CellPress

Immunity
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The Enzyme Cyp26b1 Mediates Inhibition of Mast Cell Activation by Fibroblasts to Maintain Skin-Barrier Homeostasis

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http://dx.doi.org/10.1016/j.immuni.2014.01.014

SUMMARY

Mast cells (MCs) mature locally, thus possessing tissue-dependent phenotypes for their critical roles in both protective immunity against pathogens and the development of allergy or inflammation. We previously reported that MCs highly express P2X7, a receptor for extracellular ATP, in the colon but not in the skin. The ATP-P2X7 pathway induces MC activation and consequently exacerbates the inflammation. Here, we identified the mechanisms by which P2X7 expression on MCs is reduced by fibroblasts in the skin, but not in the other tissues. The retinoic-acid-degrading enzyme Cyp26b1 is highly expressed in skin fibroblasts, and its inhibition resulted in the upregulation of P2X7 on MCs. We also noted the increased expression of P2X7 on skin MCs and consequent P2X7- and MC-dependent dermatitis (so-called retinoid dermatitis) in the presence of excessive amounts of retinoic acid. These results demonstrate a unique skin-barrier homeostatic network operating through Cyp26b1-mediated inhibition of ATP-dependent MC activation by fibroblasts.

INTRODUCTION

Mast cells (MCs) produce inflammatory mediators to initiate and exacerbate inflammation (Gilfillan and Beaven, 2011; Tsai et al., 2011). Therefore, depletion or inhibition of activated MCs attenuates the inflammatory reactions (Feyerabend et al., 2011; Otsuka et al., 2011). MCs are activated by various stimuli such as allergen-immunoglobulin E (IgE) complex and high-affinity IgE receptor (FcεRI) pathway, and molecules released from necrotic cells (e.g., IL-33) after tissue injury in various inflammatory conditions (Lunderius-Andersson et al., 2012). Furthermore, previous findings, including ours, suggest that extracellular ATP acts as a danger signal to MCs and initiates inflammation (Kurashima et al., 2012; Sudo et al., 1996). Extracellular ATP is released in response to various stresses including shear, osmolality, oxidative, and inflammatory one (Junger, 2011). Local ATP injection into the skin induces ear swelling (Mizumoto et al., 2002). Furthermore, ATP amounts are increased in the extracellular compartment in irritant contact dermatitis associated with zinc-deficiency (Kawamura et al., 2012; Mizumoto et al., 2002). In addition to skin inflammation, increased ATP concentrations are also found in asthma, graft-versus-host disease, and inflammatory bowel disease (Idzko et al., 2007; Wilhelm et al., 2010; Kurashima et al., 2012). To resolve inflammation, the extracellular ATP is degraded by the ectonucleoside triphosphate diphosphohydrolase CD39 expressed on immune cells such as Langerhans cells (LCs) and regulatory T cells (Junger, 2011). Therefore, inflammation is exacerbated in CD39-deficient mice because of increased local ATP concentration (Mizumoto et al., 2002).

As receptors for extracellular ATP, P2 purinoceptors comprise P2X1 to P2X7 and act as ATP-gated ion channels (Di Virgilio, 2007). P2X7 is involved in various inflammations and thus inflammation associated with graft-versus-host disease and colonic inflammation are ameliorated by P2X7 inhibition (Kurashima et al., 2012; Wilhelm et al., 2010). In addition,

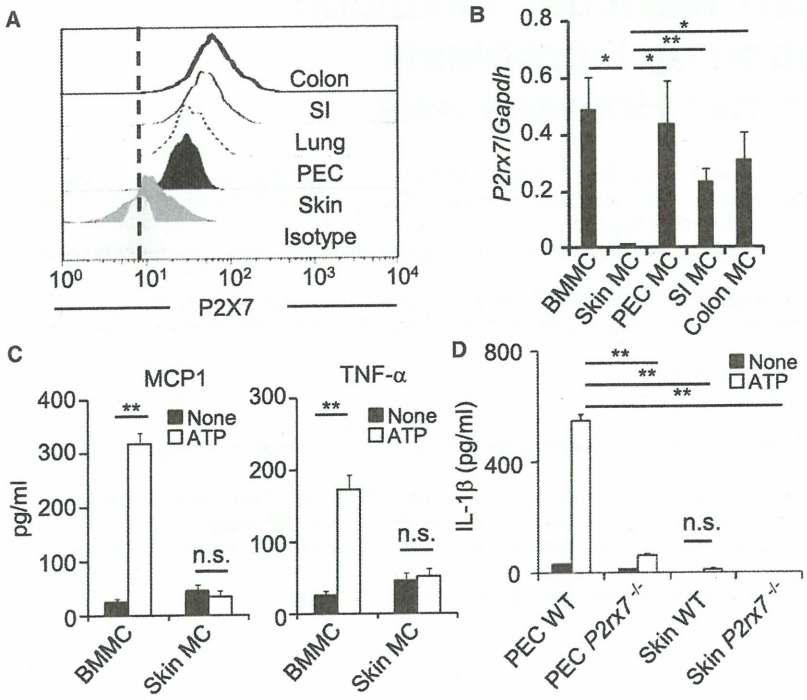

Figure 1. Low P2X7 Expression on Skin Mast Cells

(A) P2X7 expression on MCs in the colon, small intestine (SI), lung, peritoneal cavity (PEC), and skin was measured by flow cytometry. "Isotype" indicates isotype IgG2b staining as a negative control. Data are representative of at least six independent experiments.

(B) Gene expression of *P2rx7* in sorted MCs from various tissues was examined by quantitative RT-PCR. Data are means ± SEM (n = 4). *p < 0.05; **p < 0.01.

(C) Production of MCP1 and TNF-α in culture supernatant was determined after stimulation of bone-marrow-derived MCs (BMMCs) or skin MCs with 0.5 mM ATP. Data are shown as means ± SEM (n = 3). **p < 0.01; n.s., not significant.

(D) IL-1β production was measured by ELISA after sorted MCs from WT or *P2rx7*$^{-/-}$ mice were stimulated with LPS with or without ATP (n = 3). **p < 0.01; n.s., not significant.

cathelicidin-derived peptide LL37 directly stimulates P2X7 and induces skin inflammation such as psoriasis and rosacea (Elssner et al., 2004; Yamasaki et al., 2007). Despite such researches, little is understood about the regulatory mechanisms of P2X7 expression.

We previously generated an anti-P2X7 monoclonal antibody and revealed that P2X7 is highly expressed on colonic MCs, which is associated with the aggravation of intestinal inflammation (Kurashima et al., 2012). We simultaneously observed that skin MCs have lower—or almost no—expression of P2X7 (Kurashima et al., 2012). Intriguingly, it was reported that excessive production of IL-1β in skin MCs as a result of constitutive activation of NOD-like receptor family, pyrin domain-containing 3 (one of the signal pathways of P2X7), causes skin inflammation (Nakamura et al., 2012). These observations suggest that ectopic expression and aberrant activation of P2X7 in MCs might elicit skin inflammation. Here, we identified the unique regulatory function of skin fibroblasts in producing the retinoic acid (RA)-degrading enzyme Cyp26b1 to inhibit P2X7 expression on MCs for maintaining the skin homeostasis. Furthermore, we provide evidence that disruption of Cyp26b1-mediated regulatory function of skin fibroblasts, together with commensal microbial stimulation, induced the development of P2X7- and MC-mediated severe dermatitis.

RESULTS

Low P2X7 Expression by Skin MCs Accounts for Insensitivity to Extracellular ATP

We initially confirmed that skin c-kit$^+$ FcεRIα$^+$ cells were MCs by their selective depletion by diphtheria toxin (DT) treatment of MaS-TRECK mice where DT receptor (DTR) was specifically expressed on MCs (see Figure S1A available online) (Sawaguchi et al., 2012). In our previous study, P2X7 is expressed on MCs in the colon but low or undetectable in skin MCs (Kurashima et al., 2012). When we further compared P2X7 expression on MCs among colon, small intestine, lung, peritoneal cavity (PEC), and skin, it was lower on skin MCs than on MCs in the other tissues (Figure 1A). To assess whether the lower P2X7 expression on skin MCs was due to low expression at transcription or posttranscriptional events, we performed RT-PCR and intracellular flow cytometry analysis. Gene expression encoding P2X7 (*P2rx7*) was low on skin MCs, but not on MCs from the other tissues (Figure 1B). Consistently, the intracellular expression of P2X7 protein was also low in skin MCs (Figure S1B).

MCs can be categorized into two types—connective tissue and mucosal—in terms of protease phenotype. Connective-tissue-type MCs are located mainly in the skin and PEC and express mast cell protease (Mcpt) 4 and 5 (Gurish and Austen, 2012). Mucosal-type MCs are located in the gastroenterological mucosa and express Mcpt2 (Xing et al., 2011). Quantitative RT-PCR (qRT-PCR) and gene-microarray analyses indicated that even though the protease expression patterns were identical to those in previous observations (Figure S1C), P2X7 expression patterns were not applicable to the current two MC subtypes (Figure S1D).

To examine the reactivity of skin MCs against extracellular ATP, we stimulated them with ATP and measured the production of tumor necrosis factor alpha (TNF-α) and MCP1. In accordance with the lack of P2X7 expression on skin MCs, production of TNF-α and MCP1 upon ATP stimulation was detected in bone-marrow-derived MCs (BMMCs), but not skin MCs (Figure 1C). P2X7 plays a pivotal role in inflammasome activation along with stimulation by bacterial components such as lipopolysaccharide (LPS); these actions lead to interleukin-1β (IL-1β) production (Di Virgilio, 2007). Thus, IL-1β production was noted when PEC MCs from wild-type (WT) but not *P2rx7*$^{-/-}$ mice

were stimulated with both LPS and ATP. In contrast, skin MCs from both WT and *P2rx7*$^{-/-}$ mice did not produce IL-1β (Figure 1D).

Skin Environment-Mediated Downregulation of P2X7 Expression Is Independent of Commensal Microbiota and Immune Cells

MC progenitors differentiate into mature MCs in the local tissues (Gurish and Austen, 2012; Xing et al., 2011). We therefore considered that P2X7 expression would be affected by skin environment. To test this possibility, we transferred P2X7-expressing (WT) BMMCs directly into the skin of MC-deficient *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice. P2X7 expression on transferred MCs was gradually decreased to identical expression to those of skin resident MCs in WT mice within 10 days after adoptive transfer (Figures 2A and 2B). Furthermore, long-term reconstitution of MCs via the intravenous and intraperitoneal routes in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice led to successful reconstitution of P2X7-expressing MCs in the PEC and colon, whereas MCs in the skin showed low P2X7 expression (Figure S2A; data not shown) (Kurashima et al., 2012). These results indicated that P2X7 expression on MCs was reversible which was directly and negatively regulated by the skin environment.

It was recently shown that commensal microbiota stimulate immune responses in the skin (Naik et al., 2012), allowing us to compare P2X7 expression in specific-pathogen-free (SPF) and germ-free (GF) mice and in mice lacking MyD88, an adaptor molecule of an innate sensor for bacterial components (e.g., toll-like receptors [TLRs]). The low P2X7 expression on skin MCs was maintained in these mice (Figures 2C and D), suggesting that commensal microbiota did not directly influence P2X7 expression on skin MCs.

Various unique immune cells, such as γδ T cells and LCs, are important for maintaining skin homeostasis (Di Meglio et al., 2011). To examine the contribution of immune cells to the reduction of P2X7 on skin MCs, we analyzed mice lacking T cells (*Tcrb*$^{-/-}$*TCRd*$^{-/-}$), B cells (*Ighm*$^{-/-}$), or both (*Rag1*$^{-/-}$). Identically low P2X7 expression was seen on skin MCs of these mice (Figures 2C and 2E). To further explore the involvement of other immune cells, we analyzed DT-treated *Itgax*-DTR mice (Jung et al., 2002) and *Id2*$^{-/-}$ mice (Hacker et al., 2003), which lack dendritic cells (DCs) and LCs, respectively. No change of P2X7 expression on skin MCs was noted in the absence of DCs or LCs (Figures 2C and 2F). Also, the P2X7 expression in the colon MCs was comparable among these gene-deficient and WT mice (data not shown). Thus, T and B cells, DCs, and LCs were dispensable for the downregulation of P2X7 expression on skin MCs.

The skin possesses inhibitory cytokines, vitamins, and lipid mediators (Biggs et al., 2010; Schirmer et al., 2010). For instance, vitamin D_3 and IL-10 play regulatory roles in skin inflammation (Biggs et al., 2010). Although IL-10 receptor expression on MCs was slightly higher in skin than in colon (Figure 2G), no changes of P2X7 expression were noted on MCs supplemented with 1α,25$(OH)_2D_3$ (an active metabolite of vitamin D_3) or in *Il10*$^{-/-}$ mice (Figure S2B; Figure 2H). We also assessed the involvement of prostaglandin E2 (PGE_2), another candidate for control of skin MC functions (Gilfillan and Beaven, 2011). P2X7 expression on BMMCs was not altered when they were treated with PGE_2, indomethacin, or pertussis toxin, an inhibitor of G protein-coupled receptor pathway including PGE_2 receptors (Figure S2C). These results indicated that these mediators were redundant in regulating P2X7 expression on MCs.

Skin Fibroblasts Downregulate P2X7 Expression on MCs

It has been suggested that communication of MCs with stromal cells or fibroblasts induces optimal and tissue-dependent maturation. Indeed, coculture of skin 3T3 fibroblasts with immature MCs modulates the MC phenotypes, such as the expression of proteases and adhesion molecules (Takano et al., 2008). We confirmed that skin MCs were localized with fibroblasts in vivo (Figure S3A). To test the involvement of fibroblasts in the regulation of P2X7 expression, we isolated fibroblasts or stromal cells from the skin, lung, small intestine, and colon. We confirmed the morphological characteristics of tissue-derived fibroblasts or stromal cells (e.g., bipolar or multipolar) and their elongated shape with adherent growth together with expression of ER-TR7, a stromal cell pan-marker (Figures S3B and S3C). Coculture of BMMCs with colon stromal cells induced the expression of *Mcpt1* and *Mcpt2*, indicative of mucosal-type MCs; whereas skin fibroblasts induced *Mcpt4* expression in cocultured BMMCs, which is indicative of connective tissue MCs (Figure S3D). In contrast, the expression of FcεRIα on MCs was not changed in these conditions (Figure S3D). In addition, morphological and biochemical analyses revealed that skin fibroblasts regulated the expression of secretory granule components, such as heparin and chondroitin sulfate, in the cocultured MCs (Figure S3E); this behavior is characteristics of connective-tissue-type MCs (Gurish and Austen, 2012). These results indicated that coculture with stromal cells or fibroblasts induced the terminally differentiated and local-environment-adjusted MCs.

Under these experimental conditions, skin fibroblasts inhibited the P2X7 expression on cocultured BMMCs (Figures 3A and 3B). However, stromal cells from the colon and small intestine did not suppress their P2X7 expression (Figures 3A and 3B). In the case of coculture with lung-derived fibroblasts, P2X7 expression was partially suppressed, but the suppression was weaker than that with skin fibroblasts (Figures 3A and 3B). Interestingly, inhibition of P2X7 expression still occurred when both cell types were separately cultured in a transwell culture system (Figure 3C), suggesting that secretory factors from the skin fibroblasts are capable of reducing the P2X7 expression on MCs. Because MCs were differentiated from MC progenitors (Gurish and Austen, 2012), we cocultured BM cells containing MC progenitors with either skin fibroblasts or colon stromal cells for 2 to 3 weeks. P2X7 expression on newly differentiated MCs was detected in the presence of colonic stromal cells, whereas their P2X7 expression was decreased in the presence of skin fibroblasts (Figure 3D). Furthermore, P2X7 expression recovered when the skin fibroblasts were removed from the culture or replaced with colon stromal cells (Figures 3E and 3F). Consistent with our findings (Figure 1), the expression of mRNA encoding *P2rx7* was accordingly changed (Figure 3G), and BMMCs cocultured with skin fibroblasts did not produce MCP1 and TNF-α upon extracellular ATP stimulation (Figure 3H). Like P2X7 expression, gene expressions of *Mcpt1*, *Mcpt2*, and *Mcpt4* and the production of heparin and chondroitin sulfate induced

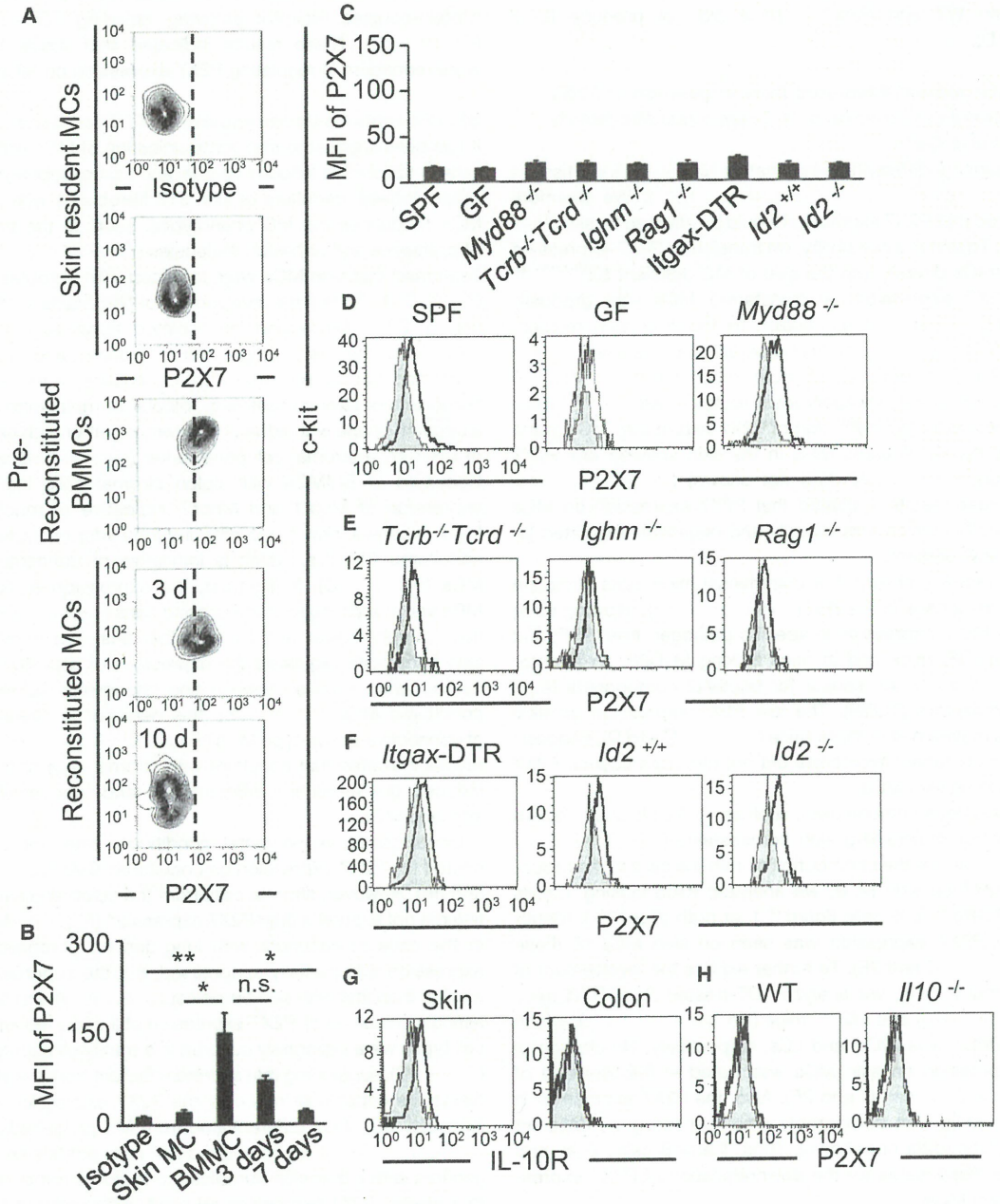

Figure 2. Skin Environment Regulates P2X7 Expression

(A and B) Flow cytometric analysis was performed to measure P2X7 expression on skin MCs from WT or $Kit^{W\text{-}sh/W\text{-}sh}$ mice receiving adaptive transfer of $P2X7^{+}$ bone-marrow-derived MCs (BMMCs) (A) and mean fluorescence intensity (MFI) was examined (B). Data are means ± SEM. *$p < 0.05$ ($n = 3$); **$p < 0.01$; n.s., not significant.

(C–F) MFI of P2X7 expression on skin MCs from various mice were examined by flow cytometry (C). Data are means ± SEM ($n = 3$ to 8). (D-F) P2X7 expression was measured by flow cytometry on skin MCs from specific-pathogen free (SPF), germ free (GF), and *Myd88*$^{-/-}$ mice (D), and *Tcrb*$^{-/-}$*Tcrd*$^{-/-}$, *Ighm*$^{-/-}$, and *Rag1*$^{-/-}$ mice (E), diphtheria-toxin-treated *Itgax*-DTR transgenic, *Id2*$^{+/+}$, and *Id2*$^{-/-}$ mice (F). Control staining with isotype control is shown as gray.

(G) IL-10 receptor (IL-10R) expression on MCs from skin and colon was analyzed by flow cytometry.

(H) P2X7 expression on skin MCs was measured in WT and *Il10*$^{-/-}$ mice. Control staining with isotype control is shown as gray. All data are representative of at least three independent experiments.

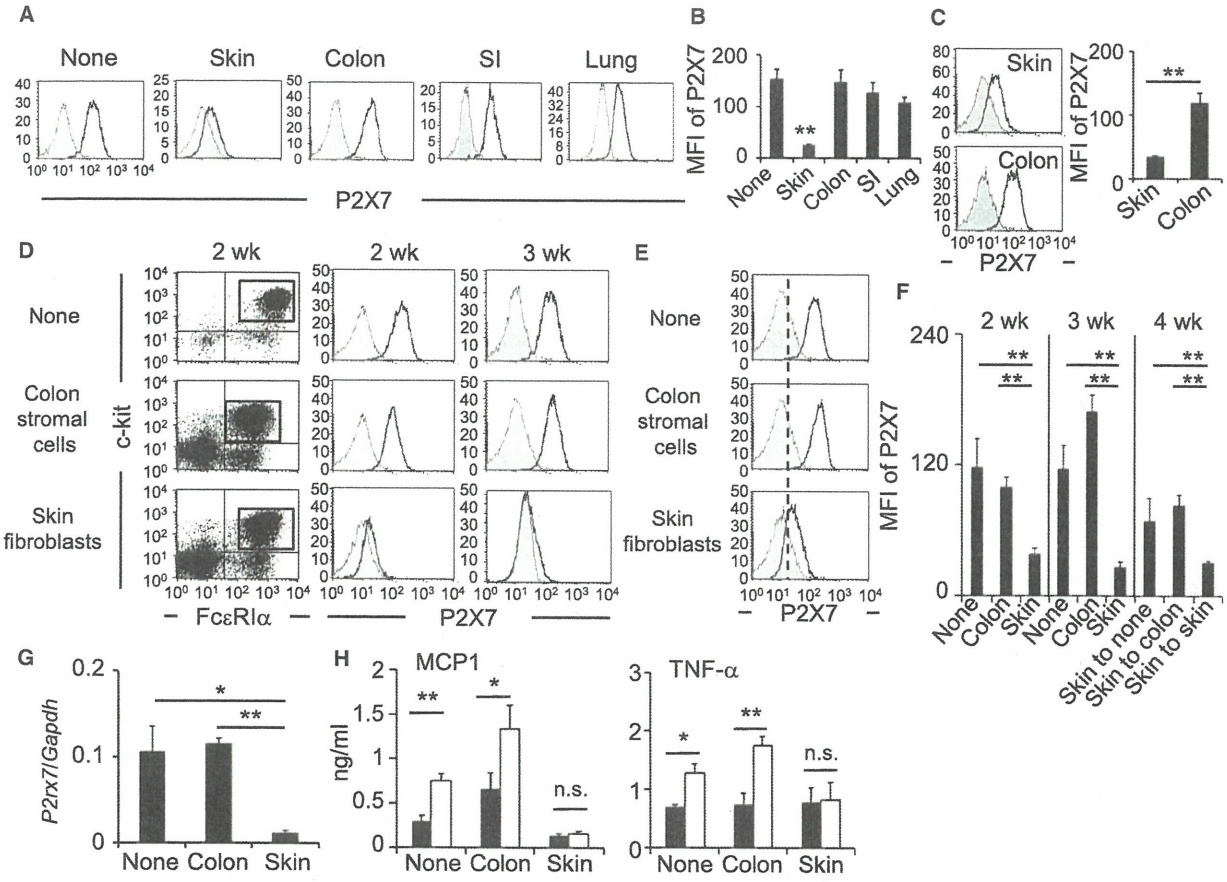

Figure 3. Skin Fibroblasts Regulate P2X7 Expression on Mast Cells

(A) BMMCs were cultured with or without skin fibroblasts, lung fibroblasts, or small intestine (SI) or colon stromal cells and stained for P2X7. Control staining with rat IgG2b is shown as gray.

(B) Mean fluorescence intensity (MFI) of P2X7 expression is shown. Data are means ± SEM. $^{**}p < 0.01$, one-way ANOVA and Tukey's method (n = 5 to 8).

(C) BMMCs and colon stromal cells or skin fibroblasts were separately cultured in the transwells for 3 weeks. P2X7 expression BMMCs was measured by flow cytometry. Control staining with rat IgG2b is shown as gray. Data are means ± SEM (n = 6). $^{**}p < 0.01$.

(D) Bone marrow cells were cultured with or without skin fibroblasts or colon stromal cells, together with IL-3 and stem cell factor for 3 weeks. Expression of c-kit, FcεR1α, and P2X7 was measured by flow cytometry. Control staining with rat IgG2b is shown as gray.

(E and F) Bone-marrow cells were cocultured with skin fibroblasts for 3 weeks and then cultured with or without skin fibroblasts or colon stromal cells for an additional 4 days. P2X7 was measured by flow cytometry (E) and MFI of P2X7 expression is shown (F). Data are means ± SEM. $^{**}p < 0.01$, (n = 4 to 6). All data are representative of at least three independent experiments.

(G) BMMCs were sorted after coculture with skin fibroblasts and colon stromal cells, and *P2rx7* expression was examined by quantitative RT-PCR. Relative expression was normalized against *Gapdh*. Data are means ± SEM. $^{**}p < 0.01$, $^{*}p < 0.05$ (n = 4).

(H) BMMCs were sorted after coculture with skin fibroblasts and colon stromal cells and then stimulated with 0.5 mM ATP. Production of MCP1 and TNF-α in culture supernatant was determined. Black bars, no treatment; white bars, ATP stimulation. Data are means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, n.s. not significant.

by coculture with skin fibroblasts were reversed by removal of skin fibroblasts or replacement with colon stromal cells (Figures S3E and S3F). These results suggest that skin fibroblasts play a pivotal role in the downregulation of P2X7 expression on MCs, leading to the blockade of their reactivity to extracellular ATP.

Cyp26b1 Plays a Critical Role in Negative Regulation of P2X7 Expression on MCs

Gene expression was compared between skin fibroblasts and colon stromal cells, as an example of P2X7-inhibitor and noninhibitor cells, respectively. Gene microarray analysis identified several genes expressed more highly in skin fibroblasts than in colonic stromal cells, including gene encoding the retinoic-acid (RA)-degrading enzymes *Cyp26a1* and *Cyp26b1* (Figure 4A). Quantitative RT-PCR analysis confirmed the higher expression of *Cyp26b1* in the skin fibroblasts than colonic stromal cells (Figure 4B), whereas *vimentin*, a stromal cell pan-marker, was identically expressed in both cell types (Figure 4C). It was reported that Cyp26b1 is involved in skin homeostasis and thus increases in RA concentrations through disruption of *Cyp26b1* cause abnormalities in embryonic skin barrier formation (Okano et al., 2012). In addition, in vitro culture of $CD8^+$ T cells with RA induces