adjacent to the site of a wound is retraction of their dendritic processes and assumption of a round morphology within hours of keratinocyte damage (Chodaczek et al., 2012; Havran and Jameson, 2010; Komori et al., 2012). In stark contrast, DETCs in CD100-deficient animals retained their dendritic morphology after wounding and were incompletely activated by keratinocytes, highlighting this morphology change as a crucial step in the complete activation of epidermal γδ T cells.

In many contexts of γδ T cell dysfunction, one common element is apparent. Whether the γδ T cells are inactive because of exposure to immunosuppressive agents, such as rapamycin (Mills et al., 2008), or because of the complex factors surrounding obesity and type 2 diabetes (Taylor et al., 2010), it is very clear that these cells do not round in response to wounding. Experiments designed to assess whether plexin B2 triggering of CD100 played a direct role in this rounding response revealed that ligation of CD100 with either a mAb specific for CD100 or a soluble plexin B2-Fc fusion protein did, indeed, induce the morphology change in DETCs that was dysregulated in vivo in the case of CD100 deficiency. Rounding of DETCs was enhanced by simultaneous signals through CD100 and the TCR; however, signals through the TCR alone were unable to induce substantial rounding in these cells. This observation is consistent with recent evidence that signals through the TCR alone cause clustering of γδ TCRs on DETCs and phosphorylation of CD3ζ, but are unable to induce dendrite retraction and rounding of DETCs (Chodaczek et al., 2012).

Although much work has been done on the signaling molecules activated by CD100 ligation of plexins, very little is known about the intracellular signals transduced through the CD100 intracellular domain. Early work suggested that a serine kinase pathway was probably associated with CD100 (Elhabazi et al., 1997), supporting the idea that CD100 ligation would generate intracellular signaling events in DETCs. In keeping with the similar functional outcomes of signals through plexins in the nervous system and CD100 in the skin, intracellular events also correlated. Both cofilin and ERK were rapidly and transiently activated after CD100 ligation, consistent with a role for this receptor-ligand pair in DETC responses during wound repair. Activation of ERK was required for the effects of CD100 on DETC morphology. In human T cells, cofilin undergoes activation and dephosphorylation in response to signals through CD2 or CD28 costimulation, but not through TCR stimulation alone (Samstag et al., 1991; Samstag et al., 1994; Samstag et al., 1992). Similarly here we show accessory molecule activation, in this case CD100, of cofilin independent of TCR stimulation. Whether rapamycin and/or obesity and type 2 diabetes directly impact CD100, as well as the signaling pathways that are activated by CD100, ligation is unknown at this time. In human T cells, however, rapamycin has no effect on the CD2 or CD3/CD28 induced cofilin dephosphorylation (Ambach et al., 2000), suggesting that the effect of rapamycin on DETC rounding may well be indirect. Nevertheless, the rounding of epidermal γδ T cells in response to cutaneous wounds is clearly both a consequence of their activation and a fundamental requirement for their activation. This rounding can be achieved through direct interaction between CD100 and plexin B2.

αβ T cell activation involves multiple cytoskeletal rearrangements (Samstag et al., 2003). These differ somewhat from the DETC morphology changes, given that circulating αβ T cells, unlike resting DETCs, are round and reorganize their cytoskeleton upon activation to direct the T cell response toward the APCs. DETCs, on the other hand, are highly dendritic stationary cells under homeostatic conditions (Chodaczek et al., 2012; Gray et al., 2011; Komori et al., 2012) and rapidly retract their dendrites upon activation (Komori et al., 2012), presumably in order to proliferate and to migrate to the wound site to direct their growth factor production for proliferation of keratinocytes and re-epithelialization of the wound area. Nevertheless, it is clear that both αβ and γδ T cells rely on accessory molecule-dependent cytoskeletal changes for complete activation.

Although a function for plexin B2 had previously only been described in the nervous system, the immune and nervous systems do share many common features. In the developing nervous system, plexin B2 is required for both proliferation and migration (Deng et al., 2007). In the wound healing response in the skin, activated γδ T cells proliferate and migrate to the site of tissue damage (Jameson et al., 2002). The subsequent re-epithelialization phase of repair involves keratinocyte proliferation, migration, and differentiation (Jameson et al., 2002). This process is severely impaired in mice lacking γδ T cells (Jameson et al., 2002). Here we show that CD100 deficiency leads to an impaired DETC response to keratinocyte damage resulting in wound healing delays that are similar to those seen in γδ T cell-deficient animals. By analogy with plexin B2 in the nervous system, the plexin B2-CD100 receptor ligand pair probably functions to regulate migration during wound healing in the skin; such a notion is supported by the intracellular signaling cascades activated by CD100 ligation.

Although we have shown here a crucial role for plexin B2 and CD100 in wound repair in the skin, these molecules also

probably function in other epithelial tissues, such as the intestine and lung, where γδ T cells play a central role in tissue maintenance. The identification of plexin B2-CD100 interactions in the immune system adds to the growing body of evidence that many molecular interactions directing neuronal guidance cues also function to initiate immune responses (Khan et al., 2001; Tordjman et al., 2002). Furthermore, identification of new plexin partners for semaphorins has broader implications for other processes where semaphorins have been shown to play a vital role, such as in vascular growth and tumor progression (Kruger et al., 2005).

EXPERIMENTAL PROCEDURES

Animals

C57BL/6, B6.PL (Thy1.1), B6.SJL (Ly5.1), *Sema4d*$^{-/-}$ (Shi et al., 2000), and clone 4 TCR transgenic (Redmond et al., 2008) mice were bred and maintained under specific pathogen-free conditions in the Scripps Research Institute's animal facility. All animal protocols were in accordance with the Scripps Research Institute Institutional Animal Care and Use Policy.

Cell Lines

PAM 2-12, CHO-K1, HEK293, and hybridoma cell lines were maintained in complete DMEM (Invitrogen) supplemented with 10% FCS (Omega Scientific) or 20% FCS (for hybridomas). The 7-17 DETC cell line was maintained in complete RPMI 1640 (Invitrogen) supplemented with 10% FCS and 20 U/ml IL-2. All cells were maintained at 37°C and 5% CO_2.

Monoclonal Antibody Production

A female 8-week-old Armenian hamster (Cytogen Research and Development) was immunized multiple times with the PAM 2-12 cell line and spleen cells were fused with X63Ag8.653 myeloma cells with PEG 4000 (Sigma). Hybridoma tissue culture supernatants were collected and mAbs purified on Protein A-sepharose (Sigma-Aldrich). Antibodies were initially screened for their ability to block or augment the activation of γδ T cells by stressed keratinocytes. From this initial screen, 12 antibodies were chosen for further characterization. The proteins recognized by these Abs were then immunoprecipitated from keratinocyte lysates. On the basis of SDS-PAGE motility, any proteins that were probably known, and well established as playing a role in T cell activation, were not further characterized. Any duplicate antibodies appearing to recognize the same protein were excluded from further analysis. Five or six unique immunoprecipitation patterns were identified. The corresponding Abs were either frozen down for later analysis or subcloned and retested for functionality and further characterized. One such antibody was identified as recognizing CD81 (Boismenu et al., 1996) and another recognized CD63 (unpublished data).

Mass Spectrometry and Protein Sequencing

The ligand of the 3E7 mAb was immunoprecipitated from lysates prepared from 2 × 10^{10} PAM 2-12 cells as described above. Immunoprecipitated proteins were resolved by SDS-PAGE, transferred to PVDF membrane, and visualized with Coomassie Brilliant Blue. The 80 kDa protein band was excised and subjected to N-terminal protein sequencing. To identify the 160 kDa protein, we first deglycosylated the immunoprecipitated proteins with PGNase F, then resolved them by SDS-PAGE and visualized them with GelCode Blue (Pierce). The resulting 120 kDa protein band was excised and digested with Trypsin overnight. Protein digests were separated by reversed phase HPLC and fractions analyzed by peptide mass fingerprinting. A number of peptides were further analyzed by chemical sequence analysis and mass spectrometry. All sequences matched those of plexin B2 fragments.

Production of Plexin B2 Fc Fusion Protein

The cDNA encoding the extracellular domain of plexin B2 was generated from PAM 2-12 RNA by RT-PCR with the following primer set; sense: 5′-CTTCTGGTACCTTGAGTTCTGGGGGCAAT-3′ and antisense: 5′-TGAGCGG CAGATCTCTCGCACGCGT-3′. KpnI and BglII restriction sites were added to the sense and antisense primers, respectively, for inframe insertion into a modified pcDNA3 vector containing coding sequence for the Fc region of human IgG1 (Chen et al., 1996). The resulting vector was stably transfected into HEK293 cells, supernatants collected, and plexin B2-Fc purified on protein A-sepharose.

Flow Cytometry

Cells were stained with unconjugated mAbs or Fc-fusion proteins and subsequently stained with fluorescent labeled secondary reagents as described previously (Jameson et al., 2004; Kumanogoh et al., 2000).

Epidermal Cell Isolation and Culture

DETC were isolated from murine skin as described (Havran et al., 1991). For production of short term cell lines, epidermal cells were cultured as described previously (Mallick-Wood et al., 1998).

Isolation of Intestinal Cells

Intestinal epithelial cells were isolated essentially as described (Chen et al., 2002).

Activation Assays

DETC activation assays were performed as described (Havran et al., 1991). For CD8 T cell activation assays, clone 4 T cells were isolated from spleen and lymph nodes of clone 4 TCR transgenic animals by negative selection on magnetic beads. mAb 3E7, control mAb (1A7), or culture medium were added to cultures for 2 hr at 37°C prior to the addition of DETC or clone 4 cells. HA peptide was added to clone 4 cultures, 1 hr prior to the addition of T cells. Twenty-four hours after initiation of cultures, supernatant was removed from wells and IL-2 quantitated with the IL-2-sensitive CTLL cell line and MTT assay (Heeg et al., 1985) or by ELISA.

Immunoprecipitation and Immunoblotting

Cells were either lysed directly or surface-labeled with biotin then lysed in 10 mM Tris (pH 7.6), 150 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 1% NP-40, and protease inhibitors (Roche). Proteins were then immunoprecipitated with 5 μg 3E7 or control mAb and protein A-sepharose. For analysis of signaling pathways, cells were treated with 10 μg/ml CD100 mAb for various times, washed with PBS, and lysed in 10 mM Tris (pH 7.6), 150 mM NaCl, 1 mM EDTA, 5 mM $MgCl_2$, 0.5% NP-40 containing protease, and phosphatase inhibitors (for ERK analysis) or lysed in 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT, and 0.01% bromophenol blue (for cofilin analysis). Total cell lysates or precipitated proteins were separated by SDS-PAGE, transferred to nitrocellulose, and visualized with peroxidase-conjugated secondary reagents and chemiluminescence (SuperSignal; Pierce).

Surface Plasmon Resonance Analysis

Mouse plexin B2-Fc and CD100-Fc fusion proteins were expressed in HEK293 cells and purified by Protein A affinity chromatography. HL4E10 IgG was expressed and purified as described previously (Witherden et al., 2010). Proteins were purified by size exclusion chromatography (Superdex 200 HR10/30) immediately prior to performing the SPR experiments. SPR measurements were performed on a BIAcore 2000 (GE Healthcare) at 25°C with PBS (pH 7.4) as running buffer. Plexin B2-Fc and HL4E10 IgG as control protein were coupled at similar immobilization levels in two different flow cells on a BIAcore CM5 sensor chip by standard amine coupling chemistry. CD100-Fc (100 nM) was then flowed over the chip surface at a flow rate of 30 μl/min.

Analysis of Cell Morphology Changes In Vitro

For fixed-cell imaging, 10^5 7-17 cells were plated on 22 × 22 mm coverslips that were either uncoated or had been coated overnight at 4°C with 0.1 μg/ml anti-CD3 (500A2) alone or in combination with 10 μg/ml anti-CD100 (Kumanogoh et al., 2000), plexin B2-Fc, or anti-CD4 (GK1.5; BD Biosciences). Cells were incubated in RPMI containing 5% FCS and 2 U/ml IL-2 at 37°C. Cells were fixed with 4% paraformaldehyde and stained with 2 μg/ml Alexa Fluor 488 phalloidin (Invitrogen) and DAPI (Sigma) 60 and 120 min after the stimulation. In some experiments, 7-17 cells were preincubated in RPMI containing 5% FCS and 2 U/ml IL-2 and 50 μM ERK inhibitor PD98059 for 30 min prior to being plated on coated coverslips. Cell images

were captured with a Zeiss AxioCam camera attached to a Nikon Eclipse E800 microscope. The system was managed by Axiovision AC software.

For timescale monitoring, 7-17 cells were labeled with 1 μM cell-tracker CMTMR (Invitrogen). Eight-well chambered coverglass (Lab-Tek II, Nalge Nunc International) were coated as described above. A total of 10^4 cells/chamber were seeded in RPMI containing 5% FCS and 2 U/ml IL-2 and allowed to adhere at 37°C. Live-cell images were captured every 1 min for 120 min by CoolSnapHQ cameras (Roper) attached to a Zeiss 200M microscope through a beam-splitter and stationary emission filters. The system was run by Slidebook 4.0.3.9tz software (3I Corp.).

For examining rounding in vitro in intact skin, ears were excised and immediately wounded with a 1 mm punch biopsy and floated for 3 hr at 37°C in complete DMEM supplemented with 10% heat-inactivated FCS in the presence or absence of 20 μg/ml plexin B2 or plexin B1 Ab. Epidermal sheets were isolated as described (Jameson et al., 2002) and stained with PE-conjugated anti-γδ TCR (GL3) and DAPI. Digital images were acquired with 10X objective (Zeiss AxioCam HRc) and rounding was quantified using Photoshop CS2 software. At least nine images proximal to the wound were counted and at least three mice were examined per condition.

Morphometric Analysis of Cell Shape

Cell circularity factor was calculated based on the area and the perimeter as follows:

$$f = \frac{4\pi \cdot A}{P^2}$$

In this formula, the circularity factor *f* is proportional to the ratio of cell area A to the square of cell perimeter P (Cox, 1927; Wan et al., 1993; Wan et al., 1994). Use of the following formula yields a numerical assessment of the particle (cell) circularity in a range of between 1 (circular) and 0 (irregularly shaped). Both the cellular parameters (area and perimeter) and statistical analysis were performed with Matlab on 8-bit fluorescent microscopy images of epidermal ear sheets and differentially stimulated DETC cultures. Initially, the cellular boundaries were defined, allowing the determination of both perimeter and the area of a 2D fluorescent image of a cell. Care was takes to minimize microscopy artifact variations between genotypes and treatment groups. Cell edge detection threshold was kept constant between samples. The statistical comparison between the genotypes and treatment groups was assessed via one-way analysis of variance and then assessed with a Tukey test.

In Vivo Wounding

Two millimeter punch biopsy wounds were made in the ears or backs of 8- to 12-week-old C57BL/6 and CD100-deficient (Shi et al., 2000) animals as previously described (Jameson et al., 2002). Epidermal sheets were isolated from ears and stained 1 and 2 hr postwounding as described (Jameson et al., 2002). Stained epidermal sheets were examined under a Nikon Eclipse E800 microscope and digital images collected with a Zeiss AxioCam camera and Axiovision AC software. For analysis of wound closure, wound area of back wounds was measured daily with Image J software.

Bone Marrow Chimeras

For generating bone marrow chimeras, 6- to 8-week-old C57BL/6 congenic (CD45.1 or Thy1.1) and CD100-deficient mice were lethally irradiated with 10 Gy. The following day, bone marrow cells were isolated from femurs of C57BL/6, C57BL/6 congenic or CD100-deficient mice and injected intravenously into the appropriate irradiated recipient. Mice were analyzed 6–8 weeks after reconstitution for their response to in vivo wounding.

Statistical Analysis

Statistical analysis was performed with an unpaired, two-tailed Student's t test. A probability level of $p < 0.05$ was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and six movies and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2012.05.026.

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