amounts of other saturated FAs, including lauric and myristic acids (Fig. 1A). Unlike palm oil, coconut oil did not alter fecal or serum Ab production in the mice (Fig. 1B). These findings suggest that dietary PA uniquely enhances intestinal IgA production.

PA-enriched dietary oils enhance intestinal IgA production and IgA responses against oral vaccine Ag

To confirm that PA enhances intestinal IgA production, we added PA to soybean oil to adjust its PA concentration to that of palm oil (Fig. 2A, *upper bar*). Fecal IgA levels in mice maintained for 2 mo on a diet containing the PA-enriched soybean oil were increased similarly to those of mice fed a palm oil–containing diet, but serum IgA production was unchanged (Fig. 2A, *lower bar*, Supplemental Fig. 1). These data suggest that PA is a key FA in the enhancement of intestinal IgA production.

We then investigated whether the enhanced production of intestinal IgA induced by dietary PA is reflected in the responses to an orally administered vaccine. To this end, we orally immunized mice concurrently with OVA and the mucosal adjuvant CT. In agreement with levels of naturally produced IgA, OVA-specific fecal IgA responses were enhanced in mice maintained on a diet that contained palm oil (Fig. 3). In addition, similarly increased OVA-specific IgA production was noted in mice maintained on PA-enriched soybean oil (Fig. 3). These findings suggest that dietary PA enhanced not only naturally produced intestinal IgA but also Ag-specific intestinal IgA induced by oral immunization.

PA content is increased in the intestinal, but not systemic, compartment

We next measured the amounts of PA in the intestines and serum of the mice. PA concentrations in the small and large intestines were higher in the mice maintained on PA-rich soybean oil compared with soybean oil alone (Fig. 4). In contrast, PA amounts in serum were comparable between groups (Fig. 4). These findings indicate that dietary PA affects the amount of PA locally in the intestine without any influence on serum PA concentration.

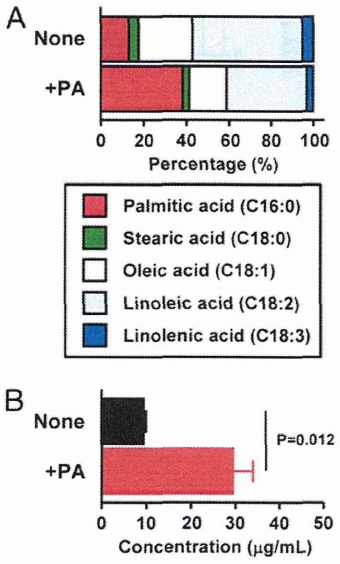

FIGURE 2. Dietary PA alone is sufficient to enhance intestinal IgA production. (**A**) Purified PA was added to soybean oil to achieve the same PA content as that in palm oil. (**B**) Mice were maintained for 2 mo on a diet containing soybean oil, with or without supplemental PA. The IgA levels of fecal extracts were measured (mean ± 1 SD, n = 16/group).

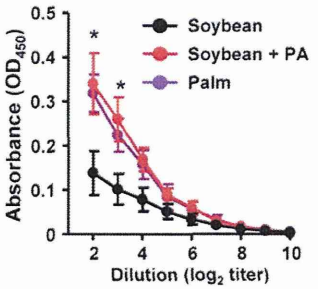

FIGURE 3. Ingestion of PA-enriched oil enhances intestinal IgA responses against orally immunized Ag. After mice were maintained for 2 mo on a diet containing soybean oil, soybean oil + PA, or palm oil, they were orally immunized with OVA plus CT on days 0, 7, and 14. On day 21, fecal extracts were collected for the determination of OVA-specific intestinal IgA by ELISA. Data are mean ± 1 SD (n = 4/group), and similar results were obtained from two independent experiments. *$p < 0.05$.

PA directly stimulates intestinal PCs to produce IgA

We then examined whether PA directly affected IgA production from PCs. To address this issue, we purified IgA^+ PCs from the intestine and cultured them with PA for 4 d. The amount of IgA in the intestinal PC culture supernatants increased in a dose-dependent manner (Fig. 5A). ELISPOT assays showed that PA did not increase the number of IgA-forming cells (Fig. 5B), suggesting that PA instead enhances Ab production from PCs. In addition, IgG production from $CD19^+$ $CD138^+$ PCs from the spleen was increased in the presence of PA (Supplemental Fig. 2), indicating that PA enhances Ab production, regardless of the Ig subtype.

PA acts as a ligand for TLR4 (24), prompting our investigation into whether TLR4 mediated the PA-induced direct activation of IgA PCs. To address this issue, we used C3H/HeN and C3H/HeJ mice. Although TLR4-mediated signaling differs between these two strains because of a spontaneous point mutation in the *Tlr4* gene of the C3H/HeJ mice (25, 26), intestinal PCs from these mouse strains produced identical levels of PA-induced IgA (Fig. 5C). Therefore, the direct effect of PA on IgA production from PCs likely is independent of the TLR4 pathway.

The PA-induced increase in IgA PCs in the large intestine is dependent on SPT

We used flow cytometry to determine the frequency of IgA^+ PCs in the small and large intestines. Although no significant difference between the control and PA-enriched diets was noted in the small intestine, the frequency of IgA^+ $B220^-$ PCs was increased in the

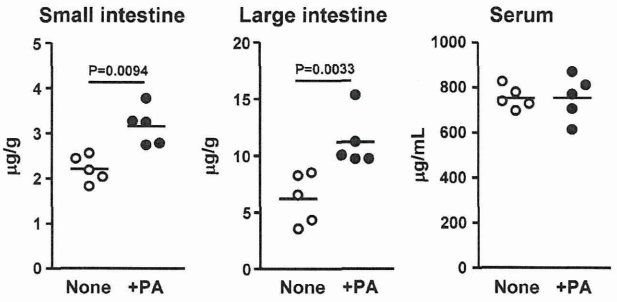

FIGURE 4. Increased PA content in the intestine of mice maintained on PA-enriched oils. Mice were maintained for 2 mo on a diet containing soybean oil, with or without PA, and small and large intestinal tissues and serum were collected for measurement of their PA contents. The graphs show data from individual mice, and the horizontal lines indicate the means; similar results were obtained from two independent experiments.

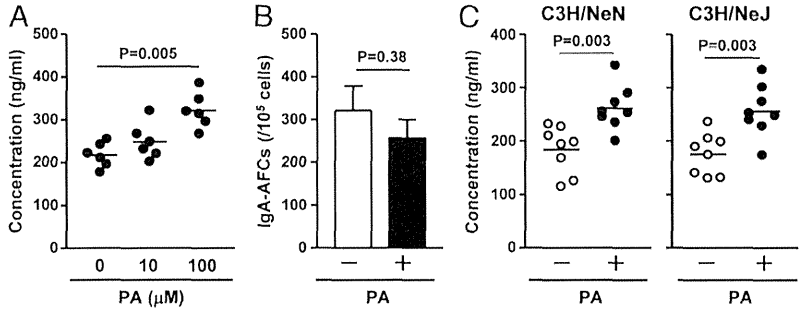

FIGURE 5. PA directly stimulates IgA-producing PCs via a TLR4-independent mechanism. IgA^+ $B220^-$ PCs were isolated from the small intestine of BALB/c (**A** and **B**) and C3H/HeN or C3H/HeJ mice (**C**) and cultured with 10 or 100 μM (A) or 100 μM (B and C) PA. The amount of IgA in the culture supernatant (A and C) and the number of IgA^+ Ab–forming cells (IgA^+-AFCs) (B) were determined by ELISA and ELISPOT, respectively. The graphs show data from individual samples, and the horizontal lines indicate the means; similar results were obtained from two independent experiments (A and C). Data in (B) are mean ± 1 SD (n = 10/group from two separate experiments).

large intestine of the PA-enriched diet group (Fig. 6A). Similarly, the number of OVA-specific IgA-forming cells was increased in the large, but not small, intestine of mice receiving oral immunization and the PA-enriched diet (Fig. 6B). In agreement with the lack of a PA-associated effect on small intestinal IgA, B cell differentiation into IgA^+ cells in the PPs, a lymphoid tissue for the initiation of small intestinal IgA responses (3), was unchanged in mice maintained on a PA-enriched diet (Supplemental Fig. 3).

PA can be converted into sphingolipids, including ceramide, sphingosine, and sphingosine 1 phosphate (S1P) (Fig. 6C); all of these lipids all known to promote cell proliferation, survival, and trafficking (27, 28). Therefore, we supposed that these PA-derived sphingolipids might be involved in the regulation of intestinal IgA PCs in the large intestine. To test this possibility, mice were treated with myriocin, an inhibitor of SPT, which is a key enzyme in the conversion of PA into sphingolipids (Fig. 6C). The PA-mediated increase in IgA PCs did not occur in the large intestine of mice that received myriocin (Fig. 6D), and myriocin had little effect on the number of $IgG1^+$ cells in the spleen (Supplemental Fig. 4), suggesting that SPT activity is required for this effect on large intestinal IgA PCs.

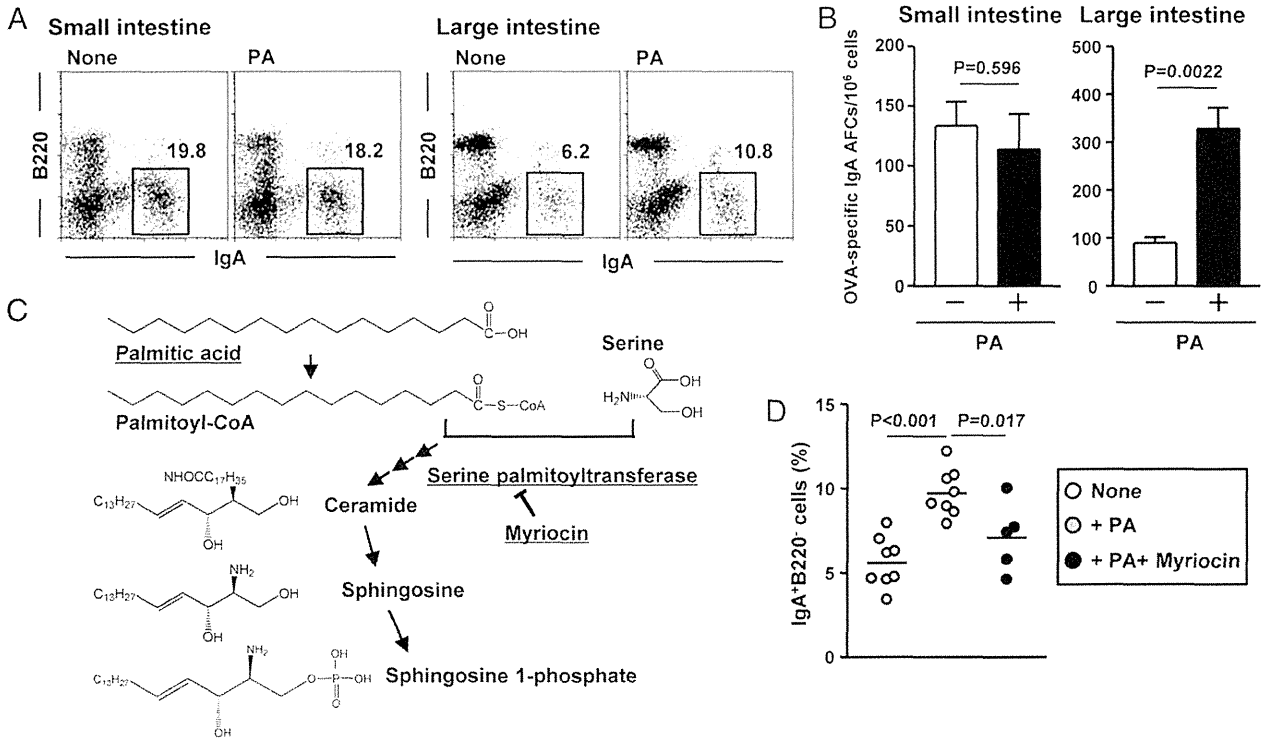

FIGURE 6. SPT is required for the increased number of IgA PCs in the large intestine. Mice were maintained on a diet containing soybean oil, with or without PA, for 2 mo. (**A**) Mononuclear cells were isolated from the small and large intestines for the flow cytometric analysis of IgA^+ $B220^-$ PCs. Data are representative of three independent experiments. (**B**) Mice were orally immunized with OVA plus CT on days 0, 7, and 14. On day 21, mononuclear cells were isolated from the small and large intestine for the detection of OVA-specific IgA Ab–forming cells by ELISPOT assay. Data are mean ± 1 SD (n = 6/group from two separate experiments). (**C**) The metabolic pathway mediated by SPT to generate sphingolipids from PA. (**D**) The frequency of IgA^+ $B220^-$ PCs in the large intestine of mice maintained on a PA-enriched diet and myriocin. The graphs show data from individual samples, and the horizontal lines indicate the means. Similar results were obtained from two independent experiments.

The increased proliferation of IgA PCs in the large intestine of mice maintained with a PA-enriched diet

We performed BrdU-uptake assays to examine the effects of PA on the proliferation and survival of IgA PCs in the large intestine. Soon after BrdU administration (day 1), the proportions of $BrdU^+$ and $BrdU^-$ IgA^+ cells in the large intestine were higher when mice were maintained on a PA-enriched diet than when they were maintained on the control diet, but the increase ratio of IgA^+ cells was higher in $BrdU^+$ IgA^+ cells than in $BrdU^-$ IgA^+ cells (Fig. 7A). These findings suggest that PA metabolites induced the proliferation of IgA^+ cells in the large intestine. In contrast, on day 4, both the control and PA-enriched groups showed similar levels of $BrdU^+$ IgA^+ cells (Fig. 7B). These data collectively suggested that PA metabolites primarily induced the proliferation of IgA^+ cells in the large intestine rather than prolonged their survival.

Discussion

In the current study, we extended our knowledge of lipid-mediated immune regulation by showing the immunologic function of dietary PA in the enhancement of intestinal IgA responses. Unlike the sterile environment of systemic immune compartments (e.g., spleen), intestinal tissues are continuously exposed to exogenous factors, including commensal bacteria and dietary materials and actively use them to establish a homeostatic inflammatory condition (6). Indeed, in contrast to the massive inflammatory responses induced by the systemic injection of bacterial products, such as LPS (e.g., sepsis), the intestinal immune system requires bacterial stimulation for its maturation (29). Our current study demonstrates that dietary PA can augment intestinal IgA responses when an appropriate amount of dietary oil (4%) is supplied. This effect contrasts sharply with the deleterious plasma PA levels that are induced by a high-fat diet and are considered to be a risk factor for inflammation and diabetes (30). Therefore, like bacterial products, PA has the opposite immunologic effect on intestinal and systemic immune compartments and actually plays a beneficial role in the maturation of the intestinal immune system.

The enhancement of intestinal IgA responses by dietary PA is mediated by at least two distinct pathways. One is PA's direct effect on IgA-producing PCs, and the other is mediated by PA-derived metabolites, sphingolipids. In addition to these two pathways, PA affects APCs (e.g., macrophages and dendritic cells) to promote Ag presentation and the production of cytokines, including IL-6 and TNF-α; this effect is at least partly mediated by TLR4 (31, 32) and represents a plausible third pathway to enhancing intestinal IgA production. Unlike the effect of PA on APCs, PA promoted IgA production from PCs in a TLR4-independent manner in our current study. In line with this finding, several groups reported that TLR2 acts as a receptor for PA or that PA enters cells where it induces signal transduction for the consequent production of inflammatory cytokines (33–36).

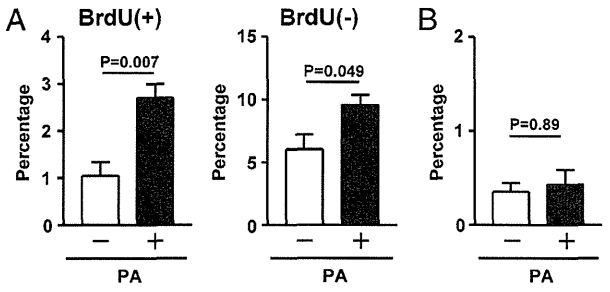

FIGURE 7. A PA-enriched diet induces the proliferation of IgA PCs. Mice were maintained on a diet containing soybean oil, with or without PA, for 2 mo, after which BrdU was injected i.p. (day 0). On day 1 (**A**) and day 4 (**B**), mononuclear cells isolated from the large intestine were analyzed by flow cytometry to determine the proportion of $BrdU^+$ IgA^+ PCs. Data are mean ± 1 SD (n = 4). Similar results were obtained from two independent experiments.

In addition to PA's direct effect on IgA-producing PCs, the SPT-mediated metabolism of PA is involved in the PA-mediated enhancement of intestinal IgA responses. SPT is an essential enzyme for the de novo pathway of sphingolipid synthesis, in which palmitoyl-CoA and serine act as substrates of SPT to generate 3-keto-dihydosphingosine, with subsequent conversion into other sphingolipids, such as sphingomyelin, ceramide, sphingosine, and S1P (37, 38). Sphingolipids are a class of membrane lipids that also are known to have biologic functions (27, 28). For example, ceramide regulates cytoskeletal changes, cell cycle, and apoptosis (27, 28). Accordingly, perhaps an increase in ceramide concentrations induced the proliferation or prolonged the survival of IgA PCs, subsequently increasing the number of IgA-producing PCs in the large intestine. In addition, in the extracellular compartment, S1P controls cell trafficking by recruiting cells toward regions with high concentrations of S1P (39). Of note, we previously reported that S1P regulates intestinal IgA responses by controlling trafficking of IgA^+ cells from inductive sites (e.g., PPs and peritoneal cavity) into the iLP (22, 40). Therefore, another possibility is that the PA-enriched diet induced an increase in the intestinal extracellular S1P concentration, resulting in the effective recruitment of IgA PCs into the intestine. Our current findings suggest that at least one of the enhancing effects of the PA-enriched diet was mediated by the induction of proliferating IgA^+ PCs in the large intestine.

PA was not only included in the diet but was also generated through de novo lipogenesis, whereby carbohydrates are converted to PA. The de novo pathway achieves stable concentrations of PA in vivo. This pathway can explain the specificity of the effect of dietary PA enrichment on the PA content in various tissues. In this study, we found that the increases in PA in the PA-enriched diet group were specific to the intestinal tissues and not the serum. This tissue specificity is consistent with the specific effect of dietary PA, which increased intestinal IgA responses without affecting serum Ab production. Mice fed high-fat diets show increased serum PA levels, which are a risk factor for inflammation and diabetes (30). However, our current findings indicate that the increased proportion of PA in the dietary oil did not affect serum PA levels when the overall amount of oil consumed was normal (4%).

Intestinal tissues contain higher levels of sphingolipids than do other tissues (41), and we found that the effect of an SPT inhibitor was selective for the large, but not small, intestine. One of the major differences between the small and large intestines is the amount of commensal bacteria. We recently reported that some lipid metabolic pathways are uniquely mediated by commensal bacteria (42), raising the possibility that commensal bacteria may affect sphingolipid metabolism. However, germ-free and specific pathogen–free rats have comparable levels of sphingolipids in the intestine (43). Therefore, it is plausible that commensal bacteria are unlikely to participate in this pathway. In contrast, the expression pattern of enzymes involved in the generation of sphingolipids differs between intestinal compartments (44, 45), indicating that the differences in sphingolipid metabolism between the small and large intestines determine their differing dependence on SPT in PA-mediated intestinal IgA responses.

Taken together, our current findings demonstrate that dietary PA and its metabolites play a critical role in the enhancement of intestinal IgA responses. This information can be applied to the development of mucosal adjuvants.

Disclosures

The authors have no financial conflicts of interest.

References

1. Macpherson, A. J., K. D. McCoy, F. E. Johansen, and P. Brandtzaeg. 2008. The immune geography of IgA induction and function. *Mucosal Immunol.* 1: 11–22.
2. Woof, J. M., and M. A. Kerr. 2006. The function of immunoglobulin A in immunity. *J. Pathol.* 208: 270–282.
3. Kunisawa, J., Y. Kurashima, and H. Kiyono. 2012. Gut-associated lymphoid tissues for the development of oral vaccines. *Adv. Drug Deliv. Rev.* 64: 523–530.
4. Fagarasan, S., S. Kawamoto, O. Kanagawa, and K. Suzuki. 2010. Adaptive immune regulation in the gut: T cell-dependent and T cell-independent IgA synthesis. *Annu. Rev. Immunol.* 28: 243–273.
5. Cerutti, A., K. Chen, and A. Chorny. 2011. Immunoglobulin responses at the mucosal interface. *Annu. Rev. Immunol.* 29: 273–293.
6. Spencer, S. P., and Y. Belkaid. 2012. Dietary and commensal derived nutrients: shaping mucosal and systemic immunity. *Curr. Opin. Immunol.* 24: 379–384.
7. Weinstein, P. D., and J. J. Cebra. 1991. The preference for switching to IgA expression by Peyer's patch germinal center B cells is likely due to the intrinsic influence of their microenvironment. *J. Immunol.* 147: 4126–4135.
8. Hamada, H., T. Hiroi, Y. Nishiyama, H. Takahashi, Y. Masunaga, S. Hachimura, S. Kaminogawa, H. Takahashi-Iwanaga, T. Iwanaga, H. Kiyono, et al. 2002. Identification of multiple isolated lymphoid follicles on the antimesenteric wall of the mouse small intestine. *J. Immunol.* 168: 57–64.
9. Kunisawa, J., M. Gohda, E. Hashimoto, I. Ishikawa, M. Higuchi, Y. Suzuki, Y. Goto, C. Panea, and I.I. Ivanov, R. Sumiya, et al. 2013. Microbe-dependent $CD11b^+$ IgA^+ plasma cells mediate robust early-phase intestinal IgA responses in mice. *Nat. Commun.* 4: 1772.
10. Maslowski, K. M., and C. R. Mackay. 2011. Diet, gut microbiota and immune responses. *Nat. Immunol.* 12: 5–9.
11. Kunisawa, J., and H. Kiyono. 2013. Vitamin-mediated regulation of intestinal immunity. *Front. Immunol.* 4: 189.
12. Hanson, L. A., A.-K. Robertson, J. Bjersing, and M. V. Herias. 2005. Undernutrition, immunodeficiency, and mucosal infections. In *Mucosal Immunology*, 3rd ed. J. Mestecky, M. E. Lamm, W. Strober, J. Bienenstock, J. R. McGhee, and L. Mayer, eds. Academic Press, San Diego, CA, p. 1159–1178.
13. Wintergerst, E. S., S. Maggini, and D. H. Hornig. 2007. Contribution of selected vitamins and trace elements to immune function. *Ann. Nutr. Metab.* 51: 301–323.
14. Galli, C., and P. C. Calder. 2009. Effects of fat and fatty acid intake on inflammatory and immune responses: a critical review. *Ann. Nutr. Metab.* 55: 123–139.
15. Margioris, A. N. 2009. Fatty acids and postprandial inflammation. *Curr. Opin. Clin. Nutr. Metab. Care* 12: 129–137.
16. Arita, M. 2012. Mediator lipidomics in acute inflammation and resolution. *J. Biochem.* 152: 313–319.
17. Jin, C., and R. A. Flavell. 2013. Innate sensors of pathogen and stress: linking inflammation to obesity. *J. Allergy Clin. Immunol.* 132: 287–294.
18. Schmitz, G., and J. Ecker. 2008. The opposing effects of n-3 and n-6 fatty acids. *Prog. Lipid Res.* 47: 147–155.
19. Nguyen, M. T., S. Favelyukis, A. K. Nguyen, D. Reichart, P. A. Scott, A. Jenn, R. Liu-Bryan, C. K. Glass, J. G. Neels, and J. M. Olefsky. 2007. A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via Toll-like receptors 2 and 4 and JNK-dependent pathways. *J. Biol. Chem.* 282: 35279–35292.
20. Håversen, L., K. N. Danielsson, L. Fogelstrand, and O. Wiklund. 2009. Induction of proinflammatory cytokines by long-chain saturated fatty acids in human macrophages. *Atherosclerosis* 202: 382–393.
21. He, Q., R. T. Riley, and R. P. Sharma. 2005. Myriocin prevents fumonisin B1-induced sphingoid base accumulation in mice liver without ameliorating hepatotoxicity. *Food Chem. Toxicol.* 43: 969–979.
22. Gohda, M., J. Kunisawa, F. Miura, Y. Kagiyama, Y. Kurashima, M. Higuchi, I. Ishikawa, I. Ogahara, and H. Kiyono. 2008. Sphingosine 1-phosphate regulates the egress of IgA plasmablasts from Peyer's patches for intestinal IgA responses. *J. Immunol.* 180: 5335–5343.
23. Schaeffler, A., P. Gross, R. Buettner, C. Bollheimer, C. Buechler, M. Neumeier, A. Kopp, J. Schoelmerich, and W. Falk. 2009. Fatty acid-induced induction of Toll-like receptor-4/nuclear factor-kappaB pathway in adipocytes links nutritional signalling with innate immunity. *Immunology* 126: 233–245.
24. Maloney, E., I. R. Sweet, D. M. Hockenbery, M. Pham, N. O. Rizzo, S. Tateya, P. Handa, M. W. Schwartz, and F. Kim. 2009. Activation of NF-kappaB by palmitate in endothelial cells: a key role for NADPH oxidase-derived superoxide in response to TLR4 activation. *Arterioscler. Thromb. Vasc. Biol.* 29: 1370–1375.
25. Poltorak, A., X. He, I. Smirnova, M. Y. Liu, C. Van Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, et al. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. *Science* 282: 2085–2088.
26. Hoshino, K., O. Takeuchi, T. Kawai, H. Sanjo, T. Ogawa, Y. Takeda, K. Takeda, and S. Akira. 1999. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J. Immunol.* 162: 3749–3752.
27. Fyrst, H., and J. D. Saba. 2010. An update on sphingosine-1-phosphate and other sphingolipid mediators. *Nat. Chem. Biol.* 6: 489–497.
28. Hannun, Y. A., and L. M. Obeid. 2008. Principles of bioactive lipid signalling: lessons from sphingolipids. *Nat. Rev. Mol. Cell Biol.* 9: 139–150.
29. Kayama, H., and K. Takeda. 2012. Regulation of intestinal homeostasis by innate and adaptive immunity. *Int. Immunol.* 24: 673–680.
30. Kennedy, A., K. Martinez, C. C. Chuang, K. LaPoint, and M. McIntosh. 2009. Saturated fatty acid-mediated inflammation and insulin resistance in adipose tissue: mechanisms of action and implications. *J. Nutr.* 139: 1–4.
31. Weigert, C., K. Brodbeck, H. Staiger, C. Kausch, F. Machicao, H. U. Häring, and E. D. Schleicher. 2004. Palmitate, but not unsaturated fatty acids, induces the expression of interleukin-6 in human myotubes through proteasome-dependent activation of nuclear factor-kappaB. *J. Biol. Chem.* 279: 23942–23952.
32. Weatherill, A. R., J. Y. Lee, L. Zhao, D. G. Lemay, H. S. Youn, and D. H. Hwang. 2005. Saturated and polyunsaturated fatty acids reciprocally modulate dendritic cell functions mediated through TLR4. *J. Immunol.* 174: 5390–5397.
33. Snodgrass, R. G., S. Huang, I. W. Choi, J. C. Rutledge, and D. H. Hwang. 2013. Inflammasome-mediated secretion of IL-1β in human monocytes through TLR2 activation; modulation by dietary fatty acids. *J. Immunol.* 191: 4337–4347.
34. Zhao, L., M. J. Kwon, S. Huang, J. Y. Lee, K. Fukase, N. Inohara, and D. H. Hwang. 2007. Differential modulation of Nods signaling pathways by fatty acids in human colonic epithelial HCT116 cells. *J. Biol. Chem.* 282: 11618–11628.
35. Erridge, C., and N. J. Samani. 2009. Saturated fatty acids do not directly stimulate Toll-like receptor signaling. *Arterioscler. Thromb. Vasc. Biol.* 29: 1944–1949.
36. Schwartz, E. A., W. Y. Zhang, S. K. Karnik, S. Borwege, V. R. Anand, P. S. Laine, Y. Su, and P. D. Reaven. 2010. Nutrient modification of the innate immune response: a novel mechanism by which saturated fatty acids greatly amplify monocyte inflammation. *Arterioscler. Thromb. Vasc. Biol.* 30: 802–808.
37. Merrill, A. H., Jr. 1983. Characterization of serine palmitoyltransferase activity in Chinese hamster ovary cells. *Biochim. Biophys. Acta* 754: 284–291.
38. Gault, C. R., L. M. Obeid, and Y. A. Hannun. 2010. An overview of sphingolipid metabolism: from synthesis to breakdown. *Adv. Exp. Med. Biol.* 688: 1–23.
39. Cyster, J. G., and S. R. Schwab. 2012. Sphingosine-1-phosphate and lymphocyte egress from lymphoid organs. *Annu. Rev. Immunol.* 30: 69–94.
40. Kunisawa, J., Y. Kurashima, M. Gohda, M. Higuchi, I. Ishikawa, F. Miura, I. Ogahara, and H. Kiyono. 2007. Sphingosine 1-phosphate regulates peritoneal B-cell trafficking for subsequent intestinal IgA production. *Blood* 109: 3749–3756.
41. Bouhours, D., and J. F. Bouhours. 1985. Developmental changes of monohexosylceramide and free ceramide in the large intestine of the rat. *J. Biochem.* 98: 1359–1366.
42. Gustafsson, B. E., K. A. Karlsson, G. Larson, T. Midtvedt, N. Strömberg, S. Teneberg, and J. Thurin. 1986. Glycosphingolipid patterns of the gastrointestinal tract and feces of germ-free and conventional rats. *J. Biol. Chem.* 261: 15294–15300.
43. Kishino, S., M. Takeuchi, S. B. Park, A. Hirata, N. Kitamura, J. Kunisawa, H. Kiyono, R. Iwamoto, Y. Isobe, M. Arita, et al. 2013. Polyunsaturated fatty acid saturation by gut lactic acid bacteria affecting host lipid composition. *Proc. Natl. Acad. Sci. USA* 110: 17808–17813.
44. Duan, R. D., L. Nyberg, and A. Nilsson. 1995. Alkaline sphingomyelinase activity in rat gastrointestinal tract: distribution and characteristics. *Biochim. Biophys. Acta* 1259: 49–55.
45. Kono, M., J. L. Dreier, J. M. Ellis, M. L. Allende, D. N. Kalkofen, K. M. Sanders, J. Bielawski, A. Bielawska, Y. A. Hannun, and R. L. Proia. 2006. Neutral ceramidase encoded by the Asah2 gene is essential for the intestinal degradation of sphingolipids. *J. Biol. Chem.* 281: 7324–7331.

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

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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,

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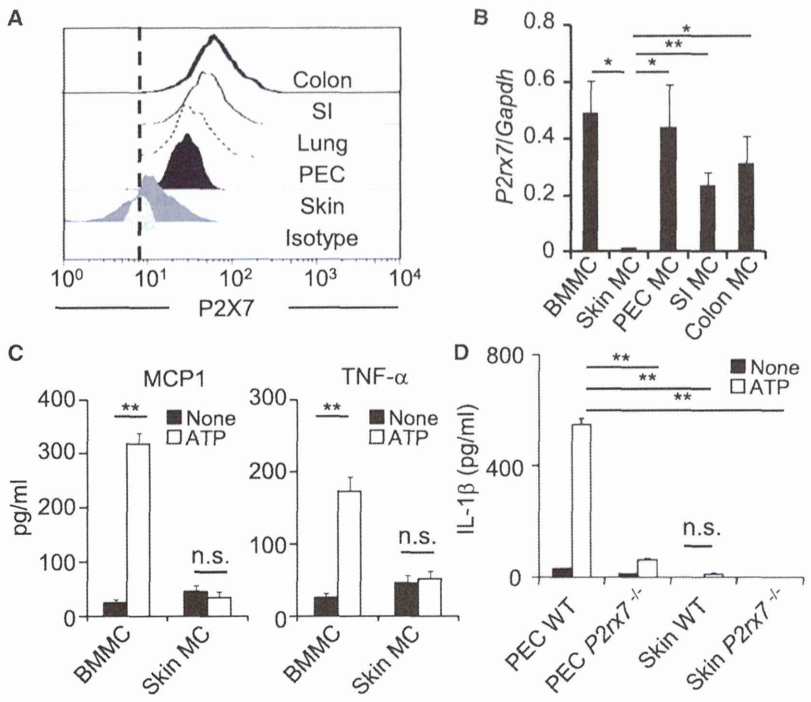

Figure 1. Low P2X7 Expression on Skin Mast Cells

(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

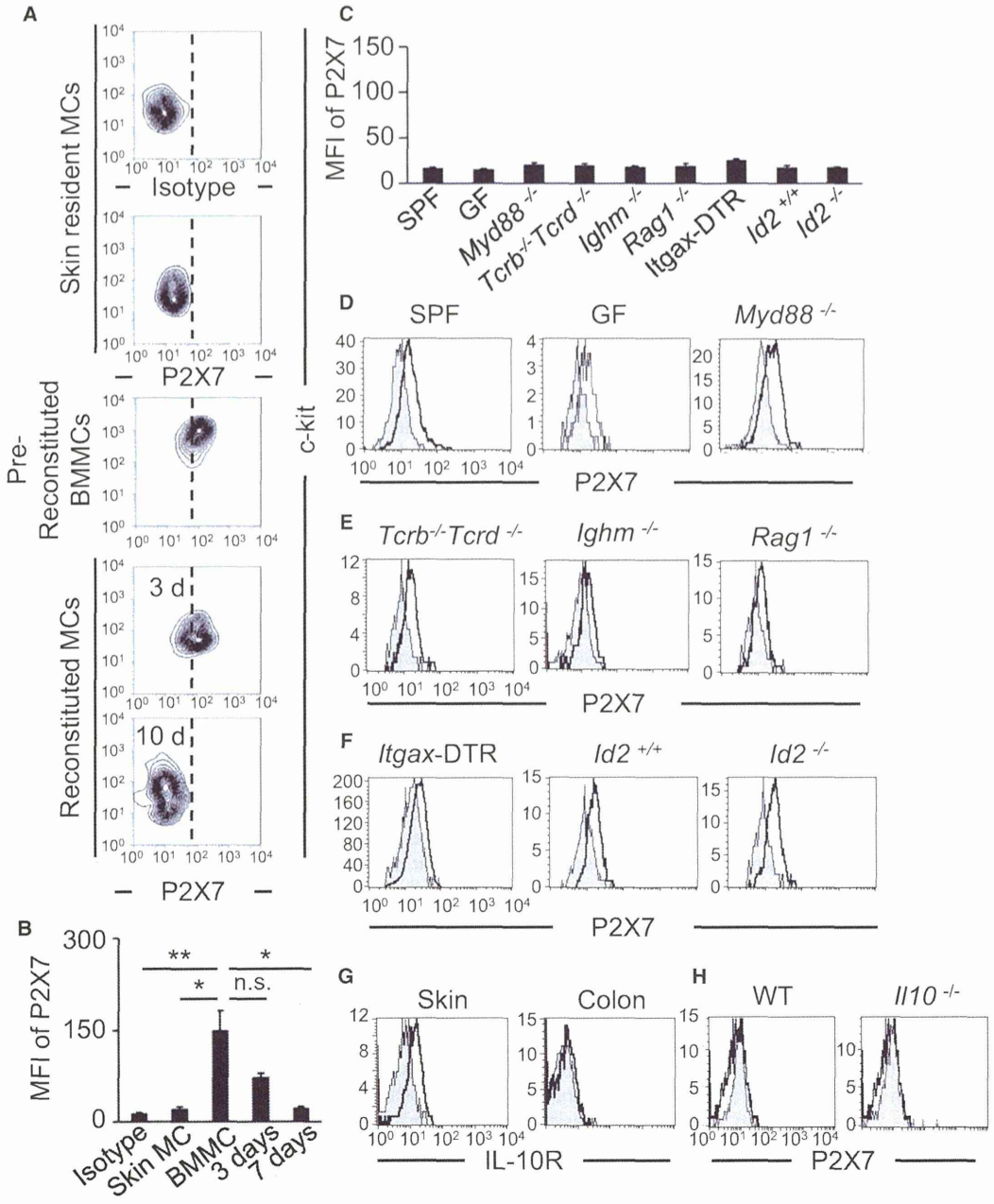

Figure 2. Skin Environment Regulates P2X7 Expression

(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.

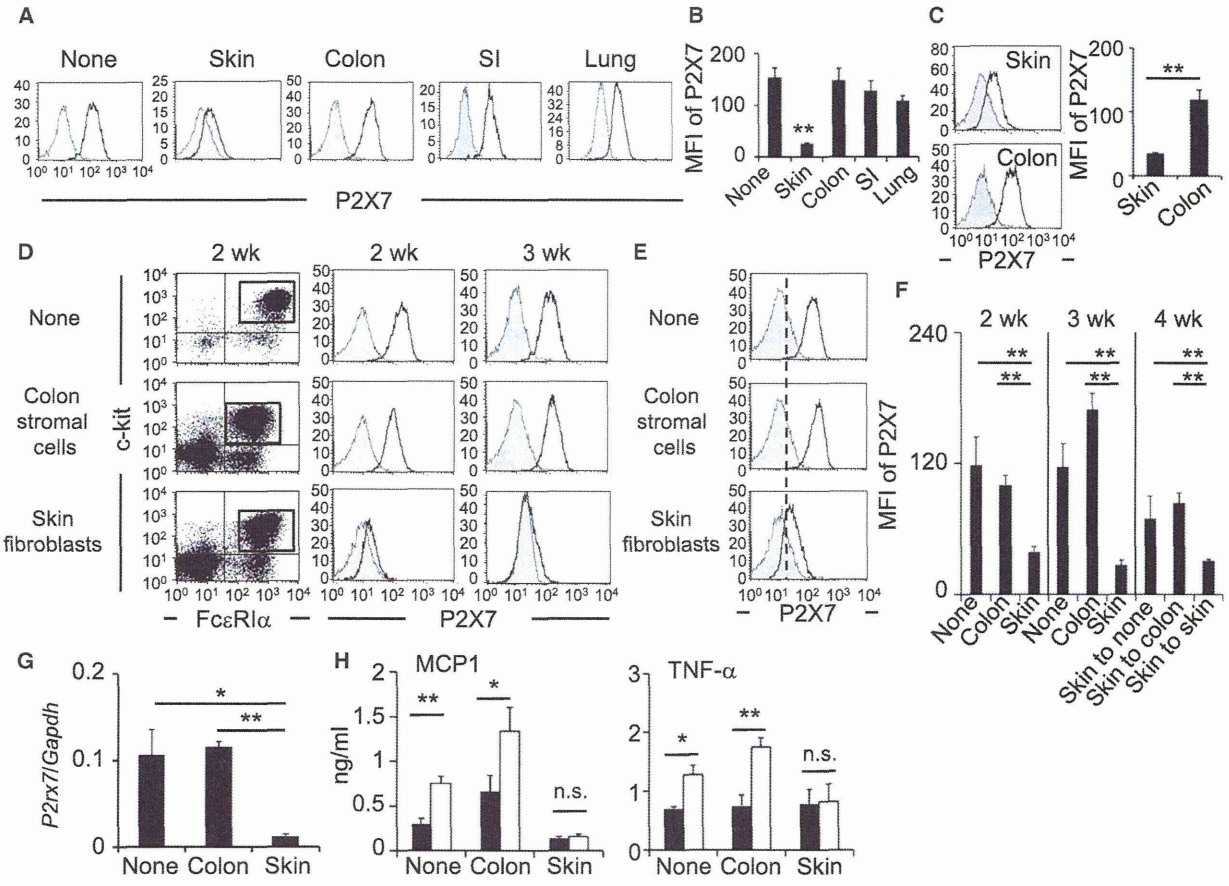

Figure 3. Skin Fibroblasts Regulate P2X7 Expression on Mast Cells

(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

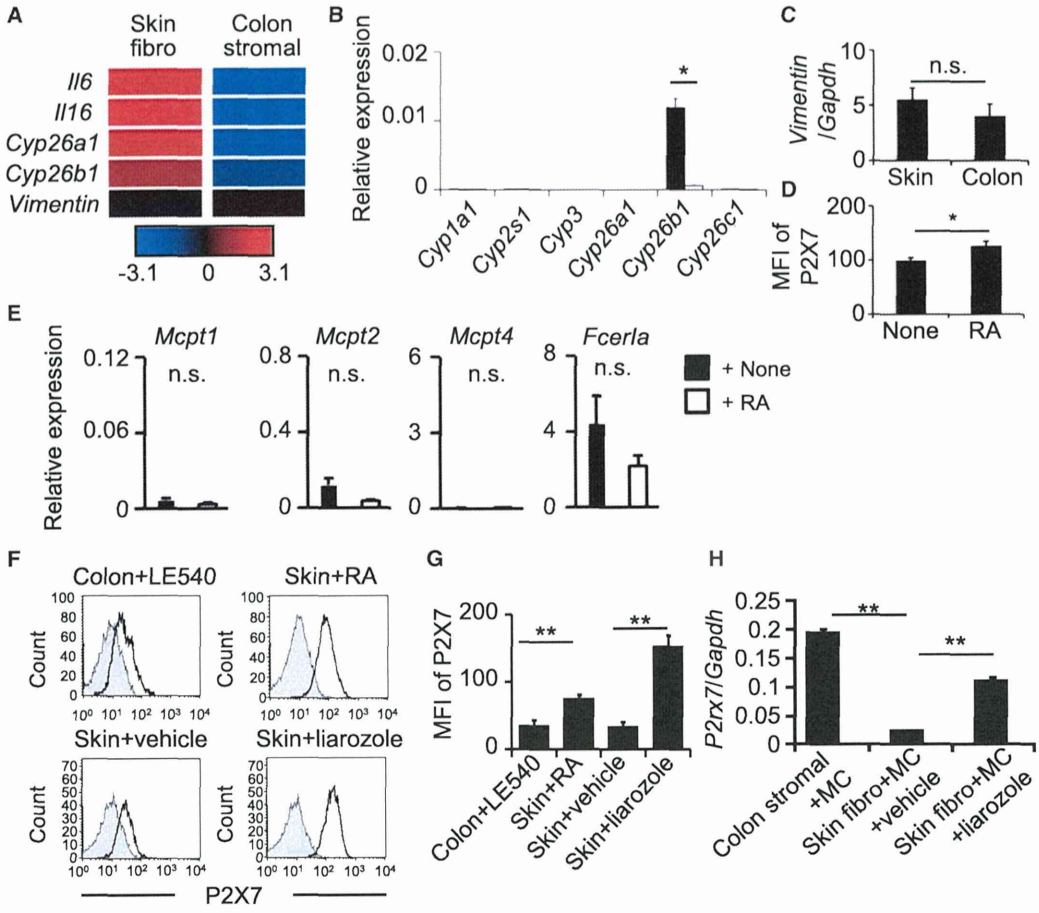

Figure 4. Critical Role of Cyp26b1 in Regulating P2X7 Expression on Mast Cells

(A) Gene microarray analysis was performed to compare the gene expression between skin fibroblasts and colon stromal cells; representative genes are shown.

(B) Gene expression of Cyp26 families was examined by quantitative RT-PCR. Gene expression relative to *Gapdh* is shown. Data are means ± SEM. *$p < 0.05$ ($n = 4$).

(C) Vimentin expression on skin fibroblasts and colon stromal cells was examined by quantitative RT-PCR (qRT-PCR). Relative expressions were normalized against *Gapdh*. Data are means ± SEM ($n = 4$). n.s., not significant.

(D and E) BMMCs were treated with 50 nM of retinoic acid (RA) for 6 days. The expression of P2X7 (D) and *Mcp1*, *Mcp2*, *Mcp4* and *Fcerla* (E) was then examined by flow cytometry and qRT-PCR, respectively. Data are means ± SEM ($n = 4$). *$p < 0.05$.

(F) BMMCs were cocultured with skin fibroblasts or colon stromal cells, with or without RA, LE540, or liarozole and stained for P2X7. Control staining with rat IgG2b is shown as gray. Data are representative of at least three independent experiments.

(G) MFI of P2X7 expression was shown ($n = 3$ to 6). Data are means ± SEM. **$p < 0.01$.

(H) *P2rx7* expression on cocultured BMMCs was examined by qRT-PCR. Data are means ± SEM ($n = 4$). **$p < 0.01$.

P2X7 expression (Heiss et al., 2008). Therefore, it is possible that Cyp26b1-mediated control of RA concentrations by skin fibroblasts regulates P2X7 expression on MCs. To test this hypothesis, we examined the P2X7 expression after adding RA to BMMCs. P2X7 expression increased in the presence of RA without affecting gene expression of *Mcpt1*, *Mcpt2*, and *Mcpt4* (Figures 4D and 4E). Similarly, adding RA to BMMCs in the presence of skin fibroblasts also increased the P2X7 expression (Figures 4F and 4G). Reciprocally, LE540, an inhibitor of retinoid X receptor and retinoid A receptor, suppressed the P2X7 expression on MCs cocultured with colon stromal cells (Figures 4F and 4G). In addition, in vitro treatment of skin fibroblasts with liarozole, a Cyp26b1 inhibitor, augmented P2X7 expression on MCs (Figures 4F–4H). These data demonstrate the critical roles of skin fibroblasts in modulation of P2X7 expression on MCs via the RA metabolic enzyme Cyp26b1.

Aberrant P2X7 Expression on MCs Induces Skin Inflammation, and Its Inhibition Ameliorates Disease Development

Retinoid and its metabolites play important roles in both pro- and anti-inflammatory responses (Fisher and Voorhees, 1996). Retinoids display key physiological roles in maintaining skin homeostasis, and dysregulation of retinoid signaling is found in various skin diseases. Indeed, topical treatment with retinoids causes epidermal hyperplasia and thickening of the differentiated