Receptor Mincle promotes skin allergies and is capable of recognizing cholesterol sulfate

Alexey V. Kostarnoy[a,1], Petya G. Gancheva[a], Bernd Lepenies[b], Amir I. Tukhvatulin[c], Alina S. Dzharullaeva[c], Nikita B. Polyakov[d,e], Daniil A. Grumov[d], Daria A. Egorova[a], Andrey Y. Kulibin[f], Maxim A. Bobrov[g], Ekaterina A. Malolina[f,h], Pavel A. Zykin[i], Andrey I. Soloviev[d], Evgeniy Riabenko[j,k], Diana V. Maltseva[l], Dmitry A. Sakharov[l], Alexander G. Tonevitsky[m], Lyudmila V. Verkhovskaya[n], Denis Y. Logunov[c], Boris S. Naroditsky[a], and Alexander L. Gintsburg[o]

[a]Laboratory of Immunobiotechnology, N. F. Gamaleya Federal Research Center of Epidemiology and Microbiology, 123098 Moscow, Russia; [b]Immunology Unit, Research Center for Emerging Infections and Zoonoses, University of Veterinary Medicine, 30559 Hannover, Germany; [c]Laboratory of Cellular Microbiology, N. F. Gamaleya Federal Research Center of Epidemiology and Microbiology, 123098 Moscow, Russia; [d]Laboratory of Detection and Ultrastructural Analysis of Microorganisms, N. F. Gamaleya Federal Research Center of Epidemiology and Microbiology, 123098 Moscow, Russia; [e]Laboratory of Instrumental Methods and Organic Reagents, Vernadsky Institute of Geochemistry and Analytical Chemistry, Russian Academy of Sciences, 119991 Moscow, Russia; [f]Laboratory of Evolutionary Developmental Biology, Koltzov Institute of Developmental Biology, Russian Academy of Sciences, 119071 Moscow, Russia; [g]Department of Pathology, M. F. Vladimirsky Moscow Regional Clinical Research Institute, 129110 Moscow, Russia; [h]Laboratory of Cell Engineering, Ivanovsky Institute of Virology, N. F. Gamaleya Federal Research Center of Epidemiology and Microbiology, 123098 Moscow, Russia; [i]Department of Cytology and Histology, St. Petersburg State University, 199034 St. Petersburg, Russia; [j]Department of Control and Applied Mathematics, Moscow Institute of Physics and Technology, 141700 Dolgoprudny, Russia; [k]Faculty of Computer Science, Big Data and Information Retrieval School, National Research University Higher School of Economics, Moscow 101000, Russia; [l]Scientific Research Centre Bioclinicum, 115088 Moscow, Russia; [m]Department of Translational Oncology, P. Hertsen Moscow Oncology Research Institute, National Center of Medical Radiological Research, Moscow 125284, Russia; [n]Laboratory of Molecular Biotechnology, N. F. Gamaleya Federal Research Center of Epidemiology and Microbiology, 123098 Moscow, Russia; and [o]Laboratory of Gene Engineering of Pathogenic Microorganisms, N. F. Gamaleya Federal Research Center of Epidemiology and Microbiology, 123098 Moscow, Russia

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Sterile (noninfected) inflammation underlies the pathogenesis of many widespread diseases, such as allergies and autoimmune diseases. The evolutionarily conserved innate immune system is considered to play a key role in tissue injury recognition and the subsequent development of sterile inflammation; however, the underlying molecular mechanisms are not yet completely understood. Here, we show that cholesterol sulfate, a molecule present in relatively high concentrations in the epithelial layer of barrier tissues, is selectively recognized by Mincle (Clec4e), a C-type lectin receptor of the innate immune system that is strongly up-regulated in response to skin damage. Mincle activation by cholesterol sulfate causes the secretion of a range of proinflammatory mediators, and s.c. injection of cholesterol sulfate results in a Mincle-mediated induction of a severe local inflammatory response. In addition, our study reveals a role of Mincle as a driving component in the pathogenesis of allergic skin inflammation. In a well-established model of allergic contact dermatitis, the absence of Mincle leads to a significant suppression of the magnitude of the skin inflammatory response as assessed by changes in ear thickness, myeloid cell infiltration, and cytokine and chemokine secretion. Taken together, our results provide a deeper understanding of the fundamental mechanisms underlying sterile inflammation.

innate immunity | sterile inflammation | allergy | Mincle | cholesterol sulfate

The recognition of tissue injury and the subsequent responses leading to (*i*) the neutralization and elimination of the agent causing the damage and (*ii*) the repair of damaged tissues are essential challenges faced by multicellular organisms. In higher organisms, injuries and infections induce inflammatory responses. Inflammation plays roles in the localization and elimination of injurious agents, the removal of damaged tissues, and the initiation of the repair process. Evolutionarily conserved pattern recognition receptors (PRRs) of the innate immune system are capable of recognizing molecules associated with pathogens (pathogen-associated molecular patterns, or PAMPs) and endogenous molecules released from stressed or damaged cells in response to injury (damage-associated molecular patterns, DAMPs) (1). Recognizing the ligands of PRRs of either exogenous or endogenous origin subsequently induces an inflammatory response. Numerous DAMPs have been proposed (1, 2). However, the ability of many of these compounds to induce sterile inflammation is still under debate because some of them display activities at nonphysiological concentrations, and others are present in high abundance as components of the extracellular matrix (3, 4).

Posttraumatic inflammation is the first necessary step of wound healing (5). Nevertheless, a prolonged sterile (noninfected) inflammatory response underlies the pathogenesis of a multitude of widespread diseases, such as allergic diseases, autoimmune diseases, atherosclerosis, and cancer. Although progress has been made in identifying potential DAMPs, the molecular mechanisms underlying the induction of sterile inflammation are still not fully understood. This knowledge gap is well illustrated by

Significance

Post-traumatic sterile inflammation is the first necessary step of wound healing. In addition, sterile inflammation underlies the pathogenesis of a multitude of common diseases, such as allergies and autoimmune diseases. The molecular mechanisms underlying sterile inflammation are still not fully understood. Here, we show that the receptor Mincle (Clec4e), the expression of which is highly induced in the skin in response to damage, recognizes cholesterol sulfate, a molecule that is abundant in the epidermal layer of the skin, subsequently inducing a pro-inflammatory response. We also identify a role for Mincle as a driving component in the pathogenesis of allergic skin inflammation. The results demonstrate a previously unconsidered important role of Mincle in mediating sterile inflammation.

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[1]To whom correspondence should be addressed. Email: kostarnoy@yandex.ru.

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the fact that for a number of the widespread diseases associated with acute or chronic sterile inflammation, endogenous molecular inductors of the inflammatory process and their receptors remain to be fully elucidated, and there are still either no or very limited effective therapies available for these diseases.

In the present study, we initially investigated changes in the gene expression profile of PRRs after skin injury in a mouse model. We determined that, among the different PRRs, the receptor Mincle was the most inducible in response to skin trauma. Mincle (also called Clec4e or Clecsf9) is a type-II transmembrane C-type lectin receptor that recognizes mycobacteria and pathogenic fungi. PAMPs recognized by Mincle are predominantly amphiphilic lipids, such as trehalose-6,6′-dimycolate (TDM) from *Mycobacterium tuberculosis* and glyceroglycolipids from *Malassezia* fungi (6). Mincle also recognizes endogenous spliceosome-associated protein 130 (SAP130), which is a constitutively expressed nuclear protein and a subunit of the histone deacetylase complex (7). The ligation of Mincle by its ligands leads to the interaction of Mincle with the adaptor protein Fc receptor γ-chain (FcRγ) and the recruitment of spleen tyrosine kinase (Syk) (7, 8). The signal is subsequently transmitted to NF-κB via the CARD9-BCL10-MALT1 pathway, leading to the production of a spectrum of proinflammatory cytokines (6, 8, 9).

We determined that after skin wounding or after a single topical application of the irritant and sensitizer 2,4-dinitrofluorobenzene (DNFB), the early secretion of proinflammatory mediators in the absence of Mincle was significantly impaired. Assuming that Mincle may be an important sensor of tissue damage, we performed a search for new endogenous ligands of this receptor. Using chromatography and mass spectrometry, we found that cholesterol sulfate, an abundant molecule in the epithelial barrier layers in the skin and gastrointestinal and respiratory tracts (10–12), is selectively recognized by Mincle. Here, we show that Mincle activation by cholesterol sulfate causes the secretion of a range of proinflammatory mediators and that s.c. injection of cholesterol sulfate results in a Mincle-mediated induction of a severe local inflammatory response.

In the study, we identified a role of Mincle in the development of the pathologic process of cutaneous allergic inflammation. In a mouse model of allergic contact dermatitis, contact hypersensitivity (CHS) was induced via the topical application of the DNFB. Using Mincle receptor-knockout (Mincle-KO) mice, we observed that the absence of Mincle leads to a strong suppression of skin inflammation as reflected by changes in swelling, myeloid cell infiltration, and cytokine and chemokine secretion.

Collectively, these findings demonstrate an important role of the receptor Mincle in mediating sterile (noninfected) skin inflammation and provide a deeper understanding of the fundamental mechanisms underlying sterile inflammation.

Results

Mincle Is a PRR in the Skin That Is Highly Inducible in Response to Trauma and Mediates the Early Inflammatory Response. We first assessed changes in the gene expression profiles of the receptors involved in innate responses to skin injuries in mice. We modeled excisional cutaneous wounds using a punch biopsy instrument. Wounds were made through the epidermis, dermis, and s.c. tissue layers, leaving the fascia intact. Skin tissue samples containing the wounds were removed 24 h and 4 d after injury, and intact skin was used as a control. RNA was extracted from samples, and a whole-genome analysis was performed using an Affymetrix microarray platform (Agilent Technologies). The results revealed that skin injury induced an increase in the expression of a number of PRR genes belonging to different families of innate immunity receptors, such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), NOD-like receptors (NLRs), and RIG-I–like receptors (RLRs). The fold-changes in the transcript levels of innate immune PRR genes in wounded skin relative to levels in intact skin are presented in Fig. 1*A* (the complete dataset has been deposited in the GEO database under accession no. GSE76144). Interestingly, one of the genes of the CLR family, *Clec4e* (*Mincle*), was extremely inducible after wounding in comparison with the changes in the other innate immune receptors. Another gene of the C-type lectin receptor family, *Clec4d* (*MCL*), which is located in the CLR cluster next to *Clec4e* and is considered to have emerged via a gene duplication of *Clec4e* (13), was the second most inducible gene at both time points among the innate immune system receptor genes.

To investigate changes in the expression of Clec4e (Mincle) upon skin injury at the protein level, we compared Mincle expression in damaged skin tissue at different stages of the wound repair process using Western blotting. In accordance with the gene array data, the expression of the Mincle protein increased quickly after injury and had decreased to baseline levels by 12 d after wounding (Fig. 1*B*). It is noteworthy that the rapid induction of Mincle expression was observed not only after the physical injury of the skin but also in response to the single topical application of the irritant and sensitizer DNFB onto the abdomen skin of mice (Fig. 1*C*). Immunohistochemical studies revealed a population of Mincle-expressing cells in the dermis that had mononuclear histiocyte-like morphological characteristics and that appeared in the damaged skin as an early response to wounding or DNFB application (Fig. 1*D*). Only a small number of weakly stained Mincle-expressing cells can be found in intact skin. Double immunofluorescent staining performed 2 h after DNFB application revealed that the Mincle-expressing cells do not express Langerin or CD11c in detectable quantities. However, these cells possess phenotype markers of plasmacytoid dendritic cells (pDC), such as BST2 (PDCA-1) and CD45R (B220) (Fig. 1*E*). Taken together, these findings indicate that Mincle is a receptor that is highly inducible in response to skin tissue damage.

To evaluate the role of Mincle in early inflammatory response, we measured concentration of proinflammatory mediators in skin at the early time point (12 h) after trauma using Mincle-deficient mice and wild-type mice. We determined that, after skin wounding or after a single topical application of DNFB, the early secretion of proinflammatory mediators in the absence of Mincle was significantly impaired (Fig. 1*F*). However, the absence of Mincle did not influence the dynamics of wound closure (Fig. S1).

The findings are consistent with those of recently reported studies that have shown increased Mincle expression in the brains of humans and rodents after brain injury (14, 15). These data, as well as the data presented here, allowed us to hypothesize that the Mincle may be an important sensor of nonphysiological cell death or cell stress that recognizes endogenous molecules released from damaged tissues and subsequently induces inflammation. The ribosomal protein SAP 130 has been reported to be an endogenous ligand for Mincle and to induce inflammation in vivo (7). We hypothesized that unknown endogenous lipid ligands for the Mincle receptor exist because the molecules of bacterial and fungal origin recognized by Mincle are amphiphilic lipids.

Isolation and Identification of Endogenous Mincle Ligands. To evaluate whether Mincle recognizes endogenous lipids, we isolated the total lipid extract from mouse skin and tested its ability to induce the Mincle-dependent transcriptional activation of NF-κB in an NF-κB–driven luciferase reporter assay using a previously developed HEK293–mMincle–NF-κB–Luc cell line. As shown in Fig. 2*A*, the total lipid extract exhibited dose-dependent Mincle-stimulating activity. Next we performed a preliminary separation of the total lipid extract using a solid-phase extraction procedure. Using a reversed-phase cartridge, we found that the active compounds could be eluted from the cartridge almost without loss in their activating effect on Mincle (Fig. 2*A*). The chromatographic retention behavior suggested that the active components of the total lipid extract were amphiphilic molecules with both hydrophilic and hydrophobic

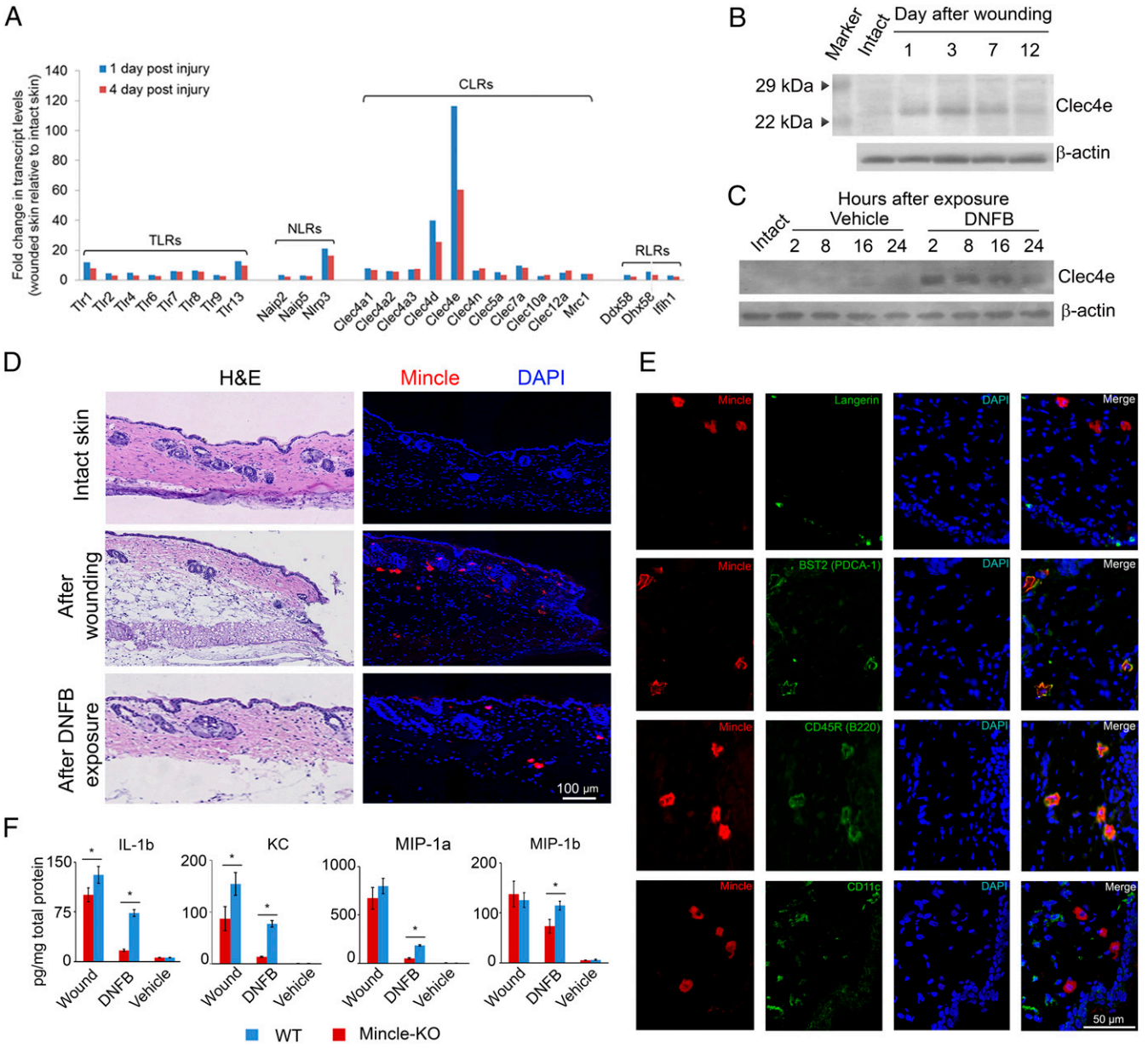

Fig. 1. Mincle (Clec4e) is a PRR in the skin that is highly inducible in response to trauma and mediates the early inflammatory response. (*A*) Whole-genome microarray analysis of the changes in gene expression of innate immune PRR genes in murine skin in response to excisional injury. The genes are grouped by families: TLRs, NLRs, CLRs, and RLRs. Fold changes in transcript levels in wounded skin relative to levels in intact skin are presented. PRR genes with absolute fold changes >3.0 at at least one time point and FDR-adjusted *P* values below 0.05 (Benjamini–Hochberg procedure) are shown. (*B*) Time course of changes in Mincle protein levels in wounded murine skin were evaluated via Western blotting. (*C*) Time course of changes in Mincle protein levels in murine skin after the single topical application of 25 μL of 0.5% DNFB compared with the Mincle protein levels in the skin after vehicle application was evaluated via Western blotting. (*D*) Evaluation of Mincle expression in intact skin and in skin after wounding or single exposure of DNFB via immunostaining. The time point 2 h after skin wounding or DNFB exposure is shown. Shown are representative results obtained from skin sections of at least six animals in each group. (*E*) Fluorescent double staining of Mincle and dendritic cell markers in murine skin 2 h after a single exposure to 25 μL of 0.5% DNFB. Representative images are shown. (*F*) The secretion of proinflammatory mediators is significantly impaired in the absence of Mincle after skin wounding and after a single topical application of 25 μL of 0.5% DNFB. The level of the mediators was measured in the skin 12 h after wounding or DNFB or vehicle exposure. $*P < 0.05$ versus wild type (WT) ($n = 5$ mice per group; representative results from one of three independent experiments are shown). Mean values ± SD are shown.

fragments. Using electrospray ionization mass spectrometry, we found, perhaps expectedly, amphiphilic lipids in the eluate, particularly, cholesterol sulfate and phosphoinositols (Fig. S2). The extract obtained from the reversed-phase cartridge was further separated via HPLC in gradient separation mode using a reversed-phase monolithic column. Fractions were collected, and each fraction was evaluated using the reporter HEK293–mMincle–NF-κB–Luc cell line. The highest activity was detected in fraction 7 (Fig. 2*B*). One of the most intense signals in the mass spectrum (negative ion detection mode) of fraction 7 corresponded to a peak with an *m/z* of 465.31 (Fig. 2*C*). To elucidate the structure of this compound, this ion was isolated and subjected to fragmentation. Along with a peak corresponding to the parent ion in the tandem mass spectrum, only two product ion peaks were detected ($m/z = 79.97$ and $m/z = 96.98$, Fig. 2*C*). This finding suggests the presence of a sulfo-group in this compound and that the peak with an *m/z* of 465.31 corresponds to a

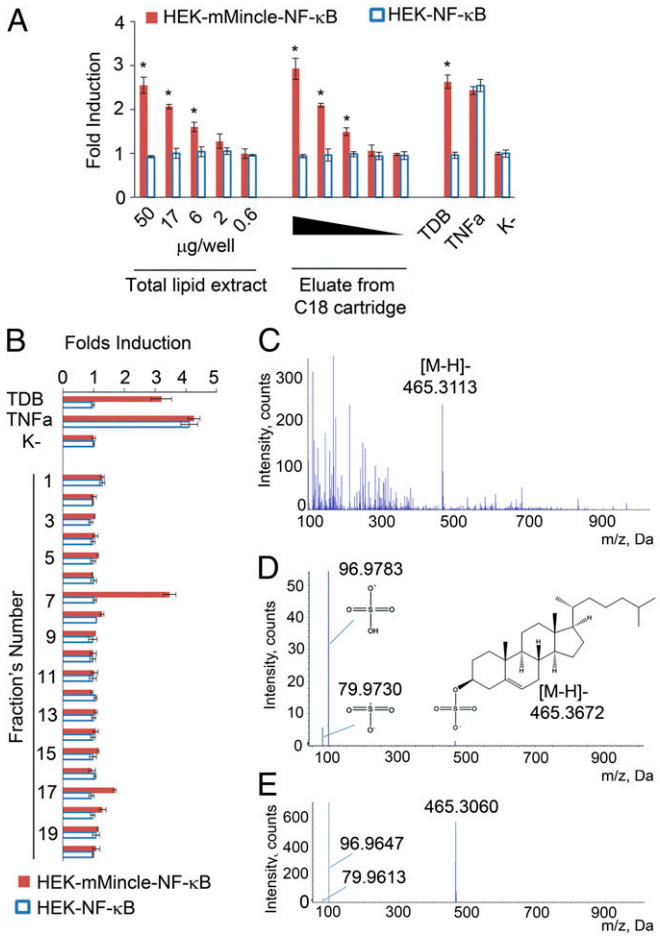

Fig. 2. Identification of a Mincle ligand of endogenous origin. (*A*) Serial dilutions of isolated total lipid extracts from murine skin samples or eluates from solid-phase extractions (reversed-phased C18 cartridge) were used to stimulate Mincle-expressing (HEK293–mMincle–NF-κB–Luc) or control (HEK293–NF-κB–Luc) reporter cell lines. TDB (2 μg/mL) and TNFa (100 ng/mL) were used as controls. Luciferase activity was measured and expressed as fold induction relative to the unstimulated control (K–). Mean values ± SD for triplicate measurements are shown. *$P < 0.05$ vs. control reporter cell line. Representative results from five independent experiments are shown. (*B*) Evaluation of the Mincle-mediated activity of fractions obtained via HPLC separation of eluates from solid-phase extractions using a Mincle-expressing reporter cell line. TDB (2 μg/mL) and TNFa (100 ng/mL) were used as controls. Luciferase activity was measured and expressed as fold induction relative to the unstimulated control (K–). Mean values ± SD for triplicate measurements are shown. Representative results from three independent experiments are shown. (*C*) Mass spectrum of fraction 7 in negative ion detection mode. (*D*) Fragmentation spectrum in negative ion mode of the ion with a *m/z* of 465.3113 from *C*. The proposed chemical structures of the parent ion and product ions are shown. (*E*) Fragmentation spectrum in negative ion mode of the synthetic cholesterol sulfate.

deprotonated molecular ion [M-H]–. According to the LIPID MAPS database and MS-LAMP software, the compound was identified as a cholesterol sulfate. To confirm the identification, synthetic cholesterol sulfate was used as a standard. Its fragmentation spectrum completely matched the fragmentation spectrum of the ion from fraction 7 that had a peak with an *m/z* of 465.31 (Fig. 2*E*). Taken together, these data show that the main active component of fraction 7 was cholesterol sulfate.

Mincle Recognizes Cholesterol Sulfate Through Direct Binding and Induces a Mincle-Dependent Proinflammatory Response Both in Vitro and in Vivo. First we performed surface plasmon resonance experiments to study the ability of cholesterol sulfate to bind with Mincle using highly purified synthetic cholesterol sulfate. An anti-histidine antibody was first covalently immobilized onto the sensor chip surface, and the histidine-tagged recombinant Mincle protein was then injected and captured by the immobilized anti-histidine antibody, after which the analytes were injected. To evaluate the specificity of the interaction, we used a negative control, synthetic 18:0–20:4 phosphoinositol. The lipid was coeluted from a reversed-phase cartridge together with cholesterol sulfate during a solid-phase extraction of skin lipids (Fig. S2), and it shared some physicochemical properties with cholesterol sulfate. We observed the direct binding of Mincle to cholesterol sulfate but not to phosphoinositol. The obtained sensorgrams are presented in Fig. 3*A*.

Next, we evaluated the ability of cholesterol sulfate to induce Mincle-dependent signaling using the reporter HEK293–mMincle–NF-κB–Luc cell line. The reporter cells showed a similar response to both cholesterol sulfate and trehalose dibehenate, an archetypical ligand of Mincle (Fig. 3*B*). Thus, this finding indicates that cholesterol sulfate binds to Mincle and induces Mincle-mediated signaling.

To determine the specificity of cholesterol sulfate signaling through Mincle under more physiologically realistic conditions and to study the relevance of the cholesterol sulfate/Mincle interaction in vivo, we used Mincle-deficient mice. To compare the ability of cholesterol sulfate and trehalose dibehenate to induce cytokine and chemokine secretion, bone marrow-derived dendritic cells (BMDCs) were isolated from Mincle-KO mice and wild-type mice and stimulated by these lipids. The levels of cytokines and chemokines in culture supernatants were measured using a multiplex immunoassay. Both cholesterol sulfate and trehalose dibehenate induced the secretion of proinflammatory mediators, such as IL-1a and IL-1b, KC, and MIP-1a and MIP-1b (Fig. 3*C*). The secretion of these proinflammatory mediators in response to both cholesterol sulfate and trehalose dibehenate was significantly reduced in BMDCs from Mincle-deficient mice, whereas the response to bacterial lipopolysaccharide (an agonist of TLR4) remained unchanged (Fig. 3*C*).

To evaluate the tissue response to cholesterol sulfate and the contribution of Mincle to this process, we subcutaneously injected a sterile aqueous solution containing micelles of cholesterol sulfate into either Mincle-deficient or wild-type mice. Sterols tend to form crystals in solution, and these crystals are potent activators of inflammatory responses (16, 17). To avoid the injection of a crystal-containing suspension, we previously developed a method using an ultrasonic treatment to produce a stable micelle solution with an average micelle diameter from 70 to 90 nm (Fig. S3). After 24 h, skin samples were excised and the local tissue response was evaluated based on a histological analysis of hematoxylin and eosin (H&E)-stained sections. The histological examination revealed a marked inflammatory infiltrate in the skin of wild-type animals injected with cholesterol sulfate, whereas, in Mincle-deficient animals, the accumulation of infiltrating cells was significantly reduced (Fig. 3*D*). The infiltrating cells were primarily neutrophils, monocytes, and eosinophils. Taken together, our findings suggest that a Mincle deficiency results in a reduced inflammatory response to cholesterol sulfate both in vitro and in vivo.

Mincle Promotes Cutaneous Allergic Inflammation. Taking into account (*i*) the significant inducibility of Mincle expression in skin to the topical skin irritation caused by DNFB, a well-known inductor of allergic skin reactions, and (*ii*) the significantly impaired secretion of proinflammatory mediators in response to a single DNFB application in Mincle-deficient animals, we examined the role of Mincle in the pathogenesis of cutaneous allergic inflammation. To address the role of Mincle in the development of cutaneous allergic inflammation, we used one of the most commonly used models of contact hypersensitivity (CHS) that adequately reflect allergic contact dermatitis (18). Mincle-KO

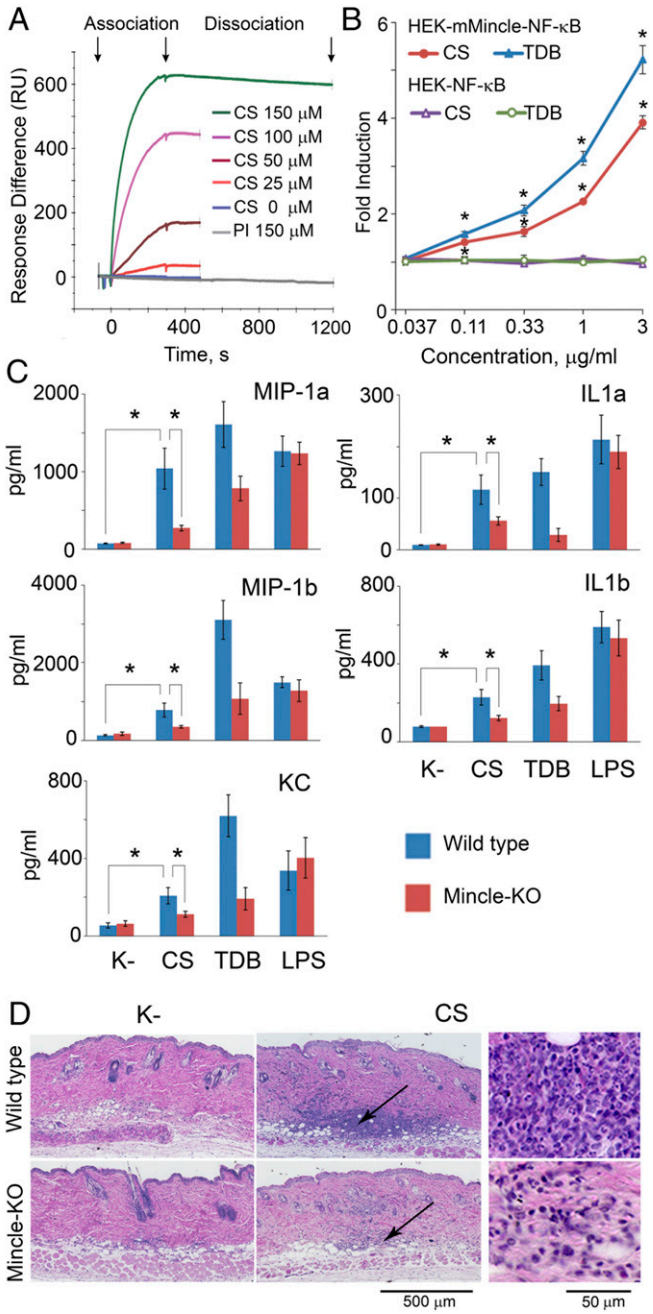

Fig. 3. Cholesterol sulfate directly binds to Mincle and induces Mincle-mediated proinflammatory signaling in vitro and in vivo. (*A*) Surface plasmon resonance analysis of the direct binding of Mincle to cholesterol sulfate. To verify binding specificity, 18:0–20:4 phosphoinositol was used. Shown are double-referenced sensorgrams (blank surface and blank buffer referencing) pertaining to the indicated concentrations of analytes. (*B*) Dose-dependent ability of cholesterol sulfate and TDB to activate Mincle-expressing reporter cells. Luciferase activity was measured and expressed as fold induction relative to the unstimulated control. Mean values ± SD for triplicate measurements are shown. *$P < 0.05$ vs. activations in the control reporter cell line. Representative results from five independent experiments are shown. (*C*) Cholesterol sulfate induces the secretion of proinflammatory mediators in a Mincle-dependent manner. BMDCs isolated from Mincle-KO mice or wild-type mice were stimulated with cholesterol sulfate (10 μg/mL). TDB (10 μg/mL) and LPS (10 μg/mL) were used as controls. Mean values ± SD for triplicate measurements are shown. *$P < 0.05$. Representative results from two independent experiments are shown. (*D*) Skin inflammatory response to a s.c. injection of cholesterol sulfate is mediated by Mincle. (*Right*) Higher magnification of areas indicated with arrows. Representative microphotographs of H&E-stained sections from one of three independent experiments are shown.

and wild-type mice were sensitized to DNFB on their abdomens, followed by challenges with the same sensitizer on the dorsum of both ears. The schedule for the mouse sensitization and challenge procedure is depicted in Fig. 4*A*. Endpoint parameters were measured 24 h after the last DNFB exposure. Under the induction protocol, Mincle-deficient mice exhibited significantly suppressed clinical symptoms of allergic contact dermatitis, such as redness and ear swelling, compared with the wild-type animals (Fig. 4 *B* and *C*). A careful review of H&E-stained stained sections of contact-allergic ear tissue at the experiment endpoint revealed a strongly reduced inflammatory response in Mincle-deficient mice in terms of ear thickness, epidermal acanthosis, and inflammatory cell infiltrate (Fig. 4*D*). A quantitative analysis using flow cytometry confirmed that there were significantly fewer infiltrating Gr1+/CD11b+ myeloid cells in the ear tissue of Mincle-deficient mice compared with wild-type control mice (Fig. 4*E*). To evaluate the differences in allergic skin inflammation at the molecular level, we measured the production of cytokines and chemokines in inflamed ear tissues. We found that a Mincle deficiency led to the suppressed production of a spectrum of mediators of inflammation (Fig. 4*F*), including proinflammatory cytokines IL-1b, TNFa, and IL12p40, as well as chemokines KC, MIP-1a, and MIP-1b. Interestingly, the production of IL-17A was also significantly suppressed in the inflamed tissues of Mincle-deficient mice compared with IL-17A's production in wild-type animals (Fig. 4*F*). Taken together, these observations demonstrate that Mincle strongly enhances the magnitude of the skin inflammatory CHS response and plays a role as a driving component in the pathogenesis of allergic skin inflammation.

Discussion

There is growing evidence that the PRRs of the innate immune system play a key role in the induction of rapid inflammation in response to infection and tissue trauma (3, 19, 20). The description of PRRs as a limited number of evolutionarily conserved receptors that recognize molecular patterns of infectious agents, the so-called PAMPs, was first developed by Janeway (21, 22). Initially, PRRs were considered as a first line of host defense during an infection, functioning in discriminating between self and nonself. Later, Matzinger proposed the existence of host molecules (DAMPs) that are released or secreted in response to tissue damage and can initiate and perpetuate an immune response (23, 24). Subsequently, it was proven that one of the functions of PRRs is the sensing of tissue damage, and many DAMPs had been identified currently (1–3, 19). Recently, Medzhitov et al. proposed that allergic reactions are a component of the host defense system against noninfectious noxious environmental factors, including venoms, noxious xenobiotics, and irritants that can induce tissue damage (25). However, despite extensive research in recent years, our understanding of the molecular mechanisms underlying the physiological inflammatory response to trauma and pathological sterile inflammation remains incomplete.

This study shows that the pattern recognition receptor Mincle (Clec4e), which is highly inducible in skin in response to injury and irritation, (*i*) recognizes cholesterol sulfate and subsequently induces a proinflammatory response and (*ii*) plays a role as a driving component in the pathogenesis of allergic skin inflammation.

Previously, it was reported that sterols are able to cause inflammation due to their interaction with the innate immune system, and some mechanisms for this effect were proposed (16, 17). The ability of cholesterol to bind to Mincle has recently been shown; however, the ability of cholesterol to cause Mincle-mediated inflammation in vivo was not demonstrated (26). Here, we show that cholesterol sulfate is an endogenous ligand for Mincle, and we studied the inflammatory response induced by the cholesterol sulfate/Mincle interaction in detail both in vitro and in vivo. Mincle recognizes cholesterol sulfate through direct binding and subsequently induces the secretion of a range of proinflammatory

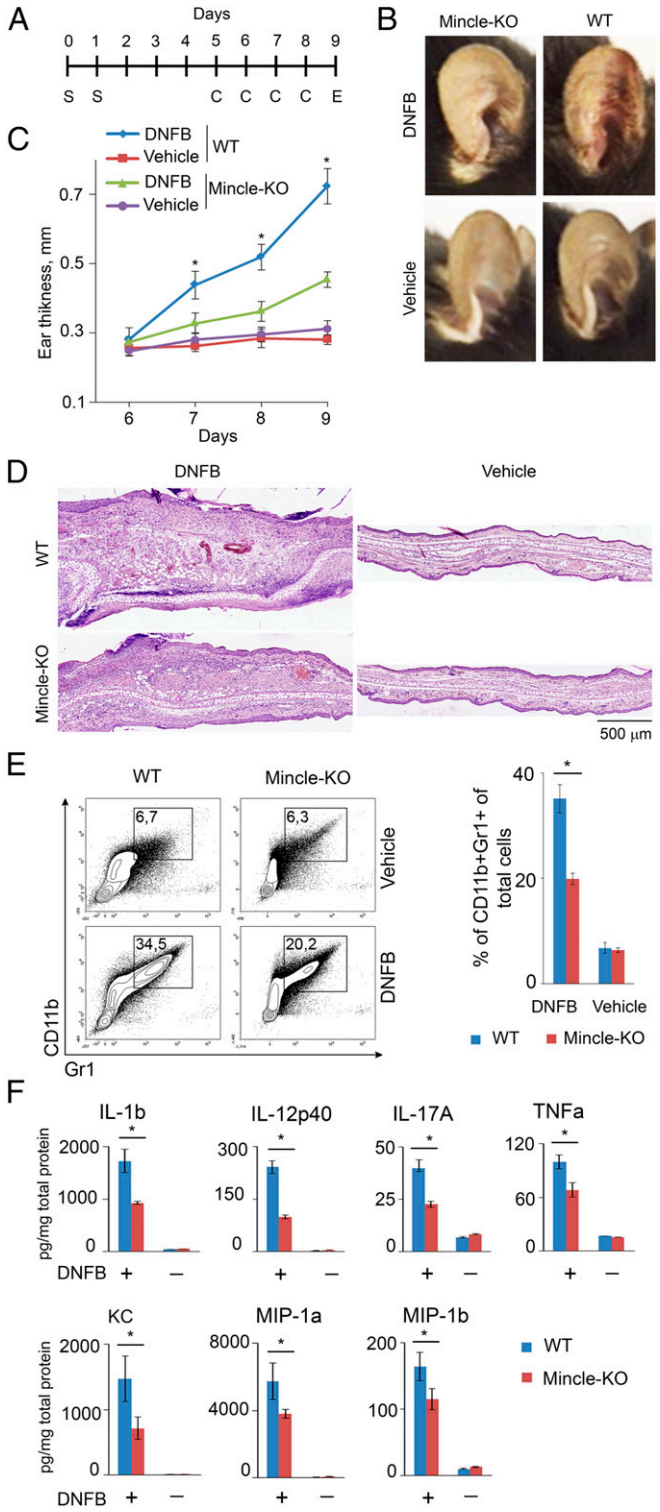

Fig. 4. Mincle promotes allergic skin inflammation in a model of allergic contact dermatitis. Mincle-KO and wild-type mice were sensitized to DNFB on their abdomens, followed by challenges with the same sensitizer on the dorsum of both ears. Endpoint parameters were measured 24 h after the last DNFB exposure. As a negative control, two other groups of Mincle-KO and wild-type mice were exposed to the vehicle without DNFB throughout the duration of experiment. *$P < 0.05$ versus WT ($n = 6$ mice per group; combined data of three independent experiments). Mean values ± SD are shown. (*A*) The schedule for mouse sensitization and challenges is shown. C, challenge; E, experimental endpoint; S, sensitization. (*B*) Dynamics of changes in ear thickness. (*C*) Representative photographs of mouse ears at the experimental endpoint are shown. The photographs illustrate decreases in certain clinical symptoms of allergic contact dermatitis, such as ear swelling and redness, in Mincle-KO mice. (*D*) Representative H&E-stained sections of ear tissue in wild-type and Mincle-KO mice at the experimental endpoint are shown. (*E*) Recruitment of Gr1+/CD11b+ myeloid immune cells in ear tissue at the experimental endpoint as assessed via flow cytometry (*Left*) and presented as percentages of cells (*Right*). (*F*) Analysis of cytokine and chemokine production in mouse ear tissue at the experimental endpoint.

mediators, such as IL-1a, IL-1b, KC, MIP-1a, and MIP-1b. In the in vivo experiments, the s.c. injection of cholesterol sulfate resulted in a Mincle-mediated induction of a severe local inflammatory response. The effects were significantly reduced when Mincle signaling was absent. However, some residual proinflammatory activity of cholesterol sulfate in the absence of Mincle was detected, suggesting the presence of compensatory mechanisms. It is possible that another CLR, MCL (also known as Clec4d or Dectin-3), is involved in the compensatory mechanisms. Mincle and MCL are homologous, and they are located next to each other in the genome, suggesting that MCL may have originated from a gene duplication of Mincle (13). Both MCL and Mincle recognize TDM and use the same FcRγ-Syk-CARD9 pathway (13, 27). We revealed that MCL and Mincle are extremely inducible in skin after trauma (Fig. 1*A*). Using the reporter HEK293–mMCL–NF-κB cell line, we found that cholesterol sulfate is able to induce MCL-mediated signaling (Fig. S4). However, additional studies are required to investigate the consequences of the interaction between cholesterol sulfate and MCL in more detail.

The post-traumatic inflammatory response is generally considered a first step in tissue repair (5). However, the signaling through Mincle induced by endogenous stimuli may also underlie the pathogenesis of certain diseases associated with sterile chronic inflammation. Recently published results indicate that signaling through Mincle plays a role in obesity-induced adipose tissue inflammation and the subsequent adipose tissue fibrosis (28). Recently, an essential role of Mincle signaling through the Syk/Card9 axis in the development of autoimmune eye inflammation in a model of experimental autoimmune uveoretinitis was revealed (29). It was shown that Mincle signaling promotes pancreatic oncogenesis, whereas the deletion of Mincle protects against oncogenesis (30). Interestingly, T cells, which are not protective against the progression of pancreatic ductal adenocarcinoma in mice with intact Mincle signaling, are reprogrammed into indispensable mediators of antitumor immunity in the absence of Mincle (30).

Here, we show the driving role of Mincle in the development of cutaneous allergic inflammation. Using a classical model of allergic contact dermatitis, we show that Mincle deficiency strongly suppresses the magnitude of the skin inflammatory CHS response, as reflected by changes in ear swelling, myeloid cell infiltration, and cytokine and chemokine production in inflamed tissues.

It was previously shown that the application of contact allergens leads to the activation of mechanisms related to innate immunity through signaling pathways that are also involved in antiinfectious immunity (31). Interestingly, it has been demonstrated that the strength of primary skin inflammation in response to contact allergens determines the strength of the subsequent CHS response and whether a response or tolerance develops (31–34). Expression of Mincle is rapidly induced in skin as a response to wounding or a DNFB single application in the cells that bear specific pDCs markers [CD45R (B220) and, importantly, BST2 (PDCA-1), which is considered to be a unique marker of pDCs]. It was previously demonstrated that pDCs are resident dendritic cells that normally occur in the dermis only rarely but can accumulate in lesioned skin (35). We have shown that the early secretion of proinflammatory mediators in response to wounding or to a DNFB single application

in the absence of Mincle was significantly impaired. Mincle uses the Syk/CARD9-signaling pathway, and the pathway in dendritic cells was recently shown to be a crucial signaling axis in induction of the skin CHS response (36). Therefore, endogenous Mincle ligands may serve as auto-adjuvants, activating the innate immune system. Interestingly, the activation of Mincle by its ligands, such as trehalose-6,6′-dimycolate from *M. tuberculosis* or the synthetic adjuvant trehalose dibehenate, promotes Th1/Th17 T-cell responses (9, 13, 37). We found in allergic inflamed ears from Mincle-KO mice a suppressed production of IL-17A, which is regarded as a signature cytokine for Th17 cells (38). Th17 cells play a crucial role as effector cells in the pathogenesis of allergic contact dermatitis, psoriasis, and autoimmune diseases (39–41). Thus, the activation of Mincle during the development of allergic contact dermatitis may lead to a bias that influences T-cell polarization. However, further studies are needed to determine the precise mechanism underlying the driving role of Mincle in cutaneous allergic inflammation.

Cholesterol sulfate is present in various tissues and body fluids, and it accumulates during squamous cell differentiation in the epithelial cells of barrier tissues, such as the respiratory tract and esophageal mucosa (11, 12, 42). Cholesterol sulfate is also found in considerable quantities in the skin, predominantly in the epidermis (10), where it acts as an important regulator during the formation of the epidermal barrier (42).

Based on our findings, it can be hypothesized that Mincle, the expression of which is rapidly increased in dermis in response to skin trauma or irritation, can recognize cholesterol sulfate released from the damaged epidermis and subsequently amplify an initial inflammatory response in the skin to activate and mobilize skin antigen-presenting cells. Although DNFB application onto the abdominal skin of mice does not lead to significant changes in the quantity of cholesterol sulfate per skin biopsy sample (Fig. S5), it could be hypothesized that the DNFB application leads to rapid cholesterol sulfate permeation from epidermis to dermis. Also, it could be hypothesized that the activity of CD8+ cytotoxic T cells during the challenge phase that causes epithelial cell damage may also lead to the release of cholesterol sulfate and subsequently Mincle activation. However, it is possible that other endogenous ligands of Mincle, such as SAP130, may play an important role in the Mincle activation during the skin inflammatory response. Additional investigations are necessary to precisely determine the role of cholesterol sulfate/Mincle interaction in the pathogenesis of allergic skin inflammation.

Taken together, our results demonstrate an important role of Mincle as a driving component in the pathogenesis of allergic skin inflammation and as a receptor of a DAMP, cholesterol sulfate. These results contribute to a deeper understanding of the fundamental innate immune mechanisms underlying sterile inflammation.

Materials and Methods

Male BALB/c mice and C57BL/6 and C57BL/6-Mincle-KO mice of both sexes were used in this study. All mice were between 9 wk and 10 wk of age. The Mincle-KO knockout mouse line was obtained from the National Institutes of Health-sponsored Mutant Mouse Regional Resource Center (MMRRC) National System and was back-crossed with C57BL/6 mice for 10 generations. All of the animal experimental procedures were in line with the Bioethics Commitee of N. F. Gamaleya Federal Research Center of Epidemiology and Microbiology guidelines.

RNA samples for microarray analysis were prepared as described previously (43, 44). Total lipids were isolated from skin samples using sequential extractions with different solvent mixtures according to a previously described procedure (45). Lipids were then fractionated according to a previously reported procedure (46). Separation via HPLC was achieved as suggested by Ikeda et al. (47) with some changes. BMDCs from Mincle-KO and wild-type mice were differentiated from proliferating mouse bone marrow progenitors according to a previously described protocol (48). A well-known murine model of allergic contact dermatitis was induced using a method described previously (18). Cholesterol sulfate quantification in skin was performed according to a protocol (49) with some modifications.

Additional information is provided in *SI Materials and Methods*.

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