Expression of CD200 on Epithelial Cells of the Murine Hair Follicle: A Role in Tissue-Specific Immune Tolerance?

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CD200 (OX-2) is a transmembrane glycoprotein that transmits an immunoregulatory signal through the CD200 receptor (CD200R) to attenuate inflammatory reactions and promote immune tolerance. CD200 expression in the skin has not been described previously. We now report that freshly isolated cells of the murine epidermis contain a subpopulation of major histocompatibility complex (MHC) class II-negative, CD3-negative keratinocytes that are CD200-positive. CD200 expression was accentuated in keratinocytes comprising the outer root sheath of the murine hair follicle (HF). When syngeneic skin grafts were exchanged between gender-matched wild-type (WT) and CD200-deficient C57BL/6 mice, significant perifollicular and intrafollicular inflammation was observed, eventually leading to the destruction of virtually all HF (alopecia) without significant loss of the CD200-negative grafts. Minimal and transient inflammation was observed in WT grafts, which persisted long term with hair. There was a 2-fold increase in graft-infiltrating T cells in CD200-deficient skin at 14 d. Alopecia and skin lesions were induced in CD200-deficient hosts by adoptive transfer of splenocytes from WT mice previously grafted with CD200-negative skin, but not from mice grafted with WT skin. Collectively, these results suggest that the expression of CD200 in follicular epithelium attenuates inflammatory reactions and may play a role in maintaining immune tolerance to HF-associated autoantigens.

Key words: alopecia/CD200/hair follicle/inflammation/keratinocyte/skin


CD200, formerly known as OX-2, is a type-1 transmembrane glycoprotein expressed in a variety of tissues including the thymus, nervous system, vascular endothelium, trophoblast, ovary, and various cells of the immune system (Bukovsky et al., 1983; Barclay et al., 1986; Barclay et al., 2002; Clark et al., 2003). CD200 mediates an immunoregulatory signal by binding to its receptor (CD200R), which is expressed on cells of the myeloid lineage as well as CD4+ and γδ T cells (Gorczynsky et al., 2000; Wright et al., 2000, 2003). CD200–CD200R interactions attenuate inflammatory reactions and promote immune tolerance in multiple settings. It is thought that CD200+ cells bind to CD200R+ macrophages and granulocytes, downregulating their basal level of activation and thus restricting their ability to initiate tissue-specific autoimmunity (Barclay et al., 2002). Consistent with this, CD200-deficient mice (CD200−/−) have increased numbers of activated CD200R+ macrophages in the steady state, chronic central nervous system (CNS) inflammation and an exaggerated inflammatory response to trauma (Hoek et al., 2000). In addition, these mice have an increased susceptibility to develop experimental autoimmune uveoretinitis, experimental allergic encephalomyelitis and collagen-induced arthritis (Hoek et al., 2000; Broderick et al., 2002; Gorczynsky et al., 2002). Abnormalities in the skin of CD200−/− mice have not been reported previously.

We recently reported that CD200−/− mice were resistant to UV-mediated induction of tolerance to hapten in a murine contact hypersensitivity model (Rosenblum et al., 2004). Since CD200 expression in the skin has not been described previously, we set out to determine which skin-resident cells expressed CD200 and whether CD200 plays a role in downregulating cutaneous immune reactions. We now provide the first evidence for expression of CD200 in the skin. We show, as expected, that some Langerhans cells (LC) express CD200, but we also report that a subset of CD3neg, major histocompatibility complex (MHC) IIneg epidermal cells (EC) express CD200 on their cell-surface. These CD200+ EC co-localized with cytokeratin-14+ (K14+) keratinocytes (KC) in the outer root sheath (ORS) of the murine hair follicle (HF). We subsequently found that syngeneic, gender-matched skin grafts lacking CD200 show inflammatory destruction of their HF (i.e., alopecia), characterized by heightened recruitment of CD4+ and CD8+ T cells into the grafts. Alopecia (and skin lesions) was induced in CD200-deficient hosts by adoptive transfer of splenocytes from syngeneic wild-type (WT; CD200+/-) mice previously grafted with CD200−/− skin, but not from those grafted with WT skin, suggesting a cell-mediated autoimmune etiology.

Abbreviations: α-MSH, α-melanocytestimulating hormone; ACTH, adrenocorticotropic; APC, antigen-presenting cells; B6, C57BL/6; BMC, bone marrow chimeras; CD200R, CD200 receptor; DC, dendritic cell; EC, epidermal cell; HF, hair follicle; K14, cytokeratin-14; KC, keratinocyte; LC, Langerhans cell; ORS, outer root sheath; SFM, serum-free medium; TGF-β, transforming growth factor-β; WT, wild-type (CD200+/-)
Results

KC express CD200. To determine whether murine EC expressed CD200 on their cell surface, we performed four-color flow cytometry on freshly isolated EC from adult ear skin. We examined CD200 expression on three major EC populations: T cells (CD3⁺, MHC II⁺), LC (CD3⁻, MHC II⁺) and keratinocytes (KC; CD3⁻, MHC II⁻). As shown in Fig 1a, epidermal T cells did not express CD200 ("R2"). Some LCs expressed low levels of CD200 ("R1"). The percentage of LC in WT and CD200⁻/⁻ mice did not differ (~2%). We consistently observed a subpopulation of KC (range 5%–15% in adult ear skin) that expressed high levels of CD200 (Fig 1a) ("R3"). When EC from murine pup trunk skin were examined, the percentage of CD200⁺ cells was slightly higher (range 15%–25%, not shown).

Subsequently, we tested murine KC cell lines for CD200 expression using flow cytometry and for mRNA using CD200-specific RT-PCR. For comparison, we also examined cultures of primary human KC derived from neonatal foreskins. The murine KC cell lines PAM212 and SP-1 did not express CD200 (Fig 1b); however, 308 cells, a KC tumor cell line initiated in vivo with 7, 12-dimethylbenz(a)anthracene (Strickland et al., 1988) expressed high levels of CD200 (Fig 1b). Although human primary KC did not show cell surface expression of CD200 (Fig 1b), CD200 mRNA was detected by RT-PCR (Fig 1c). mRNA from WT and CD200⁻/⁻ splenocytes were included as positive and negative controls, respectively.

CD200⁺ KC localize to HF epithelium. To localize CD200⁺ cells within the murine epidermis, we performed both CD200-specific immunohistochemistry and immunofluorescence on whole mounts of murine pup skin. CD200 was primarily localized to HF epithelium (Figs 2 and 3). Expression of CD200 was relatively uniform throughout the length of the follicle and was observed surrounding the bulb, isthmus, bulge, and infundibular regions. Bulb matrix cells, dermal papillary cells, and the hair shaft did not express CD200 (Fig 2). K14 is specifically expressed in KC of the basal layer of the interfollicular epidermis, the ORS and the outer layer of sebaceous glands (Braun et al., 2003). Using scanning confocal microscopy, we found that CD200 expression co-localized with the expression of K14 on KC of the ORS and on interfollicular basal KC (Fig 3). As reported by others (Clark et al., 1985), CD200 was also expressed on vascular endothelial cells.

CD200 attenuates HF-associated inflammation. HF are considered by some investigators to be sites of immune privilege (Christoph et al., 2000; Niederkorn, 2003; Paus et al., 2003). Because CD200–CD200R interactions have been shown to suppress tissue-specific inflammation in allografts and in autoimmune disease models (Gorzynski et al., 2000, 2002; Broderick et al., 2002) and because CD200⁻/⁻ mice are prone to induction of autoimmune disease (Hoek et al., 2000), we sought to determine whether CD200 might affect the fate of skin grafts taken from CD200⁻/⁻ donors and placed onto CD200⁺/⁺ WT hosts. Gender-matched (female-to-female) grafts were used to avoid male (H-Y) antigen-specific rejection. WT-onto-WT grafts were used as controls. In these studies, we observed an increased inflammatory cell infiltrate in the dermis of female CD200⁻/⁻ grafts relative to WT female grafts as early as 10 d post-transplant (Fig 4a). We observed an increased inflammatory cell infiltrate in the dermis of female CD200⁻/⁻ grafts relative to WT female grafts as early as 10 d post-transplant (Fig 4a). The infiltrate consisted of a substantial mononuclear population and some polymorphonuclear cells localized primarily in the perifollicular and intrafollicular regions of HF. By 30–40 d post-grafting, normal HF architecture in CD200⁻/⁻ grafts was distorted by inflammatory...
cells, accompanied by intrafollicular edema and intrafollicular apoptotic cells (Fig 4a inset). Inflammatory cells were rarely observed in the interfollicular dermis and non-HF associated epidermis. WT grafts showed transient inflammation and infiltration by mononuclear cells early post-transplant (e.g., 10–14 d). This was interfollicular and resolved entirely by 40 d.

**Figure 2**

Cutaneous CD200 expression localizes to hair follicle (HF) epithelium. Neonatal trunk skin was isolated from either wild-type (WT) or CD200-deficient (CD200<sup>−/−</sup>) mice. CD200-specific immunohistochemistry was performed on frozen sections as described in Materials and Methods. Scale: 1 cm = 80 μm (a,b) or 20 μm (c,d). Arrow indicates CD200<sup>+</sup> outer root sheath (ORS). Staining with isotype control IgG is shown in supplemental Fig S3.

**Figure 3**

CD200 expression co-localizes with K14 on basal keratinocytes (KC) and KC of the follicular outer root sheath (ORS). Neonatal trunk skin was isolated from either wild-type (WT) or CD200-deficient (CD200<sup>−/−</sup>) mice. CD200- and K14-specific immunofluorescence was performed on frozen sections. Images shown are z-projections obtained by sequential scanning confocal microscopy under identical optical conditions. Arrows denote the epidermis. Scale: 1 cm = 40 μm.

**Figure 4**

CD200 expression attenuates hair follicle (HF)-associated inflammation. Tail skin from either wild-type (WT) or CD200-deficient (CD200<sup>−/−</sup>) female B6 mice were grafted onto the trunks of WT female B6 recipients. (a) At various times post-grafting, punch biopsies were performed and sections were stained with H&E. Inset represents higher magnification of boxed area. Arrowheads denote HF. (b) Photograph of representative grafts at 80 d post-transplant. Results are representative of five replicate experiments. d, day. Actuarial graft survival curves are shown in supplemental Fig S1; the differences are not statistically significant (78% vs 100% 100-d survival for CD200<sup>−/−</sup> and WT grafts respectively; p > 0.05).
Beyond 30–40 d, the hair on all CD200−/− female grafts was completely lost, whereas the skin grafts remained intact (18 of 20 over five independent experiments) (Fig 4b). Histological examination confirmed loss of HF structures (Fig 4a). In some CD200−/− grafts, dermal inflammation resolved after HF loss leaving behind scarring (Fig 4a). In those grafts, non-HF-associated epidermis remained largely unaffected and hairless grafts persisted long-term (>100 d). No new hair growth was observed. In contrast to the CD200-deficient grafts, no hair loss was observed in WT female-to-female grafts (n=19), and all WT grafts followed for >100 d persisted with hair. Two of 20 CD200−/− grafts were lost at 27 and 42 d; three other grafts were lost at 6, 23, and 53 d after being biopsied. No WT-onto-WT grafts were lost, even after biopsy. The actuarial 100-d survival rate for CD200−/− grafts was not statistically different from that of WT grafts (78% vs 100%, respectively; p>0.05; supplemental Fig S1).

We next grafted gender-matched WT skin and CD200−/− skin onto a small number of CD200−/− hosts (three each) to determine whether the nature of the host (WT vs CD200−/−) altered the fate of the graft. As with WT recipients, WT grafts were accepted by CD200−/− hosts (>100 d) with no gross evidence of hair loss, whereas all three of the CD200−/− grafts developed alopecia. Notably, alopecia was confined to the grafted CD200−/− skin and did not spread to involve intact skin of the CD200−/− hosts suggesting a graft-localized effect and, possibly, other mechanisms that protect CD200−/− HF outside the graft.

Phenotype of graft-infiltrating T cells To define the phenotype of graft-infiltrating T cells, we collected pairs of WT and CD200−/− skin grafts from the same WT hosts at 14 d after grafting, collagenase digested the skin, and stained the pooled graft-cell suspensions for CD4 and CD8 T cells in the presence of 7-amino-actinomycin D (7AAD) to exclude dead cells. The results are shown in Fig 5. Both CD4 (2.7%) and CD8 (8.7%) T cells were detected in the CD200−/− grafts; the CD4:CD8 ratio was 1:3. The T cells appeared to be lymphoblasts based on increased forward light scatter (supplemental Fig S2). The percentage of T cells (11.4%) in CD200−/− grafts was almost double that of the paired WT grafts taken from the same mice (6.0% = 1.4% CD4 and 4.6% CD8).

Adoptive transfer of alopecia to CD200-deficient hosts Next we sought to determine whether alopecia could be induced in the skin of CD200-deficient B6 mice. Unseparated splenocytes from “HF-sensitized” mice (i.e., WT mice grafted with CD200−/− skin >3 mo earlier) were mixed with normal WT bone marrow and given to irradiated CD200-deficient mice. Creation of bone marrow chimeras (BMC) ensured engraftment of the infused WT spleen cells. Control
BMC received spleen cells from WT donors that had been grafted earlier with WT skin. Beginning approximately 2 wk post-transplant, we observed patches of alopecia in BMC given HF-sensitized syngeneic splenocytes (Fig 6), but not those given cells from naïve donors. In comparison to recipients of naïve cells, hair was more easily removed from affected CD200−/− mice by gentle plucking. In an extreme case, the epidermis separated from the dermis in a small patch on the scruff of the neck as the mouse was being examined (shown in Fig 6d). Mononuclear infiltrates were noted in histological examination (Fig 6e and f), but their nature has yet to be determined. These lesions were variable in their severity and did not spread beyond initial boundaries. With time, hair growth resumed in affected areas.

Discussion

Our interest in CD200 expression in the skin was prompted by the observation that cutaneous UVB-mediated tolerance to hapten could not be induced in mice that were deficient in CD200 (Rosenblum et al, 2004). As a prelude to studies on the mechanism involved, we looked at CD200 expression in murine skin. As expected, a portion of the LC population expressed CD200, but there also was a previously unidentified subpopulation of CD3neg, MHC IIneg EC that expressed cell-surface CD200 (Fig 1). Most of these intrafollicular and perifollicular inflammation upon grafting of gender-matched skin from CD200−/− donors onto syngeneic WT hosts (Fig 4), including a doubling in the number of graft-infiltrating T cells (Fig 5). This eventually led to complete and permanent hair loss (Fig 4), but not loss of most CD200+/− grafts (supplemental Fig S1). In contrast, WT grafts remained intact with continued hair growth despite evidence of early T cell infiltration (Fig 5).

Given the extensive literature implicating the CD200–CD200R pathway in immune tolerance and the fact that the HF is regarded by many as a site of immune privilege, these findings are of significant interest.

Mammalian HF are evolutionarily conserved structures which play a major role in many important biological processes such as thermal protection, camouflage, tactile sensation, and mating behavior. Since epidermal stem cells reside in HF, follicular units may play an important role in the regenerative capacity of the epidermis (Niederkom, 2003; Tumbar et al, 2004). It has been proposed that the HF is distinct sites of immune privilege with a heightened level of protection from potentially damaging cutaneous inflammatory immune reactions (Paus et al, 2003). Several lines of evidence support this view: (i) Anagen stage HF have absent or a very low level of MHC class I expression (Harrist et al, 1983; Christoph et al, 2000); (ii) anagen hair bulbs express immunosuppressive factors, such as TGF-β, ACTH, and γ-MSH (Welker et al, 1997; Slominski et al, 1998; Botchkarev et al, 1999); (iii) anagen hair bulbs have a markedly reduced number of antigen-presenting cells (APC) which do not appear to express MHC class II (Christoph et al, 2000); and (iv) HF epithelium express non-classical MHC class I molecules (Paus et al, 1994) that promote immune tolerance (Fuzzi et al, 2002). Follicles in anagen phase have the potential to manufacture follicle-associated proteins capable of acting as autoantigens in eliciting HF-directed autoimmunity, such as that observed in alopecia areata, lupus erythematosus, morphea, lichen planopilaris, and even graft-versus-host disease (Paus et al, 2003).

The mechanism of destruction of HF in our model using CD200−/− skin grafts is not yet known. CD200 interacts directly with the CD200R on macrophages and granulocytes, suppressing their tonic activation and sparing CD200−/− tissues from the damaging effects of these cells (Nathan and Muller, 2001; Barclay et al, 2002). The transient ischemia and surgical trauma associated with skin grafting are known to induce a pro-inflammatory environment within the skin graft as well as the host. In our model, tissue trauma may incite inflammatory alopecia that fails to resolve in the absence of CD200. This is consistent with the data from others showing that inflammation is not appropriately resolved in the peripheral nervous system of CD200−/− mice after trauma from facial nerve transaction, resulting in the persistence of inflammatory cells and increased local tissue damage (Hoek et al, 2000). Mast cells are among the earliest cells that respond to tissue injury. They rapidly release pro-inflammatory mediators (Sherman, 2001) and are likely candidates for initiation of an inflammatory cascade in our skin graft model. The prominent expression of CD200R on mast cells led Wright et al (2003) to suggest that the CD200–CD200R pathway may function as a constitutive regulator of mast cell biological responses and predispose CD200−/− mice to autoimmune diseases. Direct involvement of skin-resident mast cells in our model, however, remains to be demonstrated.

CD200−/− KC or LC (or both populations) may actively protect the HF of WT skin grafts by limiting inflammatory damage. Failure to regulate inflammatory cells within the graft may lead to the enhanced recruitment of autoreactive T cells or, alternatively, a failure to recruit regulatory T cells. Gorczynski et al (2004) recently found that ligation of CD200R on dendritic cells converts them to a tolerogenic phenotype that leads to the activation of CD4+ CD25+ regulatory T cells in vitro. Since some T cells (CD4+ as well as γδ+ T cells) express CD200R (Gorczynsky et al, 2000; Wright et al, 2000, 2003), it is conceivable that CD200−/− KC or LC cells may interact directly with them. Borriello et al (1997) originally identified CD200 (OX-2) as a T cell co-stimulatory molecule, suggesting it as a member of the B7 family. We found that production of pro-inflammatory cytokines by autoreactive T cells in vitro is diminished in the presence of autologous DC that express CD200−/− (Rosenblum et al, 2004). Furthermore, we found that soluble CD200 (in the form of a CD200.FLAG fusion protein) altered the cytokine gene expression profile of murine CD4+ T cells (M.D. Rosenblum, unpublished data).

Spontaneous alopecia, including a scarring alopecia similar to human follicular degeneration syndrome, has been reported in some C57BL/6 sublines.3 Abnormalities in

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the skin or hair of CD200−/− mice have not been reported previously. We have not been successful at triggering hair loss in intact CD200−/− mice using skin irritants or cytokines, although we have not tried combinations such as the mixture of interferon (IFN)-γ, tumor necrosis factor (TNF)-α, and interleukin (IL)-1β used by Ruckert et al (2000). Other regulatory mechanisms may be involved in the control of autoreactive T cells in CD200−/− mice. The finding that lesions induced by adoptive transfer of HF-sensitized splenocytes did not spread beyond the initial affected areas indirectly supports this, as does the fact that hair loss in CD200−/− skin grafts placed on CD200−/− hosts was confined to the graft and did not involve the adjacent CD200-deficient skin.

Deficiencies in regulatory mechanisms, including regulatory T cells, have been linked to some forms of alopecia: (i) McClure and Hoffmann (2002) report an increase in expression of regulatory cytokines and suggest failure to recruit CD4 and CD8 T cells as a mechanism of resistance to alopecia areata; (ii) Zoller et al (2002) found decreased numbers of CD4+ CD25+ regulatory T cells in the skin of C3H/HeJ mice with chronic alopecia areata as well as recipients of affected skin; and (iii) Hequet et al (2004) show that depletion of CD4+ T cells in autologous bone marrow chimeras induced a skin disease with clinical and histological features of alopecia areata. Lethal total body irradiation, as used by us in creating autologous BMC, is known to transiently deplete regulatory T cells. New regulatory T cells are produced de novo and emigrate from the repopulated host thymus after 2–3 wk (Johnson et al, 1999, 2002). This coincides with the approximate time that we observed stabilization of the skin lesions after adoptive transfer of HF-sensitized splenocytes. We plan to test the hypothesis that re-emergence of regulatory T cells is responsible for limiting the extent of skin lesions induced by adoptive transfer of sensitized splenocytes and, perhaps, also in the intact skin of unmanipulated CD200−/− mice.

Collectively, our data suggests that CD200 provides immune protection to murine HF. This finding also has significant implications for immune recognition of skin cancer. Basal cell carcinoma and squamous cell carcinoma of the skin can arise from malignant transformation of epithelial cells in the HF (Oro and Higgs, 2003). As shown in Fig 1b, cell surface expression of CD200 was detected on the murine KC-derived tumor cell line 308, but not on PAM212 or SP-1 cells. Because these murine KC tumor cell lines are most likely derived from a single tumor cell clone, we speculate that 308 cells may be derived from a KC that originated from CD200+ HF epithelium, whereas PAM212 and SP-1 cells did not. Alternatively, 308 cells may have induced CD200 expression upon malignant transformation. Specific expression of CD200 on 308 cells suggests the possibility that some KC-derived tumors may exploit CD200 expression as a means to evade tumor immunity. We previously reported that several tumor lines, including C1498 (a murine NKT cell leukemia), SCC-7 (a murine squamous cell carcinoma), and U2OS (a human osteosarcoma), express CD200 either constitutively or upon induction of apoptosis (Rosenblum et al, 2004). Others have shown that CD200 is involved in suppressing tumor-specific immune responses (Gorczynski et al, 2001). Experiments are planned to determine whether CD200 alters the immune response to skin cancer cells, as it appears to do with HF.

Based on the well-documented role for CD200–CD200R interaction in tolerance induction and the data presented herein, we propose that CD200 expression on HF epithelium (or LC) directly regulates innate and/or adaptive immune reactions in suppressing HF-associated inflammation and, perhaps, plays a role in maintaining HF-specific immune tolerance. Whether cells within human hair follicles express CD200 remains to be determined. Primary human KC cultures (neonatal foreskin) expressed CD200 mRNA, but cell surface expression was not observed (Fig 1b, c). CD200 may be restricted to cells that localize to HF or basal epidermis in situ. Alternatively, CD200−/− KC may preferentially expand upon culture in vitro.

Materials and Methods

Mice C57BL/6 mice (B6) were purchased from Jackson Laboratories (Bar Harbor, Maine). CD200−/− mice (Hoek et al, 2000) were provided by Dr Jonathan Sedgwick (DNAX Research Institute, Palo Alto, California). Age-/sex-matched mice were used in all experiments. All animal experiments were done under protocols approved by the institutional animal care and use committee.

KC isolation and cell culture The murine KC cell lines, PAM212, SP-1, and 308 are kindly provided by the laboratory of Dr Stuart Yuspa (National Cancer Institute, NIH, Bethesda, Maryland). Cell lines were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) (Gibco BRL, Grand Island, New York) supplemented with 10% fetal bovine serum (FBS). Human KC were derived from newborn human foreskins and cultured in KC-defined serum-free medium (SFM; Gibco BRL) (Rheinwald and Green, 1975). Neonatal trunk or adult ear skin was excised from B6 mice and EC were isolated as previously described (Takami et al, 1979). Briefly, skin was separated from the cartilage plate and floated on 0.5% trypsin (Gibco BRL) in phosphate-buffered saline (PBS) at 37°C for 45 min. Epidermal sheets were peeled from the dermis, re-suspended in 0.05% DNAase (Sigma, St Louis, Missouri) in PBS containing 10% FBS. Single-cell suspension was obtained by vigorous passage through a 60 μm syringe. For RT-PCR analysis, cells were cultured in KC-defined SFM. For flow cytometry, freshly isolated murine and human EC were washed 1× in cold PBS and 4×10^6 cells were stained for 30 min at 4°C with the following: Alexa Fluor-647 conjugated rat anti-mCD200 antibody (OX-90), Alexa Fluor-647 conjugated rat IgG isotype control (R35-95), PE anti-hCD200 (MRC OX-104); FITC anti-I-Ab (Molecular Probes, Eugene, Oregon) conjugated rat anti-mCD200 (Molecular Probes, Eugene, Oregon) conjugated rat anti-mCD200 antibody (OX-90), Alexa Fluor-647 conjugated rat IgG isotype control (R35-95), PE anti-hCD200 (MRC OX-104); FITC anti-I-Ad (KH74); PE anti-CD3 (145-2C11), and 7AAD (Calbiochem, La Jolla, California). All antibodies were from Pharmingen (San Diego, California). Ox-90 and R35-95 mAbs were conjugated to Alexa Fluor 647 according to manufacturer’s protocol. Cells were washed twice in cold PBS and analyzed by flow cytometry on a Becton Dickinson (San Jose, California) FACScan flow cytometer.

RT-PCR Mouse and human KC were isolated as described above and cultured in KC-defined SFM. After two passages, cells were harvested, and total RNA was extracted with TRIzol according to manufacturer’s instructions (Life Technologies, Rockville, Maryland). RNA was quantified, and equal amounts (~1 μg) were reverse transcribed into cDNA with oligo(dT) primers using Thermoscript RT-PCR systems (Gibco BRL) according to manufacturer’s instructions. RT-PCR was performed using CD200 (5′-AGTGTTGACCGAGTAA-3′, 5′-TACTATGGGCTGTACATAG-3′) or β-actin (5′-GAGTCCTGTCCTCCACACG-3′, 5′-CTAGGAGCAT-TTGCGGTG-GAC-3′) primer sets.

Immunofluorescence microscopy, immunohistochemistry, and flow cytometry Neonatal skin was isolated from either...
WT (CD200+/−) or CD200−/− mice. Specimens were immediately placed in Tissue-Tek OCT Compound (Miles, Elkhart, Indiana), frozen on dry ice, and stored at −70°C. 6 μm cryosections were stained for immunofluorescence microscopy as previously described (Basset-Seguin et al. 1988). Rat anti-mCD200 (OX-90), rabbit anti-K14 (AF64; Covance Research, Berkeley, California) or rat IgG isotype control (R35-95) were used as primary antibodies; FITC-conjugated goat F(ab)2 anti-rat IgG (Jackson Immunoresearch Laboratories, West Grove, Pennsylvania) and rhodamine-conjugated goat anti-rabbit IgG (Pierce Biotechnology, Rockford, Illinois) were used for detection. All antibodies were used at a 1:1000 dilution. Confocal images were acquired using a Leica TCS SP2 microscope (Leica, Heidelberg, Germany). Approximately 20 optical sections were sequentially captured with a typical increment of 1–3 μm. Scans are presented as z-projections.

Immunohistochemistry was carried out on frozen sections as previously described (Horney et al. 2000). Anti-mCD200 mAb or rat IgG isotype control mAb binding was detected using biotinylated rabbit anti-rat IgG (Vector Laboratories, Burlingame, California) followed by streptavidin-peroxidase (Vectastain ABC kit, Vector) according to manufacturer’s instructions. Peroxidase activity was revealed using 3,3′-diaminobenzidine substrate (SK-4100, Vector) for 5–10 min at room temperature. Sections were counterstained with hematoxylin and eosin (H&E). Isotype control staining is shown in supplemental Fig S3.

Paired, gender-matched skin grafts from WT and CD200−/− were harvested 14 d after grafting onto WT recipients, finely minced using scissors and digested with collagenase D (1 mg per mL) for 90 min at 37°C. The grafts were disrupted by rapid trituration using a 5 mm syringe, pooled, filtered to remove debris, counted, and then stained with CD4- and CD8-specific antibodies for analysis by flow cytometry. 7AAD was used to exclude dead cells. Electronic gates were set on viable cells with forward- and side-scatter of light characteristic of lymphocytes and lymphoblasts as shown in supplemental Fig S2.

Skin grafting Tail skin was grafted to the dorsal trunk as previously described (Rosenberg, 1994). Briefly, tail skin was harvested from age-matched WT and CD200−/− female B6 mice and grafted onto the backs of age-matched WT B6 females. In some experiments, WT and CD200−/− skin grafts were grafted onto alternate flanks on the same recipient, and in other experiments, each recipient received only one graft. Skin grafts were observed daily. Loss of graft was defined as the day that the grafted skin was no longer measurable. At various times post-grafting, punch biopsies were taken from viable grafts. Specimens were fixed in 4% formalin in PBS, embedded in paraffin, sectioned at 5 μm thickness, and stained with H&E. Actuarial graft survival curves were generated using the pooled results of five experiments, including 19 WT-onto-WT and 20 CD200−/−-onto-WT gender-matched grafts. Graft survival curves were compared by log rank analysis. Skin grafts that were biopsied were censored from the analysis on the day that they were biopsied to avoid skewing the outcome since the process appeared to trigger rejection in a few CD200−/− grafts but not in any of the WT grafts.

Isotype control staining for immunohistochemistry with wild-type C57BL/6 neonatal pup skin (right panel) as compared to the positive control stained with CD200-specific antibody (left panel). Immunohistochemistry was performed on frozen sections as described in Materials and Methods.


