ANIMAL MODELS

Mast Cells Are Dispensable in a Genetic Mouse Model of Chronic Dermatitis

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Chronic inflammatory skin diseases, such as atopic dermatitis, affect a large percentage of the population, but the role of different immune cells in the pathogenesis of these disorders is largely unknown. Recently, we found that mice lacking fibroblast growth factor receptor 1 (Fgfr1) and Fgfr2 (K5-R1/R2 mice) in the epidermis have a severe impairment in the epidermal barrier, which leads to the development of a chronic inflammatory skin disease that shares many features with human atopic dermatitis. Using Fgfr1-/Fgfr2-deficient mice, we analyzed the consequences of the loss of mast cells. Mast cells accumulated and degranulated in the skin of young Fgfr1-/Fgfr2-deficient mice, most likely as a consequence of increased expression of the mast cell chemokine Ccl2. The increase in mast cells occurred before the development of histological abnormalities, indicating a functional role of these cells in the inflammatory skin phenotype. To test this hypothesis, we mated the Fgfr1-/Fgfr2-deficient mice with mast cell-deficient CreMaster mice. Surprisingly, loss of mast cells did not or only mildly affect keratinocyte proliferation, epidermal thickness, epidermal barrier function, accumulation and activation of different immune cells, or expression of different proinflammatory cytokines in the skin. These results reveal that mast cells are dispensable for the development of chronic inflammation in response to a defect in the epidermal barrier. (*Am J Pathol* 2015, 185: 1575–1587; <http://dx.doi.org/10.1016/j.ajpath.2015.02.005>)

A large percentage of the population has the chronic inflammatory skin disease atopic dermatitis (AD). This disease affects adults and children with worldwide prevalence rates of 1% to 20%, and the incidence has increased twofold to threefold during the last 40 years.¹ External challenges, such as exposure to allergens, irritants, and pathogens and/or genetic or epigenetic alterations in genes that are expressed in keratinocytes, can lead to skin damage, disruption of its barrier function, and subsequent development of AD. In particular, mutations that lead to a defect in the epidermal barrier strongly increase the risk of AD development. Thus, up to 15% of all patients and up to 50% of patients with severe disease have mutations in the gene encoding filaggrin, a structural protein of the epidermis.^{2–5} Furthermore, variants of the gene encoding SPINK5, a protease inhibitor involved in desquamation, are linked to AD,^{6–8} as well as mutations in genes encoding tight junction components (eg, claudin-1 and claudin-23).⁹

Alterations in tight junction components may also result from defects in the transcriptional regulation of their genes.

Thus, we recently found that loss of fibroblast growth factor receptor 1 (Fgfr1) and Fgfr2 in keratinocytes in mice caused a severe defect in the epidermal barrier. This phenotype was at least in part the consequence of reduced expression of major tight junction components, which are up-regulated in keratinocytes of normal mice by dermally derived Fgfs, in particular Fgf7 and Fgf10.¹⁰ The down-regulation of tight junction proteins in the *Fgfr* mutant mice (designated K5-R1/R2 mice) caused functional deficits of these junctions, and the mice developed a phenotype, which shares various features with the abnormalities observed in flaky tail mice (mutations in the filaggrin and matted genes)^{11–13} and in patients with chronic AD (Table 1). Consistent with our findings, down-regulation of FGFR1 and FGFR2 expression

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Table 1 Comparison of Pathological Features in Patients With Atopic Dermatitis in Flaky Tail and K5-R1/R2 Mice

Parameters	Atopic dermatitis patients	Flaky tail mice	K5-R1/R2 mice
TEWL	↑ ¹⁶	↑ ^{13,17}	↑ ¹⁰
Epidermal thickness	↑ ^{18–20}	↑ ^{11,17,21–23}	↑ ¹⁰
IgE and IgG	↑ ^{20,24,25}	↑ ^{13,17,22}	↑ ¹⁰
Epidermal γδ T cells	Not reported	Not reported	↑ ¹⁰
Epidermal αβ T cells	Not reported	Not reported	↑ This study
Langerhans cells	↑ ²⁶	↑ ²⁷	↑ This study
Neutrophils	Not present ^{24,28} ↑ Mild increase ^{29,30}	↑ ^{22,23}	↑ Mild increase and overall low abundance; this study
Eosinophils	↑ ^{18,24}	↑ ^{11,23}	No difference (data not shown)
Dermal T cells	↑ ^{31,32}	↑ ^{11,13,22,23}	↑ ¹⁰
Mast cells	↑ ^{33,34}	↑ ¹⁷	↑ ¹⁰

Flaky tail mice, which have mutations in the flaggrin and matted genes,¹² were included in the comparison because they represent an established mouse model for atopic dermatitis.¹¹ Parameters analyzed include transepidermal water loss, epidermal thickening, serum levels of IgE and IgG, and number of epidermal γδ T cells, αβ T cells, Langerhans cells, dermal neutrophils, eosinophils, T cells, and mast cells. Arrow indicates an increase in a particular parameter.

was detected in the skin of AD patients,¹⁴ and single-nucleotide polymorphisms in the *FGFR1* and *FGFR2* genes were linked to atopy.¹⁵ Therefore, K5-R1/R2 mice are useful to study the molecular and cellular mechanisms underlying chronic skin inflammation that results from a defective barrier.

Because most studies found increased numbers of mast cells in skin lesions of patients with AD, it is generally assumed that mast cells contribute to the development and/or maintenance of skin inflammation in these patients.^{33,34} Mast cells are particularly abundant at the host’s interface with the environment, such as the skin or mucosae. They are long-lived cells, which proliferate in response to appropriate stimuli.³⁵ They express a high-affinity FcεRI receptor that strongly binds IgE antibodies. Interestingly, the latter are abundant in the skin and the serum of K5-R1/R2 mice and of AD patients (Table 1). On binding of allergens to mast cell–bound IgE, degranulation of mast cells occurs, resulting in release of bioactive molecules stored in granules, including histamine, heparin, different proteases, and proinflammatory cytokines. This causes vessel dilatation, local edema, activation of other immune cells, and inflammation.³⁶

Previous studies^{37–41} using different mouse models have addressed the potential role of mast cells in AD. The allergen-induced skin inflammation that results from epicutaneous application of ovalbumin to mice and that elicits some characteristics of AD was not affected in mast cell–deficient *Kit*^{W/W[−]v} mice.³⁷ Consistent with this finding, the AD-like symptoms were only mildly reduced in FcεRI-deficient mice.³⁸ On the other hand, skin inflammation induced by epicutaneous sensitization with cedar pollen antigens was abolished in *Kit*^{W/W[−]v} and *Kit*^{Sl/Sl[−]d} mice.³⁹ Similarly, the AD-like symptoms induced by epicutaneous application of *Dermatophagoides farinae* extract and staphylococcal enterotoxin B were strongly reduced in *Kit*^{W-sh/W-sh40} and also in Cpa3-Cre transgenic, *Mcl-1*^{fl/fl} mice,⁴¹ which lack mast cells and basophils and have macrocytic anemia and neutrophilia.⁴² These findings suggest that mast cells are dispensable for cutaneous inflammation induced by

epicutaneous ovalbumin sensitization but not in *Dermatophagoides farinae* extract–, staphylococcal enterotoxin B–, or cedar pollen–induced dermatitis, which induce stronger AD-like symptoms. However, *Kit*^{W/W[−]v} or *Kit*^{W-sh/W-sh} mice should be considered to have various other hematopoietic and nonhematopoietic abnormalities,^{43,44} which may affect the result and complicate the interpretation.

Because the role of mast cells in a genetic model of chronic dermatitis with impaired barrier function has not been studied yet, we analyzed the consequences of the loss of mast cells in K5-R1/R2 mice. We mated K5-R1/R2 mice with CreMaster mice, which express Cre recombinase under the control of the endogenous *Cpa3* locus. This leads to efficient depletion of mast cells during their development due to the genotoxicity of Cre. Therefore, CreMaster mice are constitutively devoid of mast cells, and they do not reappear during postnatal development.⁴⁵ Importantly, these mice do not have additional immunological abnormalities or other pathological features, except a reduction in the number of basophils. Therefore, analysis of the progeny of this breeding allowed us to determine the function of mast cells in a genetic mouse model that exhibits important features of AD. We report that mast cells are dispensable in this genetic mouse model of chronic dermatitis despite their early increase and strong degranulation in the skin.

Materials and Methods

Mice

Mice with floxed *Fgfr1* and floxed *Fgfr2* alleles were mated with transgenic mice expressing Cre recombinase under the control of the keratin 5 (K5) promoter. The progeny were designated K5-R1/R2 mice and described previously.¹⁰ K5-R1/R2 mice were further bred to mast cell–deficient CreMaster mice⁴⁵ to obtain quadruple mutant mice (designated K5-R1/R2–CreMaster). Because of the difficult breeding scheme, each experiment included mice from different litters, but at least one to two controls; CreMaster,

Table 2 Real-Time PCR Primers

Target gene	Forward sequence	Reverse sequence
<i>Scf</i>	5'-CATTTATCTTCAACTGCTCCTATTT-3'	5'-GGTCATCCACTATTTTCCCAAG-3'
<i>Ccl6</i>	5'-TTCTGGGCGCTGCTGTTTAC-3'	5'-GAGCCAACACGTGGATGCT-3'
<i>Ccl2</i>	5'-ATCAAGCCGGGCATCATCTTTA-3'	5'-TGCCCTCCTTCTCAAGCAAT-3'
<i>Claudin3</i>	5'-GCGGCTCTGCTCACCTTAGT-3'	5'-GACGTAGTCCTTGCGGTCGTA-3'
<i>Il1f8</i>	5'-GCCTGTCATCTTAGCTTGAT-3'	5'-TGCTACTTCTTAAGCTGC-3'
<i>Tnfa</i>	5'-TGGCCCAGGCAGTCAGA-3'	5'-GTTTGCTACAACATGGGCTACA-3'
<i>Il1b</i>	5'-CTGAAAGCTCTCCACCTC-3'	5'-TGCTGATGTACCAGTTGGGG-3'
<i>Il6</i>	5'-CCGGAGAGGAGACTTCACAG-3'	5'-TTCTGCAAGTGCATCATCGT-3'
<i>Tgfb1</i>	5'-AGCCCGAAGCGGACTACTAT-3'	5'-TCCACATGTTGCTCCACACT-3'
<i>Ifng</i>	5'-TTTAACTCAAGTGGCATAGATGTG-3'	5'-ATGAATGCATCCTTTTTCGC-3'
<i>Il5</i>	5'-ACATTGACCGCCAAAAGAG-3'	5'-ATCCAGGAAGTGCCTCGTC-3'
<i>Il10</i>	5'-TGCTATGCTGCCTGCTCTTA-3'	5'-ATGTTGTCCAGCTGGTCCTT-3'
<i>Il12</i>	5'-CCATCAGCAGATCATTCTAGACAA-3'	5'-CGCCATTATGATTACAGACTG-3'
<i>Il13</i>	5'-CCTCTGACCCTTAAGGAGCTTAT-3'	5'-CGTTGCACAGGGGAGTCT-3'
<i>Il17a</i>	5'-ACCCTGGACTCTCCACCAGCA-3'	5'-GTGCAGCCACACCCACCAG-3'
<i>Il17f</i>	5'-AACAGCCATGGTCAAGTCTTTGCT-3'	5'-ACCGGTGGGGGTCTCGAGTG-3'
<i>Il22</i>	5'-ACTGTTGACACTTGTGCGATCTCTG-3'	5'-CCTTAGCACTGACTCCTCGGAACA-3'
<i>Rps29</i>	5'-GGTACCAGCAGCTCTACTG-3'	5'-GTCCAACCTAATGAAGCCTATGTCC-3'

K5-R1/R2, and K5-R1/R2—CreMaster littermates were used for a direct comparison in all experiments. All mice had a C57BL/6 genetic background. They were housed under optimal hygiene conditions and received food and water *ad libitum*. Mouse maintenance and all animal experiments had been approved by the veterinary authorities of Zurich, Switzerland.

Histologic Analysis

Skin biopsy specimens were fixed overnight with 95% ethanol/1% acetic acid followed by paraffin embedding. Sections (7 μ m) were stained with hematoxylin and eosin or with toluidine blue to detect mast cells and their degranulation as described previously.¹⁰ Sections were analyzed with an Axioskop 2 microscope equipped with a Plan-Neofluar objective (20 $\times$ /0.5NA) and photographed with an Axiocam HRc camera (all from Carl Zeiss Inc., Jena, Germany). Axiovision software version 4.6 (Carl Zeiss, Inc.) was used for data acquisition. Data analysis was performed using ImageJ version 1.48 (NIH, Bethesda, MD). All analyses were performed blinded to the genotype of the mice (J.S.).

BrdU Incorporation Studies

Mice were injected intraperitoneally with 5-bromo-2'-deoxyuridine (BrdU) (250 mg/kg in 0.9% NaCl; Sigma, Munich, Germany) and sacrificed 2 hours after injection. Skin biopsy specimens were fixed overnight with 95% ethanol/1% acetic acid followed by paraffin embedding. Sections were deparaffinized, washed in 100% ethanol for 5 minutes at room temperature, and fixed with 100% methanol for 10 minutes at 4°C. They were denatured in 2N HCl for 30 minutes at 37°C, followed by neutralization with 0.1 mol/L borate buffer (pH 8.5) for 30 minutes at room temperature. After washing, sections were blocked with

phosphate-buffered saline containing 3% bovine serum albumin and 0.025% NP-40 for 30 minutes at 37°C. Primary BrdU fluorescein isothiocyanate antibody (Roche, Rotkreuz, Switzerland) and 2 μ g/mL propidium iodide as a counterstain were applied for 4 hours at room temperature in the same buffer. After washing, slides were mounted with

Table 3 Antibodies Used for Flow Cytometry Analysis

Antigen	Clone	Fluorophor	Dilution	Source
CD32	2.4G2		1:200	BD Biosciences
CD45	30-F11	APC	1:800	BD Biosciences
CD3	145-2C11	PE-CF594/PE	1:300/400	BD Biosciences
CD4	GK1.5	PE	1:800	BD Biosciences
CD8	53-6.7	PE	1:800	BD Biosciences
CD5	53-7.3	PE	1:400	BD Biosciences
TER119	TER-119	PE	1:400	BD Biosciences
NK1.1	PK136	PE	1:400	BD Biosciences
CD19	eBio1D3	PE	1:400	BD Biosciences
Gr-1	RB6-8C5	PE	1:800	BD Biosciences
TCR β	H57-597	PE-Cy7	1:500	BD Biosciences
TCR δ	GL3	FITC	1:400	BD Biosciences
CD11c	HL3	PE-Cy7/PE	1:400/600	BD Biosciences
Ly6G	1A8	FITC	1:400	BD Biosciences
Integrin β 7	M293	FITC	1:300	BD Biosciences
I-A/I-E	2G9	PE	1:4000	BD Biosciences
CD11b	M1/70	BV711/PE	1:500/2000	Biolegend
F4/80	BM8	BV421	1:200	Biolegend
CD117	2B8	APC-Cy7	1:400	Biolegend
Sca-1	E13-161-7	PE-Cy7	1:500	Biolegend
CD69	H1.2F3	BV421	1:200	Biolegend
Fc ϵ RI α	MAR-1	PE	1:200	eBioscience
CD27	LG.7F9	APC	1:400	eBioscience
CD86	GL1	FITC	1:400	eBioscience
T1/ST2	DJ8	Biotin	1:600	MD Bioscience

APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin.

Figure 1 Early accumulation and activation of dermal mast cells in K5-R1/R2 mice. **A:** Number of mast cells as determined by toluidine blue staining of paraffin sections from back skin of K5-R1/R2 mice and control (ctrl) littermates on postnatal day 7 (P7), P9, P12, P18, and P29. At least 5 mm of the skin was analyzed per mouse. **B:** RNA from total back skin of mice on P9 was analyzed by quantitative real-time RT-PCR for the expression of *Mcp4*, *Mcp6*, and *Cpa3*. *Rps29* mRNA was used for normalization. **C:** Representative image of toluidine blue–stained paraffin sections from back skin at P9 shows enhanced mast cell degranulation in K5-R1/R2 mice. **D:** The percentage of degranulated mast cells among all mast cells was determined by light microscopy analysis of toluidine blue–stained paraffin sections from back skin. At least 5 mm of the skin was analyzed per mouse. **E:** Epidermal thickness determined in hematoxylin and eosin–stained skin sections and keratinocyte proliferation in the epidermis as determined by 5-bromo-2'-deoxyuridine staining of paraffin sections from back skin at P29 and the age of 2 months (2M). At least 5 mm of the skin was analyzed per mouse. Data are representative of two experiments and are expressed as means $\pm$ SD. $n = 17$ ctrl and 8 K5-R1/R2 mice for P7; $n = 12$ ctrl and 13 K5-R1/R2 mice for P9; $n = 18$ ctrl and 10 K5-R1/R2 mice for P12; $n = 14$ ctrl and 18 K5-R1/R2 mice for P18; $n = 16$ ctrl and 13 K5-R1/R2 mice for P29 (**A**). $n = 6$ ctrl, 6 K5-R1/R2 mice for P7; $n = 17$ ctrl and 8 K5-R1/R2 mice for P9; $n = 7$ ctrl and 11 K5-R1/R2 mice for P9; $n = 9$ ctrl and 10 K5-R1/R2 mice for P18; $n = 15$ ctrl and 9 K5-R1/R2 mice for P29; $n = 9$ ctrl and 9 K5-R1/R2 mice for 3 months; $n = 9$ ctrl and 6 K5-R1/R2 mice for 12 months (**D**). $n = 6$ ctrl and 13 K5-R1/R2 mice for P29; $n = 14$ ctrl and 9 K5-R1/R2 mice for 2 months (**E**). $**P \leq 0.01$, $***P \leq 0.001$. Scale bar = 50 μ m (the area indicated by the rectangle is shown at higher magnification) (**C**).

Mowiol (Sigma). Sections were photographed using an Imager.A1 microscope equipped with an enhanced-contrast Plan-Neofluar objective (20×/0.5NA) and photographed with an AxioCam MRm camera (all from Carl Zeiss Inc.). Axiovision software version 4.6 (Carl Zeiss Inc) was used for data acquisition. Data analysis was performed using ImageJ version 1.48.

TEWL

Mouse back skin was shaved 1 day before analysis. Transepidermal water loss (TEWL) was determined using a Tewameter (Courage and Khazaka Electronic GmbH, Cologne, Germany). The probe measures the density gradient of the water evaporation from the skin indirectly by two pairs of sensors: temperature and relative humidity. A microprocessor analyzes the values and expresses the evaporation rate in grams per hour per square meter. The Tewa probe was placed on the dorsal skin, and 20 consecutive measurements were taken from four different places on the back, in total 80 measurements per mouse.

Isolation of RNA from Mouse Skin

Mice were sacrificed and shaved, and epidermis from the back skin was separated from the dermis after heat shock treatment (30 seconds at 60°C followed by 1 minute at 4°C). Isolated epidermis and dermis were immediately frozen in liquid nitrogen. RNA isolation using TRIzol was performed as described previously.⁴⁶

Real-Time RT-PCR

Quantitative real-time RT-PCR (RT-qPCR) was performed according to the manual of the Light Cycler 480 II (Roche). The RT product obtained from 12.5 ng of RNA was used together with 6 µL of SYBR Green reaction mix, including 0.45 µmol/L forward and reverse primer mix. The reaction was performed in 50 cycles (95°C for 10 minutes for initial denaturation; 95°C for 10 seconds, 60°C for 20 seconds, and 72°C for 20 seconds for each cycle). Samples were analyzed in duplicates. The primers are listed in Table 2.

Isolation of Cells from Bone Marrow, Subcutaneous Adipose Tissue, and Skin

Mice were sacrificed and shaved, and the back skin and the bone marrow were harvested. Bone marrow cells were flushed from the femurs and tibias with RPMI 1640 medium supplemented with penicillin/streptomycin and 20 mmol/L HEPES. Erythrocytes were lysed with BD Pharm Lyse (BD Biosciences, San Jose, CA), and the remaining nucleated bone marrow cells were washed with 1× phosphate-buffered saline.

The subcutaneous fat was scraped off and treated with 0.25 mg/mL TL Liberase (Roche) in RPMI 1640 medium supplemented with penicillin/streptomycin and 20 mmol/L

Figure 2 Transient increase in mast cell chemokines and mitogens in the skin of K5-R1/R2 mice. RNA from the epidermis (A) or dermis (B) of postnatal day 7 (P7), P9, P17, and 3-month-old (3M) mice was analyzed by quantitative real-time RT-PCR for the expression of Ccl2, Ccl6, and stem cell factor. Rps29 mRNA was used for normalization. Data are representative of two experiments and are expressed as means $\pm$ SD. $n = 4$ control, 7 K5-R1/R2 mice for P7, $n = 6$ control, 6 K5-R1/R2 mice for P9, $n = 5$ control, 6 K5-R1/R2 mice for P17, and $n = 6$ control, 6 K5-R1/R2 mice for 3 months. * $P \leq 0.05$, ** $P \leq 0.01$.

HEPES for 1.5 hours at 37°C under continuous shaking. Epidermis and dermis were separated using 0.25% dispase (Gibco, Zug, Switzerland) for 50 minutes at 37°C under continuous shaking. The epidermis was treated with 100 Kunitz units/mL DNase (Sigma) in RPMI 1640 medium supplemented with penicillin/streptomycin, 10% fetal bovine serum, and 20 mmol/L HEPES for 45 minutes at 37°C under continuous shaking. The dermis was cut into small pieces and treated with 0.25 mg/mL TL Liberase in RPMI 1640 medium supplemented with penicillin/streptomycin and 20 mmol/L HEPES for 1 hour at 37°C under continuous shaking. Single-cell suspensions were prepared by passing mixtures through 70-µm cell strainers, and cells were washed with 1× phosphate-buffered saline.

Flow Cytometry

Unspecific binding sites were blocked with anti-CD16/CD32 antibody. Dead cells were stained with Zombie Aqua dye (BioLegend, San Diego, CA). Antibodies used for flow cytometry analysis are listed in Table 3. Stained cells were analyzed using BD Fortessa and BD FACSDiva software version 6.0 (both from BD Biosciences). Data analysis was performed using FlowJo software version 10 (FlowJo, Ashland, OR). Compensation was performed using single-color controls prepared from the mixture of

skin and spleen (skin analysis) or bone marrow (bone marrow analysis). Compensation matrices were calculated using BD FACSDiva software version 6.0 (BD Biosciences) and applied. Doublets and dead cells were excluded from the analysis. Fluorescence minus one controls were used for gating analyses to distinguish positively from negatively staining cell populations. This control contains all fluorochromes in a panel, with the exception of the one that is being measured.

Statistical Analysis

Statistical analysis was performed using PRISM software version 5 (Graph Pad Software Inc., La Jolla, CA). Mann-Whitney *U* test was used for comparison between

two different groups. Error bars represent SD in all graphs.

Results

Early Accumulation and Activation of Dermal Mast Cells in K5-R1/R2 Mice

To characterize the role of mast cells in the phenotype of K5-R1/R2 mice, we first determined the kinetics of postnatal mast cell accumulation in the back skin. Toluidine staining of skin sections revealed a similar number of mast cells in K5-R1/R2 and control mice at postnatal day 7 (P7). An increase in mast cell number occurred between P7 and P9 in control mice and even more in K5-R1/R2 mice.

Figure 3 Mast cell deficiency does not affect the macroscopic phenotype of K5-R1/R2 mice. **A:** Representative images of 12-month-old mice from each genotype. **B and C:** Mast cells were identified by toluidine blue staining of paraffin sections from back skin (arrows) of 3-month-old mice and quantified. At least 5 mm of the skin was analyzed per mouse. **D:** Flow cytometry analysis of dermal cells from 3-month-old mice using c-Kit and FcεR1α antibodies to detect mast cells. The percentage of mast cells among all cells is shown. Data are representative of two experiments and are expressed as means ± SD (**C** and **D**). *n* = 6 control (ctrl.), 5 CreMaster, 5 K5-R1/R2, and 6 K5-R1/R2–CreMaster mice (**D**). *n* = 9 ctrl, 11 CreMaster, 9 K5-R1/R2, and 8 K5-R1/R2–CreMaster mice (note the lack of mast cells in all mice with CreMaster genotype) (**B** and **C**). ***P* ≤ 0.01, ****P* ≤ 0.001. Scale bar = 50 μm (**B**). D, dermis; E, epidermis; HF, hair follicle.

Although the number of mast cells remained high in K5-R1/R2 mice, it decreased over time in control mice (Figure 1A). Concomitant with the increase in mast cell number in K5-R1/R2 mice, we observed an increase in the mRNA levels of *Mcp4*, *Mcp6*, and *Cpa3* (Figure 1B). In young K5-R1/R2 mice, most of the mast cells were degranulated (Figure 1, C and D), followed by a decrease in degranulated mast cells thereafter (Figure 1D). The appearance of mast cells in the skin and their degranulation preceded the onset of histopathological abnormalities in K5-R1/R2 mice, in particular the epidermal thickening and keratinocyte hyperproliferation, which progressed over time (Figure 1E). These findings raised the possibility of a causative role of mast cells in the development of the skin phenotype in K5-R1/R2 mice.

Transient Increase in Mast Cell Chemokines in the Skin of K5-R1/R2 Mice

To determine the reason for the accumulation of mast cells in the skin of K5-R1/R2 mice, we analyzed the expression of mast cell chemokines. Expression of *Ccl2*, a major mast cell progenitor attractant,⁴⁷ and of *Ccl6*, an upstream regulator of *Ccl2*,⁴⁸ was significantly higher in the epidermis and even more in the dermis of K5-R1/R2 mice compared with littermate controls (Figure 2, A and B). The increase in the epidermis is not a cell autonomous effect because these chemokines were expressed at similar levels in primary keratinocytes from control and K5-R1/R2 mice (data not shown). Highest expression levels of *Ccl2* and *Ccl6* correlated with the peak in mast cell number, and their expression

Figure 4 Loss of mast cells does not affect the loss of skin appendages, epidermal thickness, and keratinocyte proliferation in K5-R1/R2 mice. **A:** Representative photographs of hematoxylin and eosin (H&E)—stained sections from back skin of 3-month-old mice. **B:** Representative images of back skin sections from 3-month-old mice stained with a 5-bromo-2'-deoxyuridine (BrdU) antibody (green) and counterstained with propidium iodide (red). **C:** Epidermal thickness was determined on H&E-stained paraffin sections from back skin of 3-month-old mice. **D:** Quantification of BrdU-positive cells in the epidermis of 3-month-old mice. Data are expressed as means $\pm$ SD (C and D). $n = 8$ control (ctrl), 11 CreMaster, 10 K5-R1/R2, and 11 K5-R1/R2—CreMaster mice (C). $n = 10$ ctrl, 10 CreMaster, 10 K5-R1/R2, and 11 K5-R1/R2—CreMaster mice for 3-month-old mice (D). * $P \leq 0.05$, *** $P \leq 0.001$. Scale bar = 50 μ m (A and B). D, dermis; E, epidermis; HF, hair follicle.

subsequently decreased. Expression levels of other mast cell chemokines, including Ccl5, Cxcl9, Cxcl10, and nerve growth factor were similar in mice of both genotypes (data not shown). Finally, mRNA levels of stem cell factor, a major mast cell growth factor and chemokine, which is increased in the serum of patients with AD,⁴⁹ were even reduced in the epidermis of K5-R1/R2 mice and not affected in the dermis. These findings suggest that a transient increase in Ccl6 and Ccl2 could be responsible for the early increase in mast cells in the skin of K5-R1/R2 mice.

The increase in mast cell number may result from proliferation of skin-resident mast cells or from recruitment and local proliferation of bone marrow–derived mast cell progenitors (MCPs) as previously reported for inflamed skin.⁵⁰ In addition, recruitment from the white adipose tissue may occur because white adipose tissue can serve as a source of MCPs.⁵¹ Consistent with this possibility, we found a significantly higher number of MCPs in the white adipose tissue of K5-R1/R2 mice compared with control mice (Supplemental Figure S1).

Mast Cell Deficiency Does Not Affect the Macroscopic Phenotype of K5-R1/R2 Mice

To investigate the role of mast cells in the AD-like phenotype of K5-R1/R2 mice, they were mated with mast cell–deficient CreMaster mice.⁴⁵ The progeny was designated K5-R1/R2–CreMaster.

K5-R1/R2–CreMaster mice were macroscopically similar to K5-R1/R2 mice at P17, the age of 3 months (data not shown), and the age of 12 months (Figure 3A). In agreement with the phenotype of K5-R1/R2 mice, both strains were viable, all females and most males were sterile, mice of both sex lost hair, and they had a lower body weight compared with control and CreMaster mice (Figure 3A) (data not shown). Mast cell deficiency in K5-R1/R2–CreMaster and CreMaster mice was verified by toluidine blue staining of skin sections (Figure 3, B and C) and by flow cytometry (Figure 3D) at the age of 3 months. The very weak signal that we obtained with samples from CreMaster and K5-R1/R2–CreMaster mice reflects flow cytometry staining background. Mast cells also did not reappear on aging as determined by toluidine blue staining of skin sections of 12-month-old mice (Supplemental Figure S2). Therefore, the macroscopic abnormalities observed in K5-R1/R2 mice are independent of the presence of mast cells.

Loss of Mast Cells Does Not Affect Acanthosis and Keratinocyte Hyperproliferation in K5-R1/R2 Mice

Because the skin of K5-R1/R2 mice and lesional skin of AD patients are characterized by a strong increase in epidermal thickness (acanthosis),^{18–20} we investigated the role of mast cells in the acanthosis of K5-R1/R2 mice. Epidermal thickness was significantly increased in

K5-R1/R2 mice compared with controls, and there was a small decrease in the absence of mast cells at the age of 3 months (Figure 4, A and C). This was, however, no longer observed at the age of 12 months (Supplemental Figure S3, A and C). In agreement with the similar severity of the acanthosis, we did not observe obvious differences in the proliferation rate of epidermal keratinocytes between K5-R1/R2 and K5-R1/R2–CreMaster mice at the age of 3 months (Figure 4, B and D) and 12 months (Supplemental Figure S3, B and D). Therefore, the acanthosis and keratinocyte hyperproliferation that occur in the absence of Fgfr1 and Fgfr2 in keratinocytes are independent of the presence of mast cells.

Loss of Mast Cells Does Not Influence the Epidermal Barrier Defect in K5-R1/R2 Mice

One of the early and major symptoms of K5-R1/R2 mice and also of AD patients is increased TEWL,¹⁶ which reflects the impaired inside-out barrier of the epidermis and results in severe skin dryness. Similar to the other epidermal

Figure 5 Loss of mast cells does not influence the epidermal barrier defect in K5-R1/R2 mice. **A:** Transepidermal water loss (TEWL) was measured on the back skin of 3-month-old control (ctrl) and K5-R1/R2 mice. **B:** RNA from the epidermis of 3-month-old mice was analyzed by quantitative real-time RT-PCR for the expression of claudin-3. Rps29 mRNA was used for normalization. Data are representative of two experiments and expressed as means $\pm$ SD. $n = 9$ control, 10 CreMaster, 6 K5-R1/R2, and 10 K5-R1/R2–CreMaster mice (**A**). $n = 6$ control, 6 CreMaster, 3 K5-R1/R2, and 3 K5-R1/R2–CreMaster mice (**B**). * $P \leq 0.05$, *** $P \leq 0.001$.

A

Figure 6 Loss of mast cells does not affect expression of proinflammatory cytokines or number of immune cells in the epidermis. **A:** Flow cytometry analysis of dissociated epidermal cells from 3-month-old (3M) mice for quantification of total CD45⁺ immune cells, γδ T cells, αβ T cells, and Langerhans cells. **B:** RNA from the epidermis of postnatal day 17 (P17), 3-month-old, and 12-month-old (12M) mice was analyzed by quantitative real-time (RT-PCR) for the expression of *Il-1f8* and tumor necrosis factor-α. *Rps29* mRNA was used for normalization. Data are representative of two experiments and indicate means ± SD. *n* = 6 control, 5 CreMaster, 5 K5-R1/R2, and 6 K5-R1/R2-CreMaster mice for P17. *n* = 4 control, 3 CreMaster, 6 K5-R1/R2, and 5 K5-R1/R2-CreMaster mice for P17. *n* = 6 control, 6 CreMaster, 6 K5-R1/R2, and 6 K5-R1/R2-CreMaster mice for 3 months. *n* = 4 control, 6 CreMaster, 4 K5-R1/R2, and 5 K5-R1/R2-CreMaster mice for 12 months (**B**). **P* ≤ 0.05, ***P* ≤ 0.01.

B

abnormalities, the increase in TEWL that we observed in K5-R1/R2 compared with control mice was not affected by the lack of mast cells at the age of 3 months (Figure 5A) and 12 months (Supplemental Figure S4A).

We had previously found that the barrier function defect in K5-R1/R2 mice is at least in part the result of reduced expression of major tight junction components, in particular of claudin-3.¹⁰ Consistent with the TEWL data, loss of mast cells did not influence the expression of *Cldn3* in the epidermis at the age of 3 months (Figure 5B) and 12 months (Supplemental Figure S4B). This finding further suggests that the impaired barrier function in K5-R1/R2

mice is a direct consequence of the deficiency in *Fgfr* signaling in keratinocytes rather than a secondary effect that results from the increase in mast cells.

Loss of Mast Cells Does Not Affect the Extent of Cutaneous Inflammation in K5-R1/R2 Mice

The defect in the epidermal barrier of AD patients is generally associated with cutaneous inflammation, characterized by an increase in various dermal and epidermal immune cells (Table 1). Because similar alterations had previously been observed in K5-R1/R2 mice,¹⁰ we assessed the contribution

Figure 7 Loss of mast cells does not affect expression of proinflammatory cytokines or number of immune cells in the dermis. **A:** Flow cytometry analysis of dissociated dermal cells from 3-month-old mice for quantification of total CD45⁺ immune cells, αβ T cells, γδ T cells, dendritic cells, macrophages, and neutrophils. **B:** RNA from the dermis of 3-month-old mice was analyzed by quantitative real-time RT-PCR for the expression of different cytokines. Rps29 mRNA was used for normalization. Data are representative of two experiments and expressed as means ± SD. *n* = 6 control, 5 CreMaster, 5 K5-R1/R2, and 6 K5-R1/R2-CreMaster mice (**A**). *n* = 6 control, 6 CreMaster, 6 K5-R1/R2, and 6 K5-R1/R2-CreMaster mice (**B**). ***P* ≤ 0.01.

of mast cells to this inflammatory phenotype using flow cytometry analysis of different immune cells and RT-qPCR for various cytokines. A comparison of K5-R1/R2 and K5-R1/R2–CreMaster mice revealed no difference in the immune cell composition in the epidermis (Langerhans cells, $\alpha\beta$ T cells, and $\gamma\delta$ T cells) (Figure 6A) or in immune cell activation as determined by the analysis of CD69, CD86, and major histocompatibility complex II by flow cytometry (data not shown). Furthermore, epidermal expression levels of Il-1f8, a cytokine that is strongly up-regulated in the epidermis of K5-R1/R2 mice¹⁰ (Figure 6B) and of tumor necrosis factor- α was not affected by the loss of mast cells in mice of different age (Figure 6B). In the dermis, we detected a slight but nonsignificant decrease in the number of total immune cells, $\gamma\delta$ T cells, dendritic cells, macrophages, and neutrophils in K5-R1/R2–CreMaster mice compared with K5-R1/R2 mice (Figure 7A). Some of these immune cells were also slightly but nonsignificantly reduced in CreMaster mice compared with wild-type controls. The cytokine expression profile, which included the major dermal T cell cytokines, was comparable between K5-R1/R2 and K5-R1/R2–CreMaster mice at the age of 3 months (Figure 7B) and 12 months (Supplemental Figure S5). Therefore, mast cells do not or only slightly influence the inflammation in K5-R1/R2 mice.

Discussion

Because of its exposure to the environment, the skin's integrity is essential for the prevention of acute and chronic inflammation as a result of invasion of irritants, allergens, or pathogens. Furthermore, the epidermal barrier protects from severe water loss.

Interestingly, most patients with AD have a genetic defect that affects key components of the epidermal barrier: the cornified envelope of the epidermis or the underlying tight junctions.^{2–9} These mutations cause a disruption of the epidermal barrier, resulting in dry skin and diminished epidermal defense mechanisms to allergens and pathogens, followed by chronic inflammation. However, the contribution of different types of immune cells to the phenotypic abnormalities is not fully understood. We found that an increase in the number of mast cells and their rapid degranulation are some of the earliest events in the development of chronic skin inflammation in mice lacking Fgfr1 and Fgfr2 in keratinocytes.

As previously described, an increase in mast cells in the skin at the site of inflammation can result from a combination of local expansion of resident mast cells and attraction of bone marrow–derived MCPs, which subsequently proliferate in the skin.⁵⁰ Interestingly, we also found an increase in the MCPs in the subcutaneous white adipose tissue of K5-R1/R2 mice at P9. Because white adipose tissue serves as a source of hematopoietic stem/progenitor cells,^{52,53} including MCPs,⁵¹ this increase may further contribute to the increase in skin mast cells.

Independent of the origin of the MCPs in K5-R1/R2 mice, the strong up-regulation of Ccl2, a major mast cell progenitor attractant,⁴⁷ in the dermis and epidermis of K5-R1/R2 mice provides an explanation for the increased number of cutaneous mast cells. Furthermore, it may contribute to increased degranulation because *in vitro* treatment of mast cells with Ccl2 induced their degranulation.⁵⁴

The reason for the up-regulation of Ccl6 and its target Ccl2 is as yet unknown. It is obviously not a direct consequence of the Fgfr deficiency because its expression was not up-regulated in cultured keratinocytes of K5-R1/R2 mice and the increase also occurred in the dermis. Therefore, indirect effects, such as skin dryness that results in mechanical stress or alterations in the skin microbiome on development of the barrier defect, may contribute to their increased expression. The overexpression of Ccl2 and Ccl6 was only transient, possibly because of the onset of yet unknown compensatory mechanisms. This decrease may also explain why the number of mast cells did not further increase in the skin of K5-R1/R2 mice but rather decreased during aging, although the barrier function deficit persisted or was even aggravated.¹⁰ Because mast cells are able to survive for months or years,³⁵ the mast cells present in aged mice may have been attracted to the skin during early postnatal development in response to the transient up-regulation of mast cell chemokines. The subsequent decrease in Ccl2 levels may also explain why fewer mast cells were degranulated in aged K5-R1/R2 mice.

Because patients with AD have increased numbers of mast cells in the skin,^{33,34} several studies used mouse models to address the role of mast cells in AD. Interestingly, there are apparent differences in the mast cell requirement among different AD models; hence, these models might mimic different aspects of the AD phenotype. However, the role of mast cells in the chronic dermatitis that develops in response to a genetic defect in the epidermal barrier had not yet been addressed. Although a mouse model can never fully mimic the human situation, K5-R1/R2 mice exhibit several important features of AD (Table 1) and are therefore suitable to address this question. Importantly, the increase in mast cells and their activation preceded the increase in other immune cells and the development of chronic cutaneous inflammation in these animals. Surprisingly, however, loss of mast cells in a K5-R1/R2 background due to Cre-mediated constitutive eradication of these cells did not affect any of the studied pathological features. However, we cannot exclude the possibility that they contribute to the response of the skin to an additional challenge, such as treatment with irritants and allergens or exposure to various pathogens. Further investigations along these lines will, however, remain challenging because the infertility of the mutant mice requires the use of heterozygous animals for breeding and only very limited numbers of quadruple mutant mice can be obtained. Furthermore, their rather poor health status did not allow the exposure to additional challenges. Other mouse models with impaired barrier may be useful to address this important question. Nevertheless, our

genetic experiments strongly suggest that mast cells are dispensable for the development of the chronic skin inflammation in K5-R1/R2 mice and also are not major regulators of other immune cells in the inflamed skin.

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Supplemental Data

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