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Lack of the purinergic receptor $P2X_7$ results in resistance to contact hypersensitivity

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Sensitization to contact allergens requires activation of the innate immune system by endogenous danger signals. However, the mechanisms through which contact allergens activate innate signaling pathways are incompletely understood. In this study, we demonstrate that mice lacking the adenosine triphosphate (ATP) receptor $P2X_7$ are resistant to contact hypersensitivity (CHS). $P2X_7$-deficient dendritic cells fail to induce sensitization to contact allergens and do not release IL-1β in response to lipopolysaccharide (LPS) and ATP. These defects are restored by pretreatment with LPS and alum in an NLRP3- and ASC-dependent manner. Whereas pretreatment of wild-type mice with $P2X_7$ antagonists, the ATP-degrading enzyme apyrase or IL-1 receptor antagonist, prevents CHS, IL-1β injection restores CHS in $P2X_7$-deficient mice. Thus, $P2X_7$ is a crucial receptor for extracellular ATP released in skin in response to contact allergens. The lack of $P2X_7$ triggering prevents IL-1β release, which is an essential step in the sensitization process. Interference with $P2X_7$ signaling may be a promising strategy for the prevention of allergic contact dermatitis.

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Abbreviations used: ACD, allergic contact dermatitis; BMDC, BM-derived DC; CHS, contact hypersensitivity; IL-1R, IL-1 receptor; IL-1Ra, IL-1R antagonist; NLR, NOD-like receptor; TLR, Toll-like receptor; TNBS, 2,4,6-trinitrobenzene-1-sulfonic acid; TNCB, 2,4,6-trinitrochloro-1-benzene.

Allergic contact dermatitis (ACD) is a T cell–mediated inflammatory skin disease most frequently caused by low molecular weight electrophilic chemicals or metal ions. Activation of DCs by contact allergens is a prerequisite for the induction of the pathogenic skin-specific T cell response (Martin and Jakob, 2008; Sigmundsdottir and Butcher, 2008).

DC activation proceeds via a broad variety of germline-encoded innate immune receptors, in particular the pattern recognition receptors, Toll-like receptors (TLRs) and NOD-like receptors (NLRs; Kawai and Akira, 2009; Martinon et al., 2009; Palm and Medzhitov, 2009). TLR and NLR recognize pathogen-associated molecular patterns as well as endogenous danger signals (Tsan and Gao, 2004; Jiang et al., 2006; Palm and Medzhitov, 2009) associated with tissue destruction, termed damage-associated molecular patterns (Seong and Matzinger, 2004). Triggering of pattern recognition receptors by pathogen-associated molecular patterns or damage-associated molecular patterns leads to an inflammatory response involving the production of proinflammatory cytokines, including IL-1β, chemokines, and antimicrobial peptides.

Inflammatory responses induced by contact allergens in the skin share many features with the innate immune responses to pathogens (Freudenberg et al., 2009). In the mouse contact hypersensitivity (CHS) model, we have recently demonstrated a critical role for TLR2 and TLR4 in the DC-mediated sensitization process (Martin et al., 2008). Moreover, contact allergens trigger oxidative stress and antioxidant responses (Matsue et al., 2003; Kim et al., 2008; Natsch and Emter, 2008; Ade et al., 2009).

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They also activate the NLRP3 inflammasome (Sutterwala et al., 2006; Watanabe et al., 2007), a cytosolic platform which activates caspase-1 for the processing of pro–IL-1β and pro–IL-18 produced, e.g., by keratinocytes and DC in response to various stimuli, including TLR, NLR, and the purinergic receptor $P2X_7$ (Ferrari et al., 2006; Di Virgilio, 2007; Martinon et al., 2009; Surprenant and North, 2009). These cytokines play a key role in the sensitization phase of CHS and are key mediators of Langerhans cell migration from the skin to the draining lymph nodes (Shornick et al., 1996; Antonopoulos et al., 2001, 2008; Cumberbatch et al., 2001).

The molecular mechanisms by which contact allergens activate these innate immune and stress pathways are largely unknown. Recent evidence suggests that endogenous ligands such as fragments of hyaluronic acid may trigger TLR2 and TLR4 in CHS (Martin et al., 2008; Freudenberg et al., 2009). Similarly, extracellular nucleotides such as ATP released by stressed or damaged cells are endogenous danger signals that can activate innate immune responses. There are two families of purinergic receptors (P2R), i.e., P2Y and P2X (North and Surprenant, 2000; Abbracchio et al., 2006; Burnstock, 2008). P2Y receptors are G protein–coupled receptors, and P2X receptors are ligand-gated ion channels. The transmembrane ATP receptor $P2X_7$ has been implicated in the posttranslational processing of pro–IL-1β and pro–IL-18 via activation of the NLRP3 inflammasome (Solle et al., 2001; Ferrari et al., 2006; Di Virgilio, 2007; Dinarello, 2009; Martinon et al., 2009; Surprenant and North, 2009). This receptor is expressed on mouse DCs. $P2X_7$-deficient DCs are severely impaired in IL-1β release and stimulation of antigen-specific T cells (Mutini et al., 1999).

ATP is the main energy carrier in cells with a cytosolic concentration of 3–10 mM. Under homeostatic conditions, extracellular ATP levels are as low as 10 nM and are tightly regulated by ectonucleotidases such as CD39 and CD73, which dephosphorylate ATP to ADP, AMP, and adenosine (Ferrari et al., 2006; Di Virgilio, 2007; Surprenant and North, 2009). Adenosine has immunoregulatory functions in CHS (Ring et al., 2009). However, under pathological conditions such as hypoxia, trauma, viral, and bacterial infection and inflammation, extracellular concentrations of ATP can be elevated as the result of active processes or passive leakage from damaged or dying cells. The concomitant down-regulation of nucleotidases causes additional accumulation of extracellular nucleotides (Robson et al., 1997; Lazarowski et al., 2003).

ATP-mediated K^+ efflux from cells is triggered by engagement of $P2X_7$ and is crucial for LPS-mediated IL-1β release from human macrophages (Ferrari et al., 1997). Moreover, LPS-primed macrophages from $P2X_7^{-/-}$ mice fail to secrete mature IL-1β because of their inability to process the immature pro form. This defect can be repaired by treatment with nigericin (Solle et al., 2001), a $P2X_7$-independent activator of the NLRP3 inflammasome. The NLRP3 inflammasome is activated in response to ATP and is required for posttranslational IL-β processing (Mariathasan et al., 2006; Dinarello, 2009).

Thus, ATP is an important endogenous mediator in inflammation. It was recently shown that $P2X_7$ triggering on DCs by ATP derived from dying tumor cells is a prerequisite for IL-1β–dependent antitumor responses, and this response was dependent on the NLRP3 inflammasome and caspase-1 (Ghiringhelli et al., 2009). Furthermore, $P2X_7$ activation on DCs is important in DC-mediated airway inflammation (Idzko et al., 2007). A proinflammatory role for ATP in CHS was suggested by the finding that injection of nonhydrolyzable ATP-γS into mice before their sensitization resulted in increased ear swelling responses (Granstein et al., 2005). Interestingly, contact allergens can trigger the release of ATP from keratinocytes and DCs, and it was shown that ATP release from keratinocytes stimulates Langerhans cells (Mizumoto et al., 2002). However, the mechanisms by which ATP triggers inflammatory responses in CHS were unknown.

In this study, we have investigated the putative role of the ATP receptor $P2X_7$ in CHS in vivo, including its mode of action. Using mice lacking $P2X_7$ or WT mice treated with the $P2X_7$ antagonist KN-62 (Baraldi et al., 2004) or the ATP-degrading enzyme apyrase, we demonstrate for the first time that $P2X_7$ is essential for the induction of CHS by contact allergens in mice. We observed ATP release in the skin upon 2,4,6-trinitrochloro-1-benzene (TNCB) painting, indicating indirect contact allergen–dependent $P2X_7$ triggering via induction of ATP release in the skin. In addition, we show by cell transfer experiments that expression of $P2X_7$ on DCs is crucial for the sensitization but not for the effector phase of CHS. The sensitization defect of $P2X_7^{-/-}$ DCs can be bypassed by treatment with the NLRP3 inflammasome activator alum (Eisenbarth et al., 2008, Franchi and Núñez, 2008; Kool et al., 2008; Li et al., 2008). However, alum did not restore the absent sensitization capacity of $NLRP3^{-/-}$ (Martinon et al., 2006) or $ASC^{-/-}$ (Sutterwala et al., 2006) DCs, indicating a link between ATP-mediated $P2X_7$ triggering and NLRP3 inflammasome activation in IL-1β release. The crucial role of this cytokine in CHS was suggested by the prevention of CHS after injection of mice with the IL-1 receptor (IL-1R) antagonist (IL-1Ra) anakinra, which is used in the treatment of inflammatory diseases such as gout (So et al., 2007; Gabay et al., 2010), or the impaired CHS in $IL\text{-}1R^{-/-}$ mice. Most importantly, injection of IL-1β into $P2X_7^{-/-}$ mice restored their CHS response. Thus, interfering with purinergic signaling via $P2X_7$ may be a promising approach for the prevention of ACD.

RESULTS

Mice deficient for the purinergic receptor $P2X_7$ are resistant to CHS

To study the role of extracellular ATP in CHS, we tested C57BL/6 WT and $P2X_7$-deficient ($P2X_7^{-/-}$) mice for their ear swelling reaction to epicutaneous sensitization with the contact allergen TNCB. Mice were treated on the abdominal skin followed by elicitation of CHS on day 5 by application of TNCB to the ear skin. The increase in ear thickness was measured 24 h after elicitation. Although WT mice developed a normal ear-swelling response, $P2X_7^{-/-}$ mice did not develop TNCB-induced CHS

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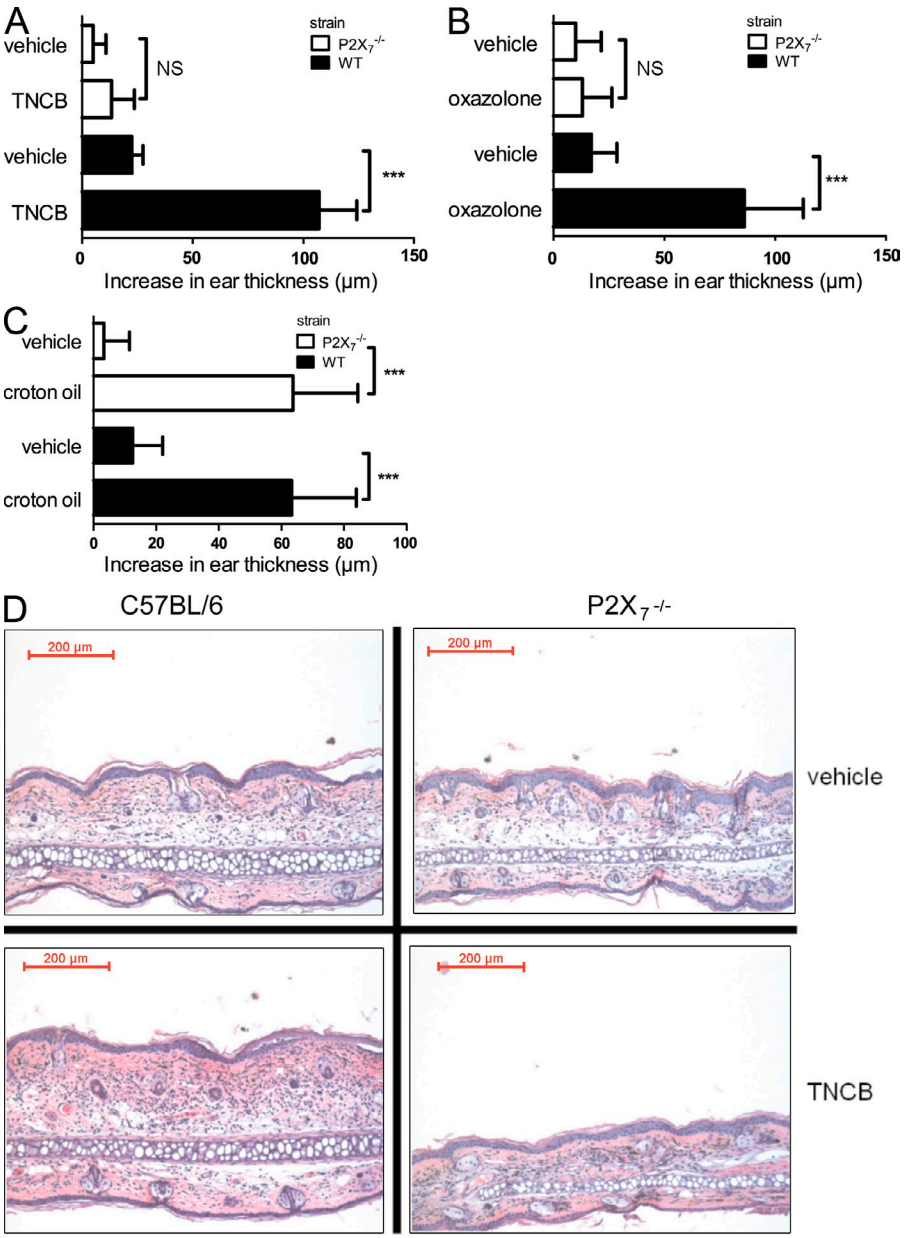

Figure 1. $P2X_7^{-/-}$ mice are resistant to allergic but not to irritant contact dermatitis. (A) C57BL/6 WT or $P2X_7^{-/-}$ mice were sensitized by application of 3% TNCB in acetone or acetone alone (vehicle) on the shaved abdominal skin. Both ears were challenged on day 5 after sensitization with 1% TNCB. Ear thickness was measured at the time of challenge and 24 h later. (B) The experiment was performed as described in A, except with 3% oxazolone in ethanol or ethanol (vehicle). Ear challenge was performed on day 6 with 1% oxazolone in ethanol. (C) WT and $P2X_7^{-/-}$ mice were treated with 1% croton oil in 4:1 acetone/olive oil or acetone/olive oil alone (vehicle) on the ear skin. The ear thickness was measured before and 24 h after treatment. (A–C) Results represent the increase in ear thickness in groups of five mice ± SD. One of two (C) or three (A and B) independent experiments is shown (***, $P < 0.001$). (D) Ear skin from TNCB-sensitized $P2X_7^{-/-}$ and WT mice was stained with H&E 24 h after challenge with vehicle or TNCB.

(Fig. 1 A). Similar results were obtained for the contact allergen oxazolone (Fig. 1 B). In contrast, $P2X_7$-deficient mice developed normal ear swelling in response to the irritant croton oil (Fig. 1 C). These results identify a crucial role for the ATP-gated cation channel $P2X_7$ in CHS induced by contact allergens. The results from ear thickness measurements are supported by histology of the ear skin. In contrast to $P2X_7^{-/-}$ mice, the ear skin of WT mice treated with TNCB shows a significant swelling and a more pronounced inflammatory infiltrate (Fig. 1 D).

2,4,6-trinitrobenzene-1-sulfonic acid (TNBS)–modified DCs from $P2X_7^{-/-}$ mice fail to sensitize WT recipients for CHS

DCs are the main interface between innate and adaptive immunity in CHS (Martin and Jakob, 2008; Freudenberg et al., 2009). Thus, the role of the $P2X_7$ receptor on DCs in the induction and/or elicitation of CHS was assessed. BM-derived DCs (BMDCs) of WT and $P2X_7^{-/-}$ mice were modified with TNBS and subsequently injected i.c. (intracutaneously) into WT recipients as described previously (Martin et al., 2008). TNBS-modified WT BMDCs effectively sensitized WT recipients (Fig. 2 A). In contrast, TNBS-modified $P2X_7^{-/-}$ BMDCs failed to sensitize WT mice under identical conditions. Together with the finding that TNBS-modified WT BMDCs were able to readily sensitize $P2X_7^{-/-}$ recipients (Fig. 2 B), these data indicate that expression of $P2X_7$ on DCs is essential during the sensitization phase but not during the effector phase of CHS.

Alum pretreatment restores the sensitization capacity of $P2X_7$-deficient BMDCs

The failure of $P2X_7^{-/-}$ BMDCs to induce sensitization may be caused by their failure to produce mature IL-1β via the NLRP3 inflammasome (Ferrari et al., 2006; Mariathasan et al., 2006; Di Virgilio, 2007; Surprenant and North, 2009). Therefore, we bypassed the need for $P2X_7$ triggering with alum, a potent $P2X_7$-independent activator of the NLRP3 inflammasome (Eisenbarth et al., 2008, Franchi and Núñez, 2008; Kool et al., 2008; Li et al., 2008). BMDCs were pretreated with alum before adoptive transfer. TNBS-modified

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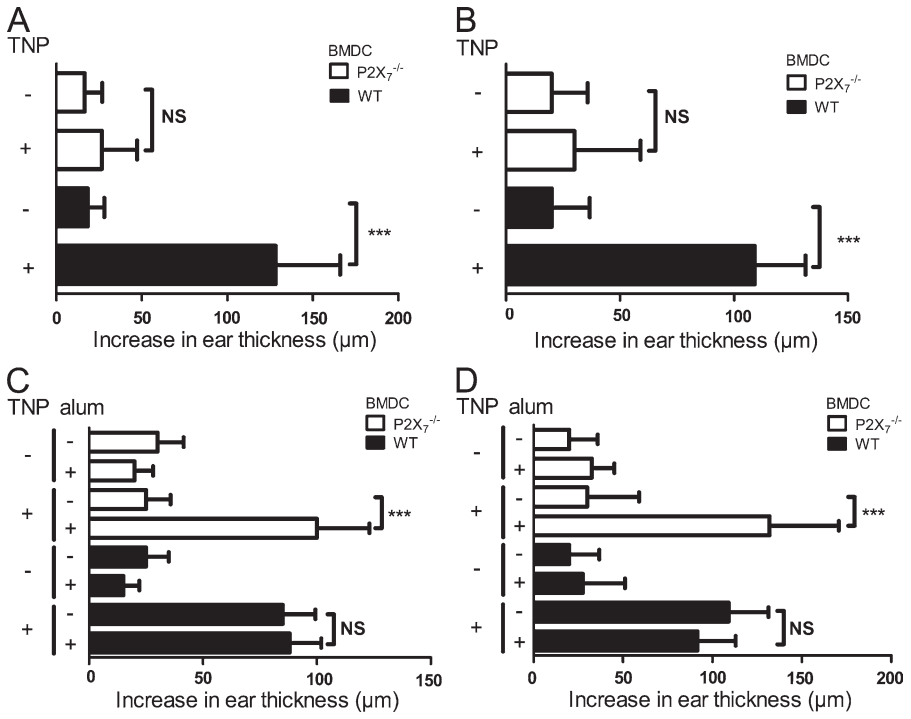

Figure 2. $P2X_7$ deficiency abrogates the sensitization capacity of BMDCs and is restored by alum pretreatment. (A and B) WT (A) or $P2X_7^{-/-}$ (B) mice were sensitized by i.c. injection of 3×10^5 unmodified or TNP-modified WT or $P2X_7^{-/-}$ BMDCs into the shaved abdominal skin. Ear challenge was performed as in Fig. 1. (C and D) The experiment was performed as described in A and B, but the indicated BMDCs were prestimulated or not with alum for 2 h before adoptive transfer. WT (C) or $P2X_7^{-/-}$ mice (D) were sensitized by i.c. injection of 3×10^5 BMDCs. (A–D) The data represent the mean increase in ear thickness of groups of five mice ± SD. One representative of three independent experiments is shown (***, $P < 0.001$).

and alum-pretreated $P2X_7^{-/-}$ BMDCs regained their sensitizing potential upon transfer into both WT and $P2X_7^{-/-}$ recipients (Fig. 2 C). Alum pretreatment of WT BMDCs did not significantly alter their sensitizing potential (Fig. 2 D). These data further support the assumption that $P2X_7$ on DCs is mandatory for efficient induction of CHS and acts via processing and release of inflammasome-dependent cytokines such as IL-1β (Ferrari et al., 2006; Mariathasan et al., 2006; Di Virgilio, 2007; Martinon et al., 2009; Surprenant and North, 2009).

Alum pretreatment fails to restore the sensitization capacity of NLRP3- or ASC-deficient BMDCs

To analyze the role of the NLRP3 inflammasome in DCs and the mechanism of the $P2X_7$ bypass with alum, WT mice were sensitized with $NLRP3^{-/-}$ (Martinon et al., 2006) BMDCs in comparison with WT and $P2X_7^{-/-}$ BMDCs with and without alum pretreatment (Fig. 3 A). No CHS was elicited by TNCB challenge in mice sensitized with unmodified control DCs or alum pretreated control DCs of all three mouse strains. Moreover, we demonstrate that the lack of NLRP3 in BMDCs modified with TNBS abrogates their sensitization capacity. This defect was not prevented by alum pretreatment, indicating that the alum bypass of $P2X_7$ is mediated by the NLRP3 inflammasome. In addition, we used BMDCs from $ASC^{-/-}$ mice (Sutterwala et al., 2006) for sensitization (Fig. 3 B). As shown for $NLRP3^{-/-}$ BMDCs, TNBS-modified $ASC^{-/-}$ BMDCs also failed to sensitize WT mice for CHS, a defect which again could not be bypassed by alum pretreatment. These data support the notion that the NLRP3 inflammasome is important in CHS (Sutterwala et al., 2006; Watanabe et al., 2007) and demonstrate that functional ASC and NLRP3 are required in DCs for successful sensitization. To analyze the role of NLRP3 in $P2X_7$-mediated IL-1β processing, WT or $NLRP3^{-/-}$ BMDCs were stimulated in vitro with LPS and alum or with LPS and ATP. Neither alum nor ATP stimulation increased the LPS-induced IL-1β secretion from $NLRP3^{-/-}$ BMDC mice in contrast to BMDCs from WT mice (Fig. 3 C). These data demonstrate that the $P2X_7^{-/-}$-dependent IL-1β release is mediated via the NLRP3 inflammasome and confirm the important role of NLRP3 in alum-mediated IL-1β processing (Eisenbarth et al., 2008; Kool et al., 2008; Li et al., 2008). IL-6 release was triggered efficiently in both WT and $NLRP3^{-/-}$ BMDCs, showing that there is no general functional defect of $NLRP3^{-/-}$ BMDCs (unpublished data).

$P2X_7^{-/-}$ BMDCs fail to process and secrete mature IL-1β

To further address the functional consequence of $P2X_7$ deficiency in DCs, WT and $P2X_7^{-/-}$ BMDCs were analyzed for the production and secretion of pro– and mature IL-1β by Western blotting (Fig. 4 A) and for secreted IL-1β by ELISA (Fig. 4 B) in response to LPS and ATP in vitro. This cytokine is essential for the sensitization process in CHS (Shornick et al., 1996; Antonopoulos et al., 2001, 2008; Cumberbatch et al., 2001). Both WT and $P2X_7^{-/-}$ BMDCs produced similar amounts of pro–IL-1β in response to LPS (Fig. 4 A), but only WT BMDCs secreted mature IL-1β when challenged with LPS and ATP, whereas $P2X_7^{-/-}$ BMDCs did not (Fig. 4 B). These data show that induction of IL-1β release by TNCB is crucially dependent on ATP-mediated triggering of $P2X_7$. It is worth noting that in both WT and $P2X_7^{-/-}$ BMDCs, ATP alone was able to efficiently induce pro–IL-1β (Fig. 4 A). This points to a $P2X_7^{-/-}$-independent effect on pro–IL-1β production. When BMDCs from both mouse strains were treated with graded concentrations of alum alone in vitro, no IL-1β release was observed (Fig. 4 C). However, the addition of 1 µg/ml LPS resulted in a dose-dependent IL-1β release from BMDCs of WT and $P2X_7^{-/-}$

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mice (Fig. 4 D). These results and the results shown in Fig. 3 show a link between LPS-induced pro–IL-1β production and ATP- and $P2X_7$-dependent IL-1β processing and release. An important finding is that ATP triggering of $P2X_7$ can be replaced by alum treatment of the BMDCs, which results in LPS-induced IL-1β production and release of processed IL-1β not only in WT but also in $P2X_7^{-/-}$ BMDCs (Fig. 4, C and D). Importantly, the alum effect is no longer visible when BMDCs from $NLRP3^{-/-}$ or $ASC^{-/-}$ mice are used for sensitization in CHS (Fig. 3, A and B). Moreover, alum also fails to restore defective LPS-induced IL-1β release in $NLRP3^{-/-}$ BMDCs in vitro (Fig. 3 C). These data support the notion that defective IL-1β release of $P2X_7^{-/-}$ DCs is responsible for their failure to sensitize mice for CHS. They also strongly suggest that contact allergen–induced ATP triggering of $P2X_7$ leads to IL-1β processing via the NLRP3 inflammasome, as demonstrated in other systems (Ferrari et al., 2006; Mariathasan et al., 2006; Di Virgilio, 2007; Idzko et al., 2007; Ghiringhelli et al., 2009; Surprenant and North, 2009).

TNCB triggers ATP release in vivo in mouse skin

We next analyzed whether ATP is released in the skin after application of the contact allergen TNCB. Bioluminescence imaging (Edinger et al., 2003) revealed that TNCB rapidly triggers ATP release, as detected by ATP-dependent luciferin-induced bioluminescence from HEK293-pmeLUC cells (Pellegatti et al., 2008) after painting of the ear skin of mice (Fig. 5). These data reveal that $P2X_7$ is triggered by the endogenous danger signal ATP that is released from cells in the skin after TNCB treatment.

$P2X_7$ antagonists or removal of extracellular ATP prevents CHS

To further investigate the role of the $P2X_7$ receptor in CHS in vivo, experiments with the $P2X_7$ receptor antagonist KN-62 (Baraldi et al., 2004) were performed in WT mice. Recipients were injected with KN-62 into the left ear pinna, followed by sensitization with TNCB on the same ear 4 h later. CHS was elicited on day 5 with TNCB on the right ear, and the increase in ear thickness was measured 24 h after elicitation. As depicted in Fig. 6 A, pretreatment with KN-62 before TNCB sensitization led to abrogation of CHS in WT mice. This effect was dose and time dependent (Fig. S1). Similar results were obtained with the purinergic receptor antagonist suramin (Fig. S2; Baraldi et al., 2004). These data again indicate an essential role for $P2X_7$ receptor signaling in CHS.

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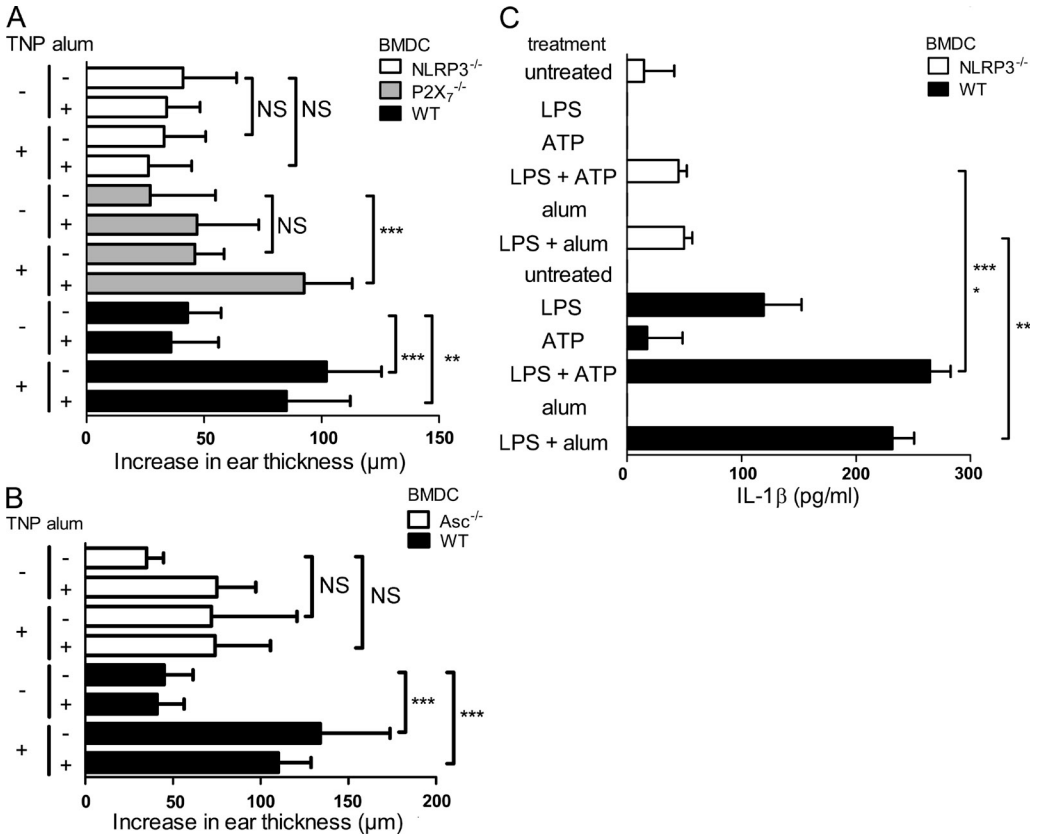

Figure 3. NLRP3 or ASC deficiency abrogates the sensitization capacity of BMDCs and is not restored by alum pretreatment.
(A and B) $NLRP3^{-/-}$, $P2X_7^{-/-}$, and WT BMDCs (A) or $ASC^{-/-}$ and WT BMDCs (B) were TNBS modified and alum pretreated where indicated, and the experiment was performed as described in Fig. 2. The data represent the mean increase in ear thickness of groups of five mice ± SD. One representative of three (NLRP3) and two (ASC) independent experiments is shown (**, $P < 0.005$; ***, $P < 0.001$). (C) $NLRP3^{-/-}$ and WT BMDCs were treated with 0.1 µg/ml LPS, 1.25 mM ATP, 80 µg/ml alum, or the indicated combination of two stimuli for 12 h. IL-1β secretion was determined by ELISA. Data represent the mean of triplicate measurements ± SD and are representative for two independent experiments (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$).

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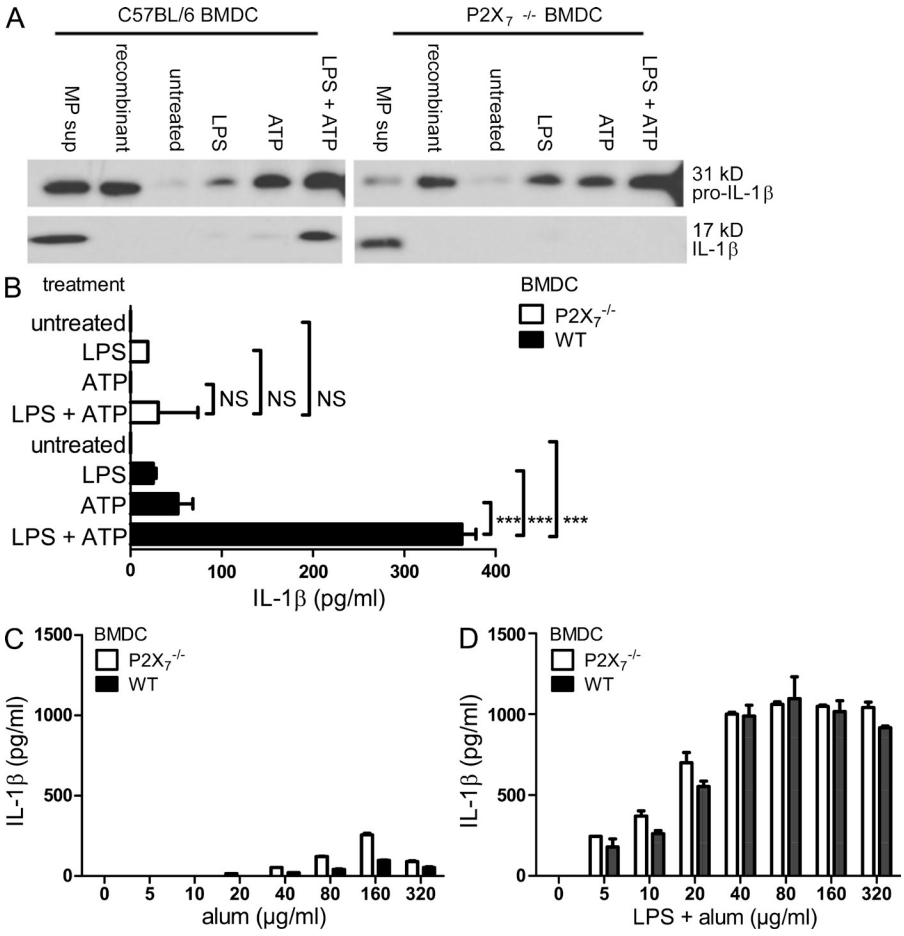

Figure 4. $P2X_7^{-/-}$ BMDCs fail to process and release mature IL-1β. (A) $P2X_7^{-/-}$ and WT BMDCs were left untreated or were treated with 1 μg/ml LPS and/or 5 mM ATP for 12 h. Western blot analysis was performed to detect pro–IL-1β and mature IL-1β in the culture supernatant. Supernatant from LPS + ATP–stimulated macrophages (MP sup) or recombinant pro–IL-1β was used as positive control. The data are representative of two independent experiments. (B) The same supernatants were used to detect IL-1β secretion by ELISA (***, $P < 0.001$). (C and D) BMDCs were treated with alum at the indicated concentrations for 12 h in the presence or absence of 0.1 μg/ml LPS. IL-1β secretion was determined by ELISA. (B–D) Data represent the mean of triplicate measurements ± SD and are representative for two (B) or three (C and D) independent experiments.

To assess the role of endogenously released ATP in CHS, WT mice were injected with apyrase, an ATP-degrading enzyme, to remove ATP from the extracellular space (Idzko et al., 2007) using the same approach as described for KN-62 (Fig. 6 A). Injection of apyrase into the ear pinna before sensitization prevented CHS in WT mice (Fig. 6 B). A dose- and time-dependent reduction of the ear swelling response was observed (Fig. S3). These results underline the crucial role for the release of extracellular ATP in the sensitization phase of CHS.

IL-1R blockade or deficiency abrogates CHS, whereas IL-1β restores CHS in $P2X_7^{-/-}$ mice

Finally, to assess the role of IL-1β in CHS, we neutralized the effects of this cytokine by i.v. injection of the recombinant IL-1Ra anakinra before sensitization of WT mice with TNCB. After ear challenge, we observed that anakinra treatment efficiently prevented the CHS response (Fig. 7 A). When we tried to induce CHS in IL-1R$^{-/-}$ mice, we observed a similar effect. In contrast to IL-18$^{-/-}$ mice, CHS was significantly reduced (Fig. 7 B). Because IL-1R signaling is not confined to IL-1β, we addressed the role of this cytokine by pretreatment of mice by IL-1β injection before sensitization. Although this treatment did not affect the CHS response in WT mice, it fully restored it in the $P2X_7^{-/-}$ mice (Fig. 7 C). These data underline the role of IL-1R signaling and the crucial pathogenic role of IL-1β for the sensitization to contact allergens (Shornick et al., 1996; Antonopoulos et al., 2001, 2008; Cumberbatch et al., 2001).

DISCUSSION

The mechanisms by which contact allergens activate innate immune and stress responses in skin are only poorly understood. We have recently demonstrated a crucial role for the innate immune receptors TLR2 and TLR4 and the cytokine receptor IL-12Rβ2 in the sensitization phase of CHS (Martin et al., 2008). These receptors are indirectly triggered by contact allergens via induction of endogenous ligands such as fragments of the extracellular matrix component hyaluronic acid. So far, only Ni^{2+} has been identified as a contact allergen that directly triggers human TLR4 by binding to conserved histidine residues that are not present in mouse TLR4 (Schmidt et al., 2010). This explains why CHS to Ni^{2+} in mice requires coapplication of adjuvants.

Besides the TLRs, a role for the NLRP3 inflammasome in CHS has been demonstrated (Sutterwala et al., 2006; Watanabe et al., 2007). In the present study, we show that $P2X_7$ signaling in DCs is a crucial step in the sensitization phase of CHS. Interestingly, irritant contact dermatitis induced by croton oil is not affected in $P2X_7^{-/-}$ mice. However, these mice are resistant to contact allergen–induced CHS. $P2X_7^{-/-}$ DCs are incapable of sensitizing WT mice for CHS, whereas WT DCs efficiently sensitize $P2X_7^{-/-}$ mice. The reconstitution of the sensitization capacity of $P2X_7^{-/-}$ DCs by pretreatment with alum, a $P2X_7$-independent activator of the NLRP3 inflammasome (Eisenbarth et al., 2008, Franchi and Núñez, 2008; Kool et al., 2008; Li et al., 2008), supports the functional role of the inflammasome in CHS (Sutterwala et al., 2006; Watanabe et al., 2007) and

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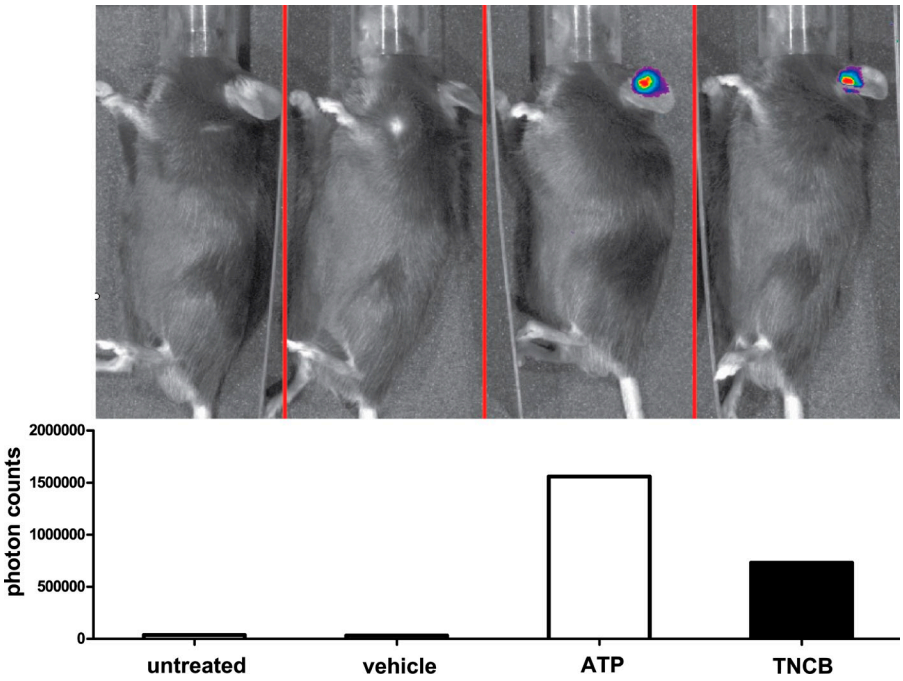

Figure 5. TNCB triggers ATP release in the skin. Mice received ATP-dependent luciferase-expressing HEK293 cells into the right ear pinna. The injected ear was painted 24 h later with acetone or 3% TNCB in acetone, and luciferin was injected i.p. immediately after painting. ATP was injected into the ear pinna as a positive control. Imaging was performed 10 min later. One out of three representative experiments is shown (top). Bioluminescence was quantified for the different conditions (bottom).

suggests a $P2X_7$-dependent pathway in the processing and secretion of the key cytokines IL-1β and IL-18 in CHS (Shornick et al., 1996; Antonopoulos et al., 2001, 2008; Cumberbatch et al., 2001). $P2X_7$-mediated K^+ efflux from the cell can activate the NLRP3 inflammasome and caspase-1, which is a requirement for IL-1β processing and secretion (Solle et al., 2001, Mariathasan et al., 2006; Dinarello, 2009). Similar effects are observed upon depletion of intracellular K^+ by nigericin and maitotoxin (Ferrari et al., 2006; Mariathasan et al., 2006; Di Virgilio, 2007; Surprenant and North, 2009). Our findings suggest that $P2X_7$ is involved in contact allergen–induced and NLRP3 inflammasome–dependent IL-1β release.

The previously reported link between $P2X_7$ and post-translational IL-1β processing, which involves the NLRP3 inflammasome (Ferrari et al., 2006; Mariathasan et al., 2006; Di Virgilio, 2007; Idzko et al., 2007; Ghiringhelli et al., 2009; Surprenant and North, 2009), is further supported by our in vitro findings. Stimulation of WT BMDCs with LPS and ATP results in production and secretion of mature IL-1β, whereas IL-1β processing and secretion is abrogated in $P2X_7^{-/-}$ BMDCs. The fact that we can restore this defect with the inflammasome activator alum, which bypasses $P2X_7$ by direct activation of the NLRP3 inflammasome (Eisenbarth et al., 2008, Franchi and Núñez, 2008; Kool et al., 2008; Li et al., 2008), and the lack of IL-1β release from $NLRP3^{-/-}$ DCs after stimulation with LPS and ATP reveal a functional link between ATP, $P2X_7$, and the NLRP3 inflammasome in IL-1β processing and secretion in the sensitization phase of CHS. Similar results were reported for LPS-primed macrophages from $P2X_7^{-/-}$ mice, which secrete IL-1β when pretreated with the inflammasome activator nigericin (Solle et al., 2001). This is further supported by our results demonstrating a failure of TNBS-modified BMDCs from $ASC^{-/-}$ and $NLRP3^{-/-}$ mice (Martinon et al., 2006; Sutterwala et al., 2006) to sensitize WT mice. In contrast to the results with $P2X_7$ BMDCs, alum pretreatment of these DCs fails to restore their sensitizing capacity.

Interestingly, our data support the notion that IL-1β as a rapidly induced pathogenic cytokine in the sensitization phase of CHS (Enk and Katz, 1992) plays a crucial role. Keratinocytes and

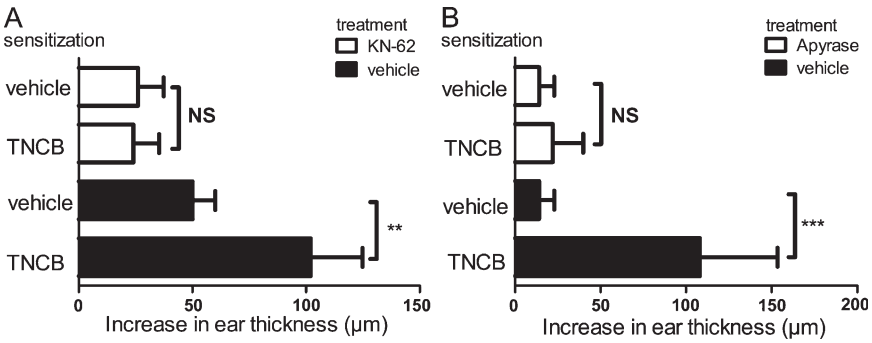

Figure 6. Purinergic receptor antagonism or ATP degradation prevents sensitization and CHS. (A) The $P2X_7$ receptor antagonist KN-62 was injected into the pinna of the left ear of WT mice 4 h before sensitization. Mice were sensitized with 3% TNCB in acetone on the left ear and were challenged 5 d later by application of 1% TNCB on the right ear. Data represent the mean ear swelling of groups of five mice ± SD. (B) Mice received apyrase in PBS or PBS as a vehicle control into the left ear pinna at the time of sensitization. Mice were sensitized with 3% TNCB or acetone as a vehicle control on the left ear for sensitization. Challenge was performed with 1% TNCB applied on the right ear 5 d later. The increase of ear thickness was determined 24 h after challenge. Data represent the mean increase in ear thickness ± SD of groups of five mice. (A and B) One representative of three independent experiments is shown (**, $P < 0.005$; ***, $P < 0.001$).

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Langerhans cells are sources for IL-1β in the skin. A recent in vivo imaging study identified $CD45^+CD11b^+$ myeloid leukocytes as primary sources for IL-1β by monitoring messenger RNA induction in vivo (Matsushima et al., 2010). In contrast to the failure to sensitize WT mice treated with the IL-1Ra anakinra or the absence of CHS in IL-$1R^{-/-}$ mice, CHS to TNCB was normal in IL-$18^{-/-}$ mice. Moreover, pretreatment of $P2X_7^{-/-}$ mice with IL-1β fully restored CHS. These results show the crucial role of IL-1β in the sensitization to TNCB. The extent of IL-1β messenger RNA induction correlates with the dose as well as with strength of the contact allergen (Lass et al., 2010). Interestingly, it was shown that CHS to DNFB (2,4-dinitrofluorobenzene) is also dependent on IL-18 (Klekotka et al., 2010). In this study, a role for IL-1R on radiosensitive bone marrow–derived cells and for IL-18 in radioresistant host-derived cells was demonstrated in bone marrow chimeras. Our findings on the role of IL-1β are in line with the study by Antonopoulos et al. (2008), who showed that deficient CHS in caspase-$1^{-/-}$ mice can be restored by pretreatment with IL-1β but not with IL-18. The authors concluded that IL-18 acts upstream of IL-1β.

In the light of recent data showing that ATP can activate regulatory T cells and control CHS (Ring et al., 2010), a dual role for ATP in inflammation and immunoregulation becomes evident. It remains to be determined how the balance between proinflammatory and regulatory immune responses is regulated. The kinetics of these responses, the target cells, and the purinergic receptors involved may all play a role.

Our study identifies ATP triggering of $P2X_7$ on DCs as a crucial step in the induction of skin inflammation by contact allergens but not skin irritants such as croton oil. Similar to our findings with indirect TLR2 and TLR4 triggering by contact allergens such as TNCB, oxazolone, and FITC, via induction of endogenous TLR ligands (Martin et al., 2008), we provide evidence that $P2X_7$ activation by contact allergens is also indirect. We show that TNCB rapidly induces the release of the $P2X_7$ agonist ATP in the skin. We demonstrate that TNCB-induced CHS can be readily prevented by injection of the $P2X_7$ antagonist KN-62 and the purinergic receptor antagonist suramin or by the ATP-degrading enzyme apyrase. Further studies will show whether $P2X_7$-specific antagonists are also effective in the control of established CHS. In this context, our finding that treatment of mice with the IL-1Ra

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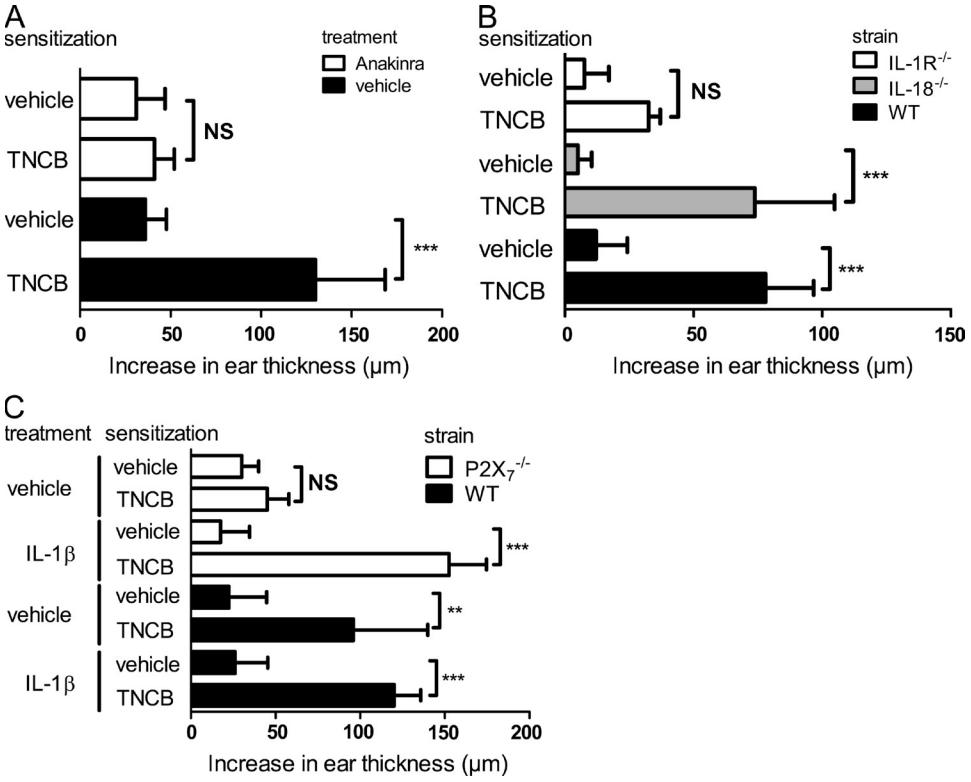

Figure 7. IL-1R antagonism or deficiency abrogates CHS, whereas IL-1β pretreatment of $P2X_7^{-/-}$ mice restores it. (A) Anakinra was injected i.p. on days 5, 3, and 1 before sensitization. Sensitization and challenge were performed as in Fig. 1. (B) Mice of the indicated strains were treated with 100 µl of 3% TNCB or acetone as vehicle control on day 0. On day 5, all mice received 20 µl of 1% TNCB on the dorsum of the ear pinna as challenge. (A and B) One representative of three independent experiments is shown. (C) WT and $P2X_7^{-/-}$ mice were injected with IL-β in PBS/0.1% BSA or vehicle control 30 min before sensitization in the dorsum of the left ear. Mice were sensitized by topical application of 3% TNCB on the dorsum of the right ear on day 0. Challenge was performed on day 5 with 1% TNCB on the dorsum of the left ear. One experiment was performed. (A–C) Data represent the mean increase in ear thickness ± SD of groups of five mice (**, $P < 0.005$; ***, $P < 0.001$).

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anakinra efficiently prevents CHS opens the possibility to test its efficiency in patients with ACD.

The fact that TLR2 and TLR4, $P2X_7$, or NLPR3 and ASC must be functional on DCs to enable them to sensitize mice for CHS and that the lack of function of one of these pathways is sufficient to abrogate their sensitization potential supports our concept of an essential collaborative action of these pathways in CHS (Freudenberg et al., 2009). The essential role of the DC is explained by its unique function as the cell type that migrates from the inflamed skin to the lymph nodes, communicates the activation of the innate immune system to contact allergen–specific T cells, and directs effector T cells to the skin (Dudda and Martin, 2004; Edele et al., 2007; Sigmundsdottir and Butcher, 2008).

Our study may open new perspectives for the treatment of ACD with available antagonists of the $P2X_7$ receptor, as recently reported for allergic airway inflammation (Idzko et al., 2007). So far, our data suggest that $P2X_7$ antagonists may prevent sensitization to contact allergens. It remains to be studied whether $P2X_7$ signaling also plays a role in the elicitation phase or in chronic contact dermatitis.

MATERIALS AND METHODS

Mice. C57BL/6 WT, C57BL/6 $P2X_7^{-/-}$ (Solle et al., 2001), and $ASC^{-/-}$ mice (Sutterwala et al., 2006) were bred at the animal facility of the University Medical Center Freiburg under specific pathogen-free conditions. $P2X_7^{-/-}$ mice were purchased from The Jackson Laboratory. $NLRP3^{-/-}$ mice (Martinon et al., 2006) were received from M. Kopf (Swiss Federal Institute of Technology Zurich, Zurich, Switzerland) with permission from J. Tschopp (University of Lausanne, Lausanne, Switzerland). $IL\text{-}1R^{-/-}$ (Labow et al., 1997) and $IL\text{-}18^{-/-}$ mice (Wei et al., 1999) on C57BL/6 background were a gift from M. Freudenberg (Max-Planck-Institute of Immunobiology, Freiburg, Germany). All of the experimental procedures were in accordance with institutional, state, and federal guidelines on animal welfare. The animal experiments were approved by the Regierungspräsidium Freiburg and supervised by the Animal Protection Representatives of the University Medical Center Freiburg.

Chemicals and reagents. TNBS, FITC, oxazolone, and croton oil were purchased from Sigma-Aldrich. TNCB was purchased from VeZerf Laborsynthesen GmbH. LPS of *Salmonella abortus equi* was purchased from Enzo Life Sciences, Inc. IL-1β ELISA was purchased from BD (OptEIA ELISA set). Recombinant IL-1β was purchased from R&D Systems. Flow cytometry was performed as described previously (Martin et al., 2008) on a FACScan instrument with CellQuest Pro software (BD).

Generation of BMDCs. BMDCs were prepared as previously described (Martin et al., 2008). Overall DC viability was not significantly influenced by the lack of $P2X_7$ as controlled by growth rate and cell yield in DC cultures from $P2X_7^{-/-}$ compared with WT mice.

Immunization and induction of CHS. Mice were sensitized by painting the abdominal skin with 100 µl of 3% TNCB/acetone or acetone alone as vehicle control on day 0. Epicutaneous application of 20 µl of 1% TNCB on the dorsum of both ears on day 5 was used for elicitation. Alternatively, mice were sensitized by i.c. injection of 3×10^5 unmodified or TNBS-modified BMDCs in 2×50 µl PBS into two sites of the shaved abdomen (Martin et al., 2008) followed by elicitation on day 5 by painting the dorsum of both ears with 1% TNCB. CHS to oxazolone was induced by sensitization with 150 µl of 3% oxazolone in ethanol, followed by ear challenge with 20 µl of 1% oxazolone/ethanol 6 d later. Ear measurement was performed before and 24 h after challenge using an engineer's thickness gauge (Mitutoyo).

The $P2X_7$ receptor antagonist KN-62 (Sigma-Aldrich) was dissolved in DMSO at 7.14 mg/ml. Mice were injected with KN-62 diluted in 71.4 µg/ml PBS, and 50 µl was injected into the pinna of the left ear 4 h before sensitization unless indicated otherwise. Apyrase (2 U potato apyrase grade 7 [Sigma-Aldrich] in 50 µl PBS) was injected into the ear pinna immediately before sensitization unless indicated otherwise. Mice were sensitized with 3% TNCB on the same ear and challenged 5 d later on the right ear with 1% TNCB. 24-h ear-swelling responses were measured. Anakinra Kineret (Amgen) was injected at a dose of 200 µg/mouse i.p. in 200 µl PBS on days 5, 3, and 1 before sensitization. The optimal doses and the timing for KN-62 and apyrase treatment were determined as shown in Figs. S1 and S3. IL-1β (50 µg in 30 µl PBS/0.1% BSA) or PBS/BSA as vehicle control was injected into WT and $P2X_7^{-/-}$ mice 30 min before sensitization in the dorsum of the left ear as described previously (Antonopoulos et al., 2008). Mice were sensitized by topical application of 20 µl of 3% TNCB on the dorsum of the left ear on day 0. Challenge was performed on day 5 with 20 µl of 1% TNCB on the dorsum of the right ear.

Histology of the skin. Ears from vehicle or allergen-sensitized mice were removed 24 h after allergen ear challenge and fixed in 3.7% buffered formaldehyde. Organ slices of 5 µm were prepared and stained with hematoxylin and eosin (H&E).

Induction of irritant contact dermatitis. Mice were painted on both ears with 20 µl of 1% croton oil (Sigma-Aldrich) in acetone/olive oil (4:1). The ear thickness was measured before and 24 h after application of croton oil.

Alum pretreatment of BMDCs before adoptive transfer. BMDCs were harvested on day 7 and modified with TNBS as described previously (Martin et al., 2008). For alum stimulation, BMDCs were incubated for 2 h at 37°C in RP-10 containing 240 µg/ml aluminum hydroxide (diluted 1:166.7; Imject Alum; Thermo Fisher Scientific) unless indicated otherwise. For $NLRP3^{-/-}$ and $ASC^{-/-}$ BMDC transfers into WT mice, 80 µg/ml alum was used to avoid stimulation of recipient WT cells caused by alum carryover. Cells were washed three times in PBS before adoptive transfer by i.c. injection.

In vivo bioluminescence imaging of ATP release. In vivo bioluminescence imaging was performed as previously described (Edinger et al., 2003). In brief, 5×10^6 HEK293-pmeLUC cells (Pellegatti et al., 2008) were injected into the ear skin in 50 µl PBS. Mice were treated 24 h after cell transfer with the contact allergen TNCB (20 µl of 3% TNCB/acetone), the vehicle control acetone, or by injection of 50 µl of 1.25 mM ATP/PBS as a positive control, followed by i.p. injection of luciferin. 10 min later, the mice were imaged with an exposure time of 5 min with an IVIS 100 charge-coupled device imaging system (Xenogen) and Igor Pro Carbon (WaveMetrics).

In vitro stimulation of DCs and IL-1β measurement. Stimulation of 10^6 BMDCs was performed in 500 µl RP-10 for 12 h with 1 µg/ml LPS in the presence or absence of 5 mM ATP or the corresponding controls. No significant differences in the viability of BMDCs from WT and $P2X_7^{-/-}$ DCs were observed by propidium iodide/annexin-V staining. Supernatants were used for Western blotting and ELISA to measure IL-1β. ELISA (OptEIA; BD) was performed according to the manufacturer's recommendations. Peritoneal macrophages were prepared and stimulated with LPS and ATP as described previously (Metkar et al., 2008), and the supernatant was used as positive control for Western blots. For alum stimulation, BMDCs were incubated for 12 h at 37°C in RP-10 containing graded concentrations of aluminum hydroxide in the presence or absence of 0.1 µg LPS.

Statistical analysis. Statistical analysis was conducted using one-way analysis of variance with Tukey's multiple comparison post test and Prism version 5.00 (GraphPad Software, Inc.). Data are shown as means ± SD. Differences between groups as marked by asterisks were statistically significant at $P < 0.05$ (*), $P < 0.005$ (**), or $P < 0.001$ (***).

Online supplemental material. Fig. S1 shows the dose titration and time kinetics for the $P2X_7$ antagonist KN-62. Fig. S2 shows suppression of the

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CHS response in WT mice treated with the purinergic receptor antagonist suramin before sensitization. Fig. S3 shows the dose titration and time kinetics for the ATP-degrading enzyme apyrase. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20092489/DC1.

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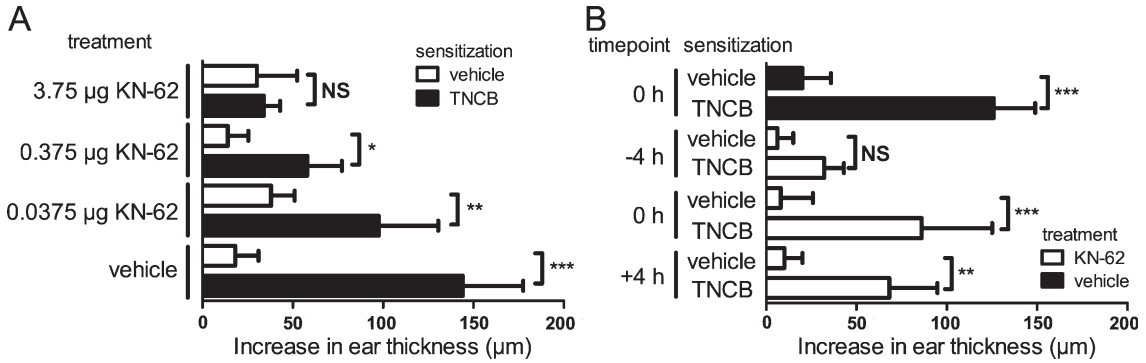

Figure S1. Dose titration and time kinetics for the $P2X_7$ antagonist KN-62. (A) Different concentrations of the $P2X_7$ receptor antagonist KN-62 were injected into the pinna of the left ear of WT mice 4 h before sensitization. Mice were sensitized with 3% TNCB in acetone on the left ear and were challenged 5 d later by application of 1% TNCB on the right ear. Data represent the mean increase in ear thickness of groups of five mice ± SD. (B) WT mice were treated with 3.75 µg KN-62 at the indicated time points. The experiment was performed as in A with groups of five mice. Ear thickness was measured before challenge and 24 h after challenge. Experiments in A and B were performed once and as in Fig. 6 A (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$).

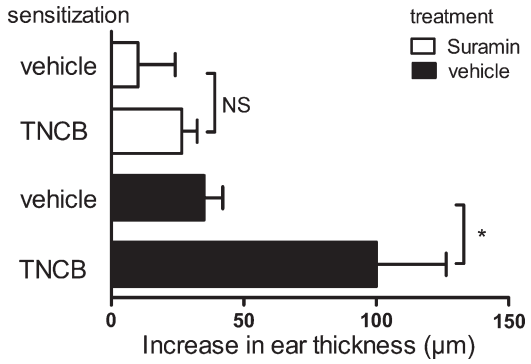

Figure S2. Purinergic receptor antagonism suppresses CHS responses in WT mice. WT mice were injected with the purinergic receptor antagonist suramin into the pinna of the left ear 4 h before sensitization. Mice were sensitized with 3% TNCB on the left ear and were challenged 5 d later by application of 1% TNCB on the right ear. Data represent the mean increase in ear thickness of groups of three mice ± SD. One representative of two independent experiments is shown. The experiment was performed as in Fig. 6 A (*, $P < 0.05$).

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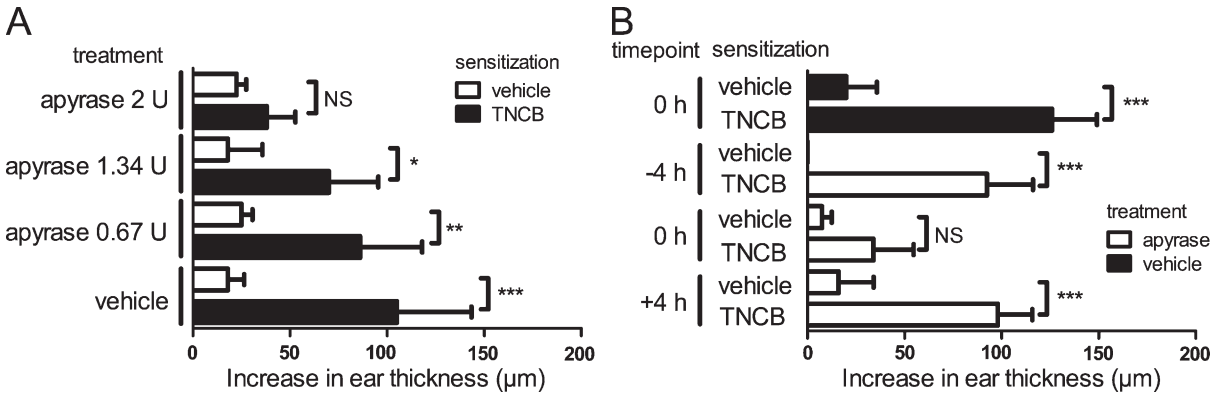

Figure S3. Dose titration and time kinetics for the ATP-degrading enzyme apyrase. (A) WT mice received different concentrations of apyrase in PBS or PBS as a vehicle control into the left ear pinna at the time of sensitization. Mice were sensitized with 3% TNCB or acetone as a vehicle control on the left ear for sensitization. Challenge was performed with 1% TNCB applied on the right ear 5 d later. The increase of ear thickness was determined 24 h after challenge. Data represent the mean increase in ear thickness ± SD of groups of five mice. (B) WT mice were treated with 2 U apyrase per mouse. The experiment was performed as in A with groups of five mice. Ear thickness was measured before challenge and 24 h after challenge. Experiments in A and B were performed once and as in Fig. 6 B (*, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.001$).

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