Toll-like receptor and IL-12 signaling control susceptibility to contact hypersensitivity

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Allergic contact hypersensitivity (CHS) is a T cell–mediated inflammatory skin disease. Interleukin (IL)-12 is considered to be important in the generation of the allergen-specific T cell response. Loss of IL-12 function in IL-12Rβ2–deficient mice, however, did not ameliorate the allergic immune response, suggesting alternate IL-12–independent pathways in the induction of CHS. Because exposure to contact allergens always takes place in the presence of microbial skin flora, we investigated the potential role of Toll-like receptors (TLRs) in the induction of CHS. Using mice deficient in TLR4, the receptor for bacterial lipopolysaccharide (LPS), IL-12 receptor (R) β2, or both, we show that the concomitant absence of TLR4 and IL-12Rβ2, but not the absence of TLR4 or IL-12Rβ2 alone, prevented DC-mediated sensitization, generation of effector T cells, and the subsequent CHS response to 2,4,6-trinitro-1-chlorobenzene (TNCB), oxazolone, and fluorescein isothiocyanate. Introduction of the TLR4 transgene into the TLR4/IL-12Rβ2 mutant restored the CHS inducibility, showing a requirement for TLR4 in IL-12–independent CHS induction. Furthermore, the concomitant absence of TLR2 and TLR4 prevented the induction of CHS to TNCB in IL-12–competent mice. Finally, CHS was inducible in germ-free wild-type and IL-12Rβ2–deficient mice, but not in germ-free TLR4/IL-12Rβ2 double deficient mice, suggesting that the necessary TLR activation may proceed via endogenous ligands.

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Abbreviations used: ACD, allergic contact dermatitis; CHS, contact hypersensitivity; HA, hyaluronic acid; i.c., intracutaneous; ODN, oligodeoxynucleotide; TLR, Toll-like receptor; TNCB, 2,4,6-trinitro-1-chlorobenzene; TNBS, 2,4,6-trinitrobenzene-1-sulfonic acid; TRIF, Toll/IL-1 receptor domain–containing adaptor-inducing interferon-β.

Allergic contact dermatitis (ACD) caused by reactive haptens and metal ions, a form of delayed type hypersensitivity, is one of the most common skin diseases (1, 2). Contact hypersensitivity (CHS), the recognized mouse model for studying human ACD, involves painting a small area of abdominal skin with the allergen (sensitization), e.g., 2,4,6-trinitro-1-chlorobenzene (TNCB), followed by an epicutaneous challenge 5 d later on a previously unexposed site, usually the ear (elicitation). The CHS response is then measured as the increase in ear thickness 24 h later. During the sensitizing contact, the hapten binds to proteins in the skin, altering their antigenic specificity, and the newly created antigens are then processed by epidermal (Langerhans cells) and dermal DCs. A crucial event in this sensitization phase is the induction of a transient skin inflammation via activation of the innate immune system, culminating in the migration of activated DC, bearing hapten–peptide complexes, to the draining lymph nodes (1, 2). During this time, DCs up-regulate costimulatory molecules on their surface, produce IL-12 (3), and polarize to a Th1/Tc1 ($CD8^+$ cytotoxic type 1 T cells)-inducing DC phenotype (1, 4). After completing their maturation, the immigrated DC prime hapten-specific naive T cells in an IL-12–dependent manner (5), giving

S.F. Martin and M.A. Freudenberg contributed equally to this paper.
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rise to allergen-specific $CD8^+$ T cells which express skin-specific homing receptors (6). More recently, however, it was suggested that both Th1 and Th2 cells play a role in CHS and that their equilibrium is dependent on the identity of the antigen. The crucial role of Th1/Tc1 cells in the induction of CHS has been questioned by a study using Tyk2-deficient mice (7). In this study, sensitization with TNCB resulted in an enhanced CHS response despite an impaired IL-12 signaling and Th2 polarization. Furthermore, the FITC-induced CHS was shown to be Th2 type driven (8, 9). Reexposure of the sensitized mice to the allergen elicits the infiltration of hapten-specific effector and memory T cells that orchestrate the characteristic local inflammatory response with edema 24–72 h later.

Although considerable information exists on several aspects of CHS development, the events leading to the skin inflammation that is necessary for both sensitization and elicitation with allergen are not well understood (1, 2). A striking observation is that contact allergens, similar to the highly conserved microbial constituents (pathogen-associated molecular patterns) which are sensed by Toll-like receptors (TLR; for review see reference 10), activate NF-κB and MAP kinases (11–14). Notably, the inflammatory reaction resembles the local inflammatory response to pathogen-associated molecular patterns (15, 16). This response is accompanied by the production of proinflammatory cytokines and chemokines, up-regulation of costimulatory molecules, and an influx of blood cells into the inflamed tissue. The skin, as the site of exposure to contact allergens, is also continuously exposed to environmental microbial flora and, thus, to TLR ligands. Consequently, participation of TLRs in the CHS induction may be envisaged.

In mammals, the TLR family consists of at least 11 members (for review see reference 10). TLR4 acts as the receptor for a most potent activator of the innate immune system, Gram-negative bacterial LPS (17, 18). Another member of the family, TLR2, recognizes a variety of microbial components, including bacterial lipopeptides (for review see reference 10). In addition to microbial ligands, there is evidence that endogenous substances may also act as ligands for TLR4 and TLR2. We demonstrated earlier that oligomeric degradation products of hyaluronic acid (HA) activate human and mouse DCs via TLR4 (19). More recently, HA fragments generated in vitro (135 kD) and those purified from human serum (peak 200 kD) were shown to activate mouse macrophages via TLR4 and TLR2 (20). Furthermore, HA degradation products and the two TLR receptors were reported to participate in the regulation of noninfectious mouse lung injury (20, 21). In addition, several other endogenous factors, including proteins or peptides such as heat shock proteins, have been reported to stimulate TLR4 (22, 23).

In this paper, we investigated the possible involvement of TLRs in the development of CHS to contact allergens. The results reveal a novel role of TLR4 in susceptibility to CHS of mice with impaired IL-12 function. We show further that the role of IL-12 and TLR4 signaling in CHS is interchangeable and that IL-12 deficiency enhances the allergic response. Finally, we show that the development of CHS is abolished in mice with an intact IL-12 function but lacking both TLR4 and TLR2. CHS was also not induced in germ-free IL-12Rβ2–deficient mice lacking TLR4. The results suggest that the TLR activation necessary for sensitization to contact allergens may proceed via endogenous TLR ligands.

RESULTS

Requirement for TLR4 in IL-12–independent CHS

To assess the possible role of TLR4 in the induction of CHS, we investigated the response of three genetically distinct strains of TLR4-deficient mice (C57BL/10: $H\text{-}2^b$, BALB/c: $H2^d$, and 129Sv: $H\text{-}2^b$) to an epicutaneous application of the contact allergen TNCB. Because the Th1/Tc1 polarizing cytokine IL-12 is still regarded as a dominant factor in CHS induction (3), we included in the investigation TLR4 and IL-12Rβ2 single- and double-deficient mice on the same C57BL/10 background (24, 25). The TLR4 single-deficient mice and the corresponding WT controls were painted with TNCB and rechallenged with the allergen 5 d later. 24 h after rechallenge, all mice developed comparable ear swellings (Fig. 1 A and Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20070509/DC1), indicating that the sole absence of TLR4 had no influence on the allergic response. In a similar experiment performed in TLR4/IL-12Rβ2–deficient mice, a CHS response to TNCB was completely absent (Fig. 1 A). Furthermore, we found that T cells from the skin-draining lymph nodes of TNCB-painted TLR4/IL-12Rβ2 double-deficient mice, in contrast to T cells from WT and TLR4 single-deficient animals, exhibited no IFN-γ responses to an in vitro restimulation with syngeneic spleen cells modified with 2,4,6-trinitrobenzene-1-sulfonic acid (TNBS; a water-soluble analogue of TNCB; Fig. 1 B). The absence of CHS correlated with the absence of significant cellular infiltration of the ear skin (see Fig. 3 B). An absence of CHS in these mice was also observed when the contact sensitizers oxazolone and FITC were used (Fig. 1, C and D). Similarly, lymph node cells of oxazolone-sensitized TLR4/IL-12Rβ2 double-deficient mice, in contrast to those of WT mice, exhibited no enhanced IFN-γ responses upon stimulation with anti-CD3 (Fig. S2). Interestingly, the same stimulation of cells from FITC-sensitized WT and double-deficient mice resulted neither in an increased IFN-γ response (Fig. S2) nor in detectable IL-4 production (not depicted). Furthermore, although edema and cellular infiltration was present in the skin of FITC-challenged WT mice after multiple rounds of sensitization, eosinophilia, which is typical for a Th2-mediated CHS, was completely absent (Fig. S3). These data suggest that the CHS response to FITC in C57BL/10 mice, unlike in BALB/c and C57BL/6 mice (8, 9), is not Th2 dependent.

Finally, lymph node cells from TNCB-painted TLR4/IL-12Rβ2 double-deficient mice were unable to adoptively transfer CHS to naive WT recipients (Fig. 1 E). These data indicate that mice with a combined TLR4 and IL-12Rβ2 deficiency fail to develop the characteristic allergen-specific type 1

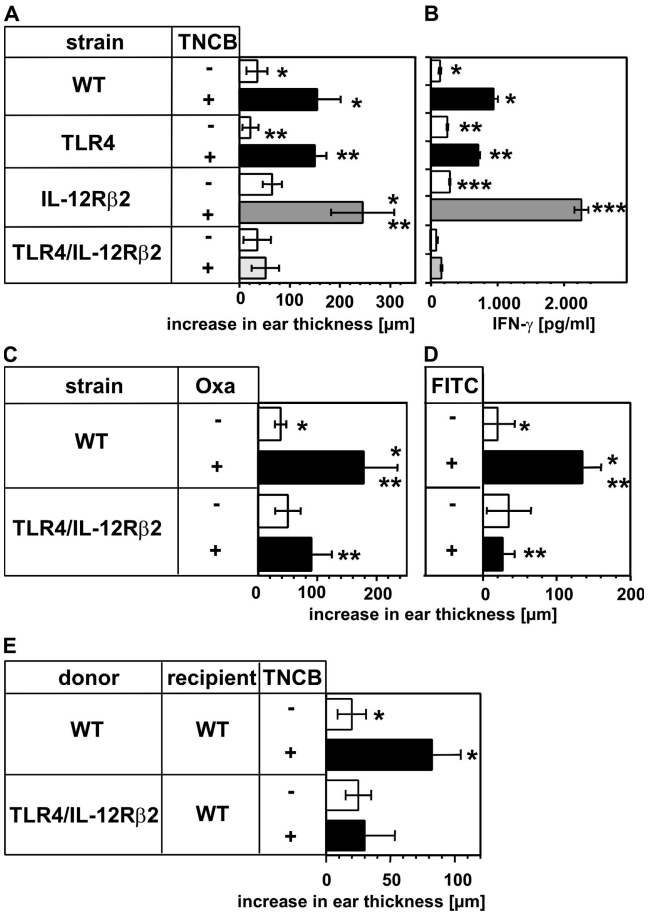

Figure 1. Differential CHS response to TNCB in TLR4−/− and IL-12Rβ2−/− mice. (A) Different CHS responses in mutant mouse strains. Mice of the indicated strains were sensitized on the shaved abdominal skin with TNCB or acetone as vehicle control. Ear challenge with TNCB was performed 5 d later and the 24-h ear swelling response was measured. The results represent the increase in ear thickness for groups of three mice ±SD. Differences were statistically significant at $P > 0.05$ for groups compared (marked by *, **, or ***, respectively). One of six independent experiments is shown. (B) IFN-γ production by lymph node cells from mice after CHS. Lymph node cells were isolated 3 d after ear challenge and stimulated in vitro for 36 h with irradiated spleen cells with or w/o TNBS modification. IFN-γ production was measured by ELISA. The values represent the mean of triplicate measurements ±SD for lymph node cells from pooled mice ($n = 3$). One of three independent experiments is shown. (C and D) Lack of CHS to oxazolone and FITC in TLR4/IL-12Rβ2 double-deficient mice. The indicated mouse strains were sensitized by abdominal painting with oxazolone (C) or FITC (D) solutions. Ear challenge was done on day 6. The results represent the increase in ear thickness for groups of three mice ±SD. Data are representative for three independent experiments. (E) Inability of lymph node cells from sensitized mutant mice to passively transfer CHS sensitization. The indicated mouse strains were sensitized with TNCB by painting of the abdominal skin and ears. Skin-draining lymph nodes were pooled on day 5 and lymph node cells were adoptively transferred into WT recipients. Ear challenge was performed on day 6, and the 24-h swelling reaction was measured. The results represent the mean of three mice per group ±SD. Data are representative of three independent experiments.

CD8+ effector T cells (Tc1) in response to sensitization. The evidence that the TNCB-induced CHS in mice on C57BL/10 background is mediated by Tc1 cells (like that in C57BL/6 mice [references 26, 27]) is presented in Fig. S4 (available at http://www.jem.org/cgi/content/full/jem.20070509/DC1). It shows that skin-draining lymph node cells of TNCB-painted C57BL/10 mice exhibited enhanced IFN-γ responses to an in vitro restimulation with allergen when depleted of CD4+ T cells and no responses when depleted of CD8+ T cells.

Interestingly, TLR4-competent mice, which are deficient for IL-12Rβ2 (C57BL/10; Fig. 1 A), exhibited enhanced CHS to TNCB. The same was true for all mice tested with an impaired IL-12 function, including knockouts for IL-12p35/p40 (129Sv; Fig. 2 A) or the signal transducer and activator of transcription, STAT4 (BALB/c; Fig. 2 B). Furthermore, cells isolated from the draining lymph nodes of TNCB-sensitized IL-12Rβ2 single-deficient mice exhibited strongly increased IFN-γ (Fig. 1 B) and no detectable IL-4 responses (not depicted) to an in vitro restimulation with syngeneic spleen cells modified with TNBS. Thus, our results show that the loss of IL-12 signaling did not prevent a Tc1 response or lead to Th2/Tc2 polarization of the CHS response to TNCB.

On the whole, the data indicate the existence of two distinct pathways of sensitization, one IL-12 dependent and the other IL-12 independent, and that induction of CHS via the IL-12–independent pathway requires TLR4. To test this, we used TLR4/IL-12Rβ2 double-deficient mice carrying a functional mouse TLR4 as a transgene (28). The presence of the transgene in these mice rescued the inducibility of CHS by TNCB (Fig. 3 A). Histology of skin slices shows that the ear of the TLR4 transgenic mouse is noticeably thicker because of an edema and a lymphocyte-rich exudate (Fig. 3 B, slices 1–4), compared with minimal changes in the TLR4/IL-12Rβ2 double-deficient mouse (Fig. 3 B, slices 5–8). These data provide evidence for the essential role of TLR4 in the IL-12–independent pathway of CHS induction.

Failure of CHS induction by TNBS-modified DC from TLR4/IL-12Rβ2 mice

The results obtained so far indicate an impaired generation of allergen-specific T cells in mice with a combined TLR4 and

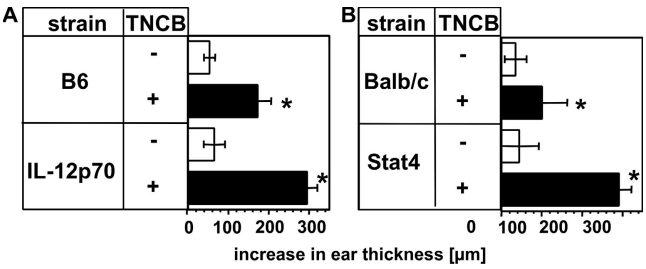

Figure 2. Increased CHS responses to TNCB in IL-12p70−/− and STAT4−/− mice. CHS was induced in IL-12p70 (A) or STAT4-deficient (B) mice as described in Fig. 1 A. The results represent the increase in ear thickness for groups of three mice ±SD. The data are representative of three independent experiments.

IL-12 receptor deficiency. To test the impact of this deficiency on the function of the allergen-presenting DC, we replaced the initial TNCB painting with an intracutaneous (i.c.) injection of in vitro TNBS-modified myeloid DC (DC-TNP) from TLR4/IL-12Rβ2–deficient mice. Injection of such cells, in contrast to DC-TNP from WT (Fig. 4 A) and TLR4 single-deficient mice (Fig. 4 B), failed to sensitize WT mice to TNCB challenge. Furthermore, the presence of the TLR4 transgene restored the sensitizing capability of DC-TNP from TLR4/IL-12Rβ2–deficient mice (Fig. 4 C). Moreover, the injection of DC-TNP from WT mice successfully sensitized the double-deficient mice (Fig. 4 D). These findings provide evidence that the combined absence of TLR4 and IL-12Rβ2 on the allergen-presenting DC is responsible for the absence of CHS induction in double-deficient mice.

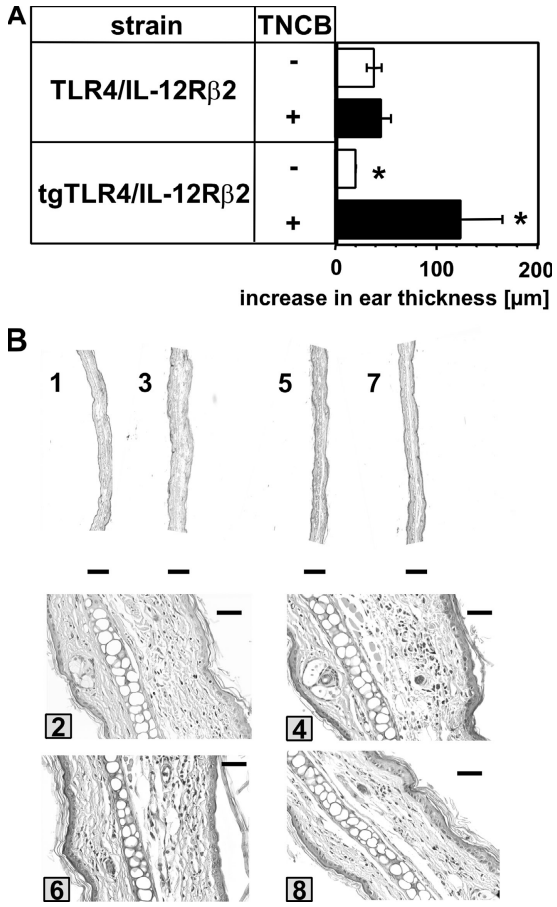

Figure 3. Restoration of CHS response in TLR4/IL-12Rβ2−/− mice by TLR4 transgene. (A) Ear swelling response. CHS was induced as described in Fig. 1 A. The results represent the increase in ear thickness for groups of three mice ±SD. Data are representative of three independent experiments. (B) Histopathology of the ear. Ear skin from TNCB-sensitized TLR4 transgenic (slices 1–4) and nontransgenic (slices 5–8) TLR4/IL-12Rβ2−/− mice 24 h after challenge with vehicle (control: 1, 2, 5, and 6) or TNCB (3, 4, 7, and 8). hematoxylin and eosin staining. Bars: (1, 3, 5, and 7) 400 μm; (slices 2, 4, 6, and 8) 50 μm.

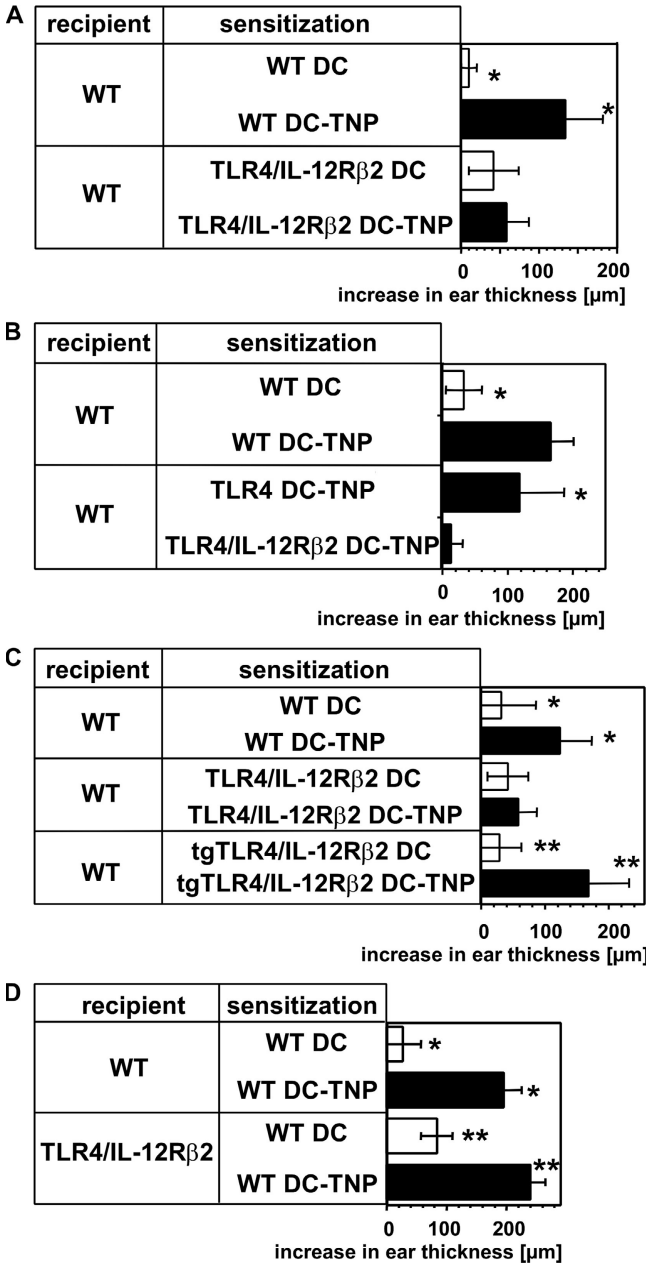

Figure 4. Absence of sensitization for CHS by TNP-modified BM-DC from TLR4/IL-12Rβ2−/− mice. (A) TLR4/IL-12Rβ2−/− DC fail to sensitize WT mice. WT mice were sensitized by i.c. injection of TNP-modified BM-DC from the indicated mouse strains into the shaved abdominal skin. Ear challenge and CHS measurement were performed as in Fig. 1 A. The data represent the mean ear swelling for groups of three mice. One representative of two experiments is shown. (B) WT and TLR4-deficient DC efficiently sensitize WT mice. WT or TLR4-deficient DC-TNP were injected into WT recipient mice. Unmodified WT DC or TLR4/IL-12Rβ2−/− DC-TNP were used as controls. The data represent the increase in ear thickness for groups of three mice ±SD. One representative of three experiments is shown. (C) Transgenic TLR4 expression in TLR4/IL-12Rβ2−/− DC restores sensitizing potential. WT mice were sensitized for CHS by i.c. injection of DC from the indicated strains and CHS elicitation was done as in A. The results represent the increase in ear thickness for groups of three mice ±SD. The data are representative of three independent

Like TLR4, TLR9 is known to drive the maturation of DC and Th1 responses (29–31). We therefore investigated whether triggering via TLR9 might rescue the dysfunction of TLR4/IL-12Rβ2–deficient DC. DC from WT and double-deficient donors were therefore incubated for 1 h with CpG–oligodeoxynucleotides (ODN) before TNBS modification and i.c. injection into WT mice. Preincubation of TLR4/IL-12Rβ2–deficient DC with control ODN failed to reverse their inability to induce CHS (Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20070509/DC1). As shown in Fig. 5 and Fig. S5, the activation via TLR9 endowed the deficient DC with excellent sensitizing properties, suggesting that other members of the TLR family may also support the IL-12–independent CHS.

Requirement of additional cytokine-inducing signals for the full activation of DC by allergen

To examine whether the absence of CHS in TLR4/IL-12Rβ2-deficient mice might be a result of an impaired migration of allergen-loaded DC to the draining lymph nodes, we painted the ears of WT and TLR4/IL-12Rβ2–deficient mice with the allergen FITC. Using flow cytometry, we observed a similar increase in the number of FITC-positive WT and deficient DC into auricular lymph nodes, excluding impaired migration as the reason for the impaired generation of allergen-specific effector T cells (Fig. 6).

Induction of IL-12 and of costimulatory molecules on DC after exposure to allergen are considered to be crucial factors in the efficient generation of contact allergen-specific Tc1 cells. To find out if there was a defect in the activation of double-deficient DC by contact allergen, we stimulated DC from WT and TLR4/IL-12Rβ2–deficient mice for 24 h with TNBS (water-soluble form of the allergen) or TNCB in vitro. This stimulation resulted in the efficient up-regulation of CD40, CD86, I-A^b, and CCR7 (Fig. 7 A) on both WT and double-deficient cells. Surprisingly however, both DC types failed to produce IL-12p40, IL-6, or IFN-αβ (Fig. 7 B) and did not express enhanced levels of IL-23 mRNA (Fig. 7 C) when stimulated with TNBS. In contrast, WT cells stimulated with LPS (TLR4 ligand) and both DC types stimulated with CpG-ODN (TLR9 ligand) did so (Fig. 7, B and C). The control ODN CpG-GC showed no cytokine-inducing activity (Fig. 7 B). Furthermore, DC from IL-12Rβ2 single-deficient mice, like those from WT animals, produced cytokines when stimulated with LPS or CpG ODN but not with TNBS or control ODN (Fig. S6, available at http://www.jem.org/cgi/content/full/jem.20070509/DC1).

These findings indicate that allergen stimulation alone is insufficient for a full activation of allergen-presenting DC and that an additional signal is required. This could be provided by activation via TLR.

experiments. (D) WT DC sensitize TLR4/IL-12Rβ2−/− mice. The experiment was performed as in Fig. 4 A. One representative of three experiments is shown.

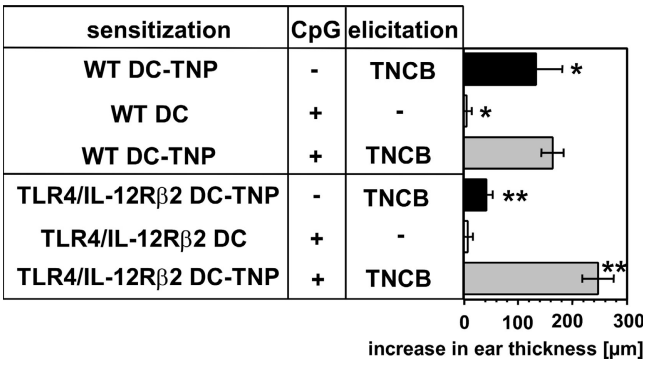

Figure 5. Restoration of the sensitizing capacity for CHS of TLR4/IL-12Rβ2−/− DC-TNP by CpG-ODN pretreatment. The experiment was performed as in Fig. 4 A. However, where indicated, DC were pretreated with CpG-ODN for 1 h before i.c. injection. The data represent the increase in ear thickness for groups of three mice ±SD. One representative of three experiments is shown.

TLR2 and TLR4 requirement for induction of CHS in IL-12–competent mice

The finding of the involvement of TLR4 in the IL-12–independent induction of CHS led us to speculate that TLR signaling is required for the induction of CHS, irrespective of whether an IL-12–dependent or –independent mechanism is available for its development. If this were indeed the case, another TLR must compensate for the loss of the TLR4 function in IL-12–competent TLR4-deficient mice (Fig. 1 A). TLR2 appeared to be a suitable candidate because, like TLR4, it is expressed on the surface of innate immune cells, recognizes

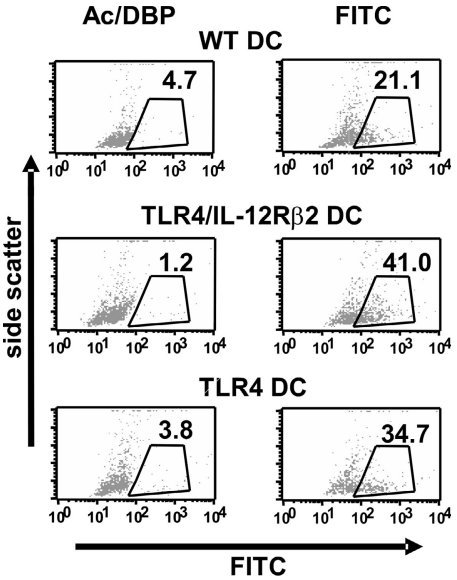

Figure 6. Normal DC migration in FITC-treated TLR4/IL-12Rβ2−/− mice. Mice of the indicated strains received FITC/DBP on both ears. Both auricular lymph nodes were removed 24 h later and FITC-positive DC were detected by flow cytometry. The gate was set on $CD11c^{hi}$/$I\text{-}A^{b\,hi}$ cells. The values for a single lymph node are shown and are representative for three experiments with two individual mice per group.

both microbial and endogenous ligands, and exhibits many overlapping activities with TLR4 (for review see reference 10). As shown in Fig. 8 A, the ear swelling responses of mice with disrupted *TLR2* were slightly, but significantly, reduced when compared with those of WT controls. The combined

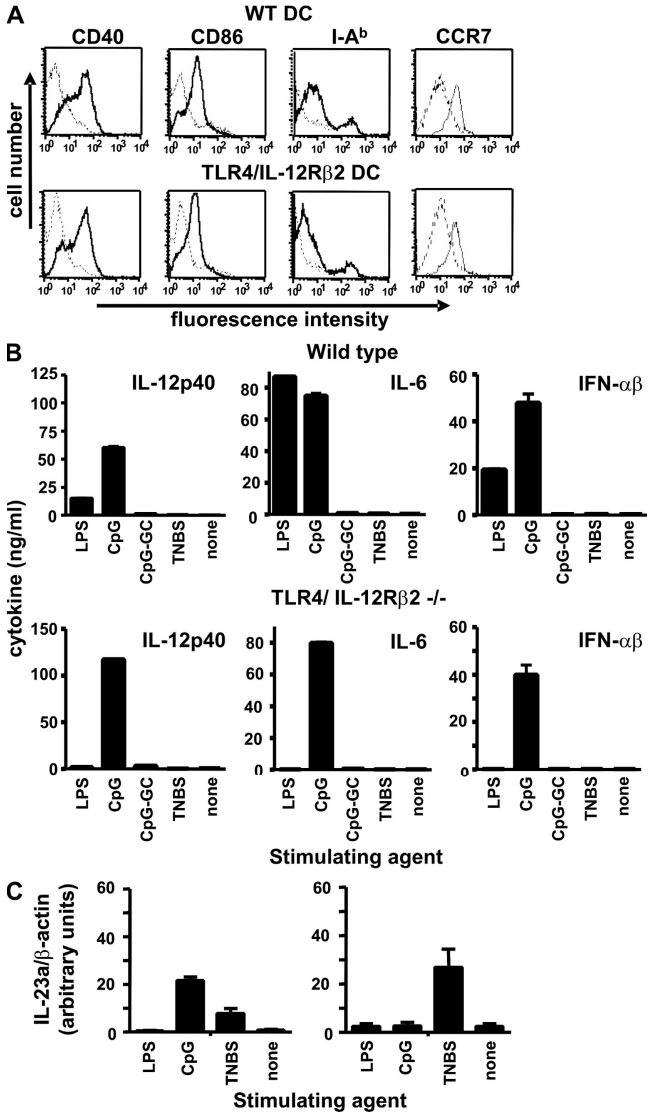

Figure 7. Partial activation of DCs by TNCB. (A) TNCB induces up-regulation of surface markers on DC. BM-DC from WT or TLR4/IL-12Rβ2−/− mice were stimulated for 24 h with 100 μM TNCB. Surface expression of the indicated markers was determined by flow cytometry. Dashed lines represent untreated control DC and solid lines represent TNCB-treated DC. (B) TNBS fails to induce cytokine production by DC. DC from WT or TLR4/IL-12Rβ2−/− mice were stimulated for 24 h with 0.75 mM TNBS. IL-12, IL-6, and IFN-αβ production in culture supernatants was determined by ELISA. As a control, DC were treated with LPS CpG-ODN or control ODN (CpG-GC). The values represent the mean of triplicate measurements ±SD. (C) TNBS fails to up-regulate IL-23 mRNA. The total RNA was isolated from DC of WT or TLR4/IL-12Rβ2−/− mice, stimulated for 3 h with the indicated agents. The levels of mRNA for IL-23 were analyzed by quantitative RT-PCR. The values represent the mean of triplicate measurements ±SD.

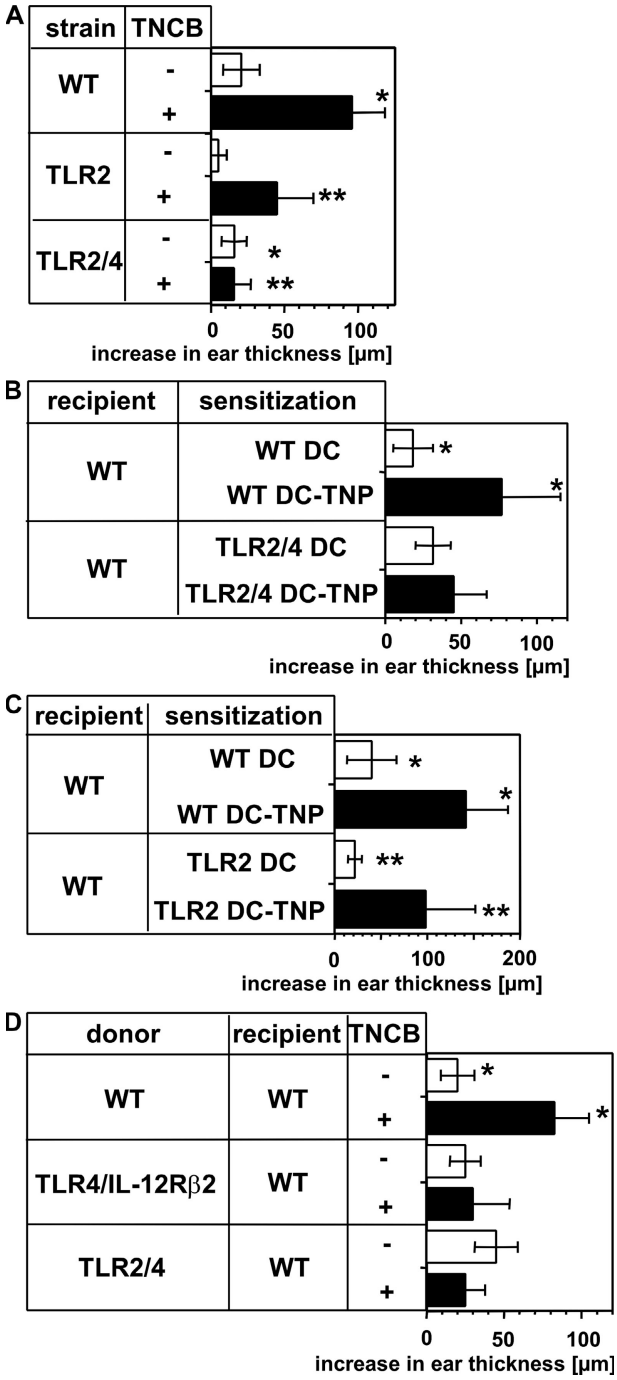

Figure 8. Lack of CHS induction in mice lacking TLR2 and TLR4. (A) Absence of ear swelling reaction in TNCB-sensitized TLR2/TLR4 double-deficient mice. CHS was induced as described in Fig. 1 A. Data are representative of three independent experiments. The data represent the increase in ear thickness for groups of three mice ±SD. (B) Absence of sensitization for CHS by TNP-modified BM-DC from TLR2/TLR4-deficient mice. The experiment was performed as described in Fig. 4 A. The data represent the increase in ear thickness for groups of three mice ±SD. One representative of two experiments is shown. (C) TLR2 single-deficient DC sensitize WT mice for CHS. The experiment was done as in Fig. 4 A. The data represent the increase in ear thickness for groups of three mice ±SD. (D) Inability of lymph node cells from sensitized TLR2/TLR4-deficient mice

absence of both TLR2 and TLR4, however, resulted in the complete absence of CHS to TNCB (Fig. 8 A), indicating that in mice with an intact IL-12–IL-12R system, TLR2 and TLR4 are, to a substantial degree, interchangeable with regard to their contribution to the development of CHS.

To test the impact of the combined TLR2/TLR4 deficiency on the function of the allergen-presenting DC, we replaced the initial TNCB painting by an i.c. injection of in vitro TNBS-modified myeloid DC (DC-TNP) from double-deficient mice. Injection of such cells, in contrast to DC-TNP from WT or TLR2 single-deficient mice (Fig. 8, B and C), failed to sensitize WT mice to TNCB. Finally, lymph node cells from TNCB-painted TLR2/TLR4 double-deficient mice were unable to adoptively transfer CHS to naive WT recipients (Fig. 8 D). These findings indicate that in IL-12–competent mice, expression of at least one of the two receptors TLR2 or TLR4 on DC is required for generation of allergen-specific T cells.

Presence of CHS in mice lacking microbial flora

Because LPS-containing Gram-negative bacteria are part of the skin flora, we examined the possible involvement of this TLR ligand in the induction of CHS. For this purpose, we used germ-free mice and investigated their CHS response to endotoxin-free TNCB under germ-free conditions. Normal ear swelling responses were observed in germ-free TLR- and IL-12–competent NMRI (Fig. 9 A), as well as IL-12Rβ2 single-deficient mice (Fig. 9 B), showing that both IL-12–dependent and IL-12–independent CHS proceed also in a microbe-free environment. In contrast, in this environment IL-12–independent CHS was absent in TLR4/IL-12Rβ2 double-deficient mice (Fig. 9 C). In view of this finding, we hypothesize that the TLR4 activation, which is essential for the induction of both IL-12–dependent and –independent CHS, may proceed via endogenous ligands. This notion is further supported by the data shown in Fig. S7 (available at http://www.jem.org/cgi/content/full/jem.20070509/DC1). In contrast to BMDC from WT mice (Fig. S7 B), those from TLR4/IL-12Rβ2 double-deficient mice failed to induce CHS in germ-free TLR4/IL-12Rβ2 double-deficient mice (Fig. S7 A). Interestingly, pretreatment of germ-free NMRI mice with an inhibitor of HA (32) before sensitization significantly reduced the CHS response (Fig. 10). These data indicate a role of HA in the skin as an endogenous TLR ligand contributing to sensitization for CHS.

DISCUSSION

Our investigation of the role of TLR4 in the induction of CHS has revealed an as yet unknown TLR4-dependent mechanism of CHS induction to contact allergens that is independent of IL-12. The finding that mice lacking both TLR4 and IL-12 receptors are completely resistant to CHS, whereas mice lacking only one of the two receptors remain susceptible, shows that TLR4 and IL-12 receptor can mutually replace each other in the induction of CHS. Interestingly, we found that loss of IL-12 function predisposed the TLR4-competent mice to CHS, leading to an enhanced allergic response. An enhanced response to TNCB has been reported in mice with an impaired IL-12 function caused by the disruption of *Tyk2* (7). In that study however, T cells from sensitized Tyk2−/− mice exhibited a high IL-4 and decreased IFN-γ production (Th2 polarization), whereas in our study, T cells of similarly sensitized IL-12 receptor-deficient mice exhibited no IL-4 but a high IFN-γ production (Th1/Tc1 polarization) after rechallenge with the allergen. This may be because of the existence of more than one IL-12–independent pathway, all leading to an enhanced CHS response, regardless of the type of T cell polarization. The augmentation of CHS in mice with an impaired IL-12 function could be explained by a loss of the inhibitory effect of IL-12 on IL-17–producing $CD8^+$ T cells (33), which, according to recent reports, participate in the elicitation of CHS (33, 34). The impaired generation of allergen-specific T cells in TNCB-treated TLR4/IL-12Rβ2–deficient mice suggested a defective cross talk between DC and T cells. The inability of

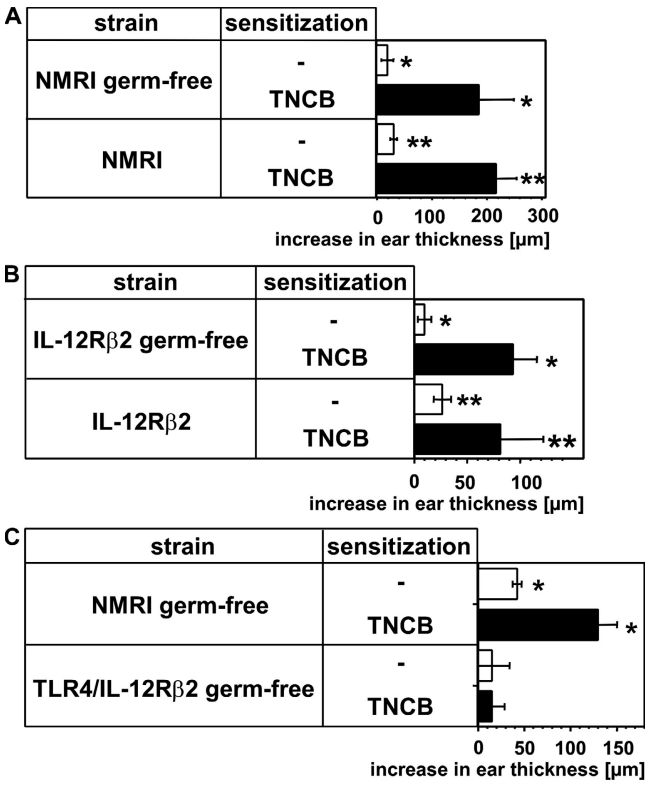

Figure 9. CHS induction in germ-free mice. CHS was induced as described in Fig. 1 A. Germ-free and conventionally housed NMRI (A), IL-12Rβ2 (B), or TLR4/IL-12Rβ2−/− (C) mutant mice were used. The ear swelling reaction was measured 24 h later. Results represent the mean of three mice per group ±SD. Data are representative of two experiments.

to passively transfer CHS sensitization. The data represent the increase in ear thickness for groups of three mice ±SD. The experiment was performed as described in Fig. 1 E.

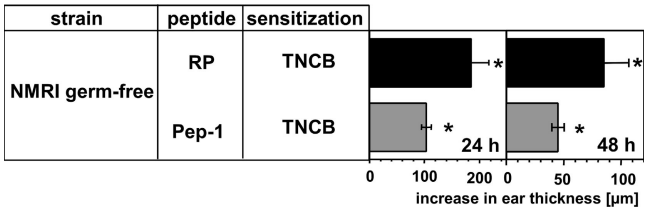

Figure 10. HA inhbitor Pep-1 reduces CHS in germ-free WT mice. Germ-free NMRI mice were injected with 40 µg Pep-1 or the control random peptide RP in 20 µl PBS in the right ear pinna 24 and 1 h before sensitization. Mice were sensitized by painting of 20 µl of 3% TNCB/acetone on the right ear. Mice were challenged on day 5 on the left ear with 1% TNCB/acetone, and ear thickness was measured 24 and 48 h after challenge. Data represent the ear swelling reactions for groups of six (RP) and eight (Pep-1) mice ±SD. The peptide sequences were as previously published (32).

allergen-loaded TLR4/IL-12Rβ2–deficient DC to sensitize WT or TLR4/IL-12Rβ2–deficient recipients and its full restoration by the presence of the TLR4 transgene in double-deficient DC is evidence that the induction of IL-12–independent CHS requires TLR4 and that it is sufficient when this receptor is present only on DC.

Although the exact role of TLR4 in the induction of IL-12–independent CHS remains to be elucidated, our study offers a possible explanation. We showed in this paper that DC stimulated in vitro with TNCB efficiently up-regulated costimulatory molecules MHC class II and CCR7 but failed to elicit cytokine production, which is a prerequisite for a successful priming of T cells to contact allergens (1, 2). This function, however, can be provided by stimulation with TLR ligands (for review see reference 10; this study). Therefore, TLR4 might be required for the induction of cytokines in DC of mice with impaired IL-12 function. Furthermore, our study demonstrates that activation through TLR9 can replace TLR4 triggering for the generation of functional TNP-presenting TLR4/IL-12Rβ2–deficient DC. TLR9, the receptor for microbial DNA, is important for recognition of certain viruses and bacteria (for review see reference 10; 25). It is therefore possible that, particularly in the skin, infections may provide TLR ligands that support or replace the function of TLR4 during sensitization to contact allergens.

A possible candidate cytokine induced via TLR4 activation is IL-23, a member of the IL-12 family (35, 36). IL-23 is inducible via TLR4 (37) (this study) and, like IL-12, participates in DC activation and in the enhancement of delayed-type hypersensitivity responses (3). IL-23 stimulates, whereas IL-12 inhibits, IL-17–producing CD8+ T cells in CHS (33). In our study, IL-23 would come in question for the induction of CHS observed in IL-12Rβ2– and STAT4-deficient mice but not for the CHS observed in mice deficient in IL-12p40 and IL-12p35/p40 or IL-12Rβ1 because the p40 subunit and the receptor IL-12Rβ1 chain is shared by both the cytokine IL-23 and IL-12 and their receptors, respectively (35, 36). A further candidate for IL-12 replacement is IFN-αβ. It shares several relevant activities with IL-12, such as the generation of activated DC (38–42), stimulation of T cell proliferation, and generation of IFN-γ–producing $CD8^+$ T cells (43–45). In this study, LPS-activated TLR4 competent DC, as well as both CpG-activated TLR4 competent and deficient DC, produced high levels of IFN-αβ in vitro. Furthermore, preliminary experiments indicate a substantial induction of IFN-β mRNA expression in regional lymph nodes of mice between 4 and 18 h after the primary exposure to TNCB (unpublished data). Another candidate of interest is CD40L. Gorbachev and Fairchild (46) reported that engagement of CD40 promotes CHS responses and development of IFN-γ–producing T cells, independently of IL-12. We show in this study that exposure to TNCB alone enhances CD40 expression on DC. In this connection, the induction of CD40/CD40L ligand by LPS (i.e., via TLR4) has been reported (47, 48).

The inability of TNCB to induce cytokines in DC observed in this study also raised the question of how IL-12 is induced in CHS. The finding that TLR2/TLR4 double-deficient mice are completely resistant to CHS, whereas mice lacking only one of the two receptors remain susceptible, shows that these receptors can substitute for each other in the induction of CHS in IL-12–competent mice. It is well documented that activation of TLR2 and TLR4 on DCs results in overlapping, but also distinct, cellular responses (49). This is in part because of the fact that both receptors use the adaptor MyD88 for signaling, whereas TLR4 also uses the adaptor Toll/IL-1 receptor domain–containing adaptor-inducing interferon-β (TRIF). The TRIF-dependent activation up-regulates the MyD88-dependent production of proinflammatory cytokines and exhibits additional effects, including induction of IFN-αβ (for review see reference 10). The inability of TLR2 ligands to activate the TRIF-dependent signaling pathway may explain why this receptor is incapable of substituting for TLR4 in the IL-12 independent CHS.

Most interestingly, the development of CHS to TNCB in germ-free WT and IL-12Rβ2–deficient mice, but its absence in germ-free TLR4/IL-12Rβ2–deficient mice, i.e., in the absence of microbial flora, provides strong support for the hypothesis of an involvement of endogenous TLR ligands (22, 23) in the sensitization to allergens. Although the identity of these ligands was not addressed in this study, degradation products of HA seem to be suitable candidates because they can activate TLR4 and TLR2 (20, 21). HA is abundant in the skin as a high molecular mass polymer. It facilitates migration of skin DC and its fragments can deliver maturational signals to DC (32, 50). Interestingly, DC themselves synthesize and possibly also degrade HA (50, 51). It is worth mentioning that shortly after TNCB painting we identified biochemical and histochemical changes in HA skin content (i.e., a subcutaneous accumulation of HA and its concomitant disappearance in the epidermis [reference 1] [unpublished data]). HA fragments were shown to elicit various proinflammatory processes (22) and to be responsible for TLR2 and TLR4 stimulation in bleomycin-induced lung injury (20, 21). A recent publication reported that the inflammatory response to sterile skin injury is weaker in TLR4-deficient mice, probably

because of a partial loss of HA signaling (52). These authors further demonstrated that a receptor complex consisting of TLR4, CD44, and MD-2 is required for full inflammatory response of cultured cells to HA fragments. Importantly, CD44−/− mice exhibited an impaired CHS response, which is in agreement with the involvement of HA in the induction of CHS. In line with this study is the inhibition of sensitization for CHS by the HA inhibitor Pep-1 (32). Interestingly, in our study this inhibitor also reduced the CHS response in germ-free NMRI mice. This points to a contribution of HA as a potential TLR2/4 ligand involved in the sensitization for CHS.

Apart from HA degradation products, several endogenous ligands of TLRs, especially of TLR4, have been reported (22, 23). These also come into question as potential endogenous ligands participating in the induction of CHS.

In the past, the search for a genetic predisposition to ACD concentrated on the MHC linkage but failed to find one. By using mice with natural mutations of *IL-12Rβ* and/or *TLR4*, our study shows that a genetic predisposition to CHS can depend on mutations in innate immune receptors that critically affect DC function. In humans, TLR and IL-12/IL-12R polymorphisms affecting the function of these molecules have been described (53–60). The present study, therefore, encourages the search for a linkage between polymorphisms or mutations in these genes and susceptibility to CHS.

MATERIALS AND METHODS

Mice. All mice were bred at the Max Planck Institute under specific pathogen-free conditions. The following animals of both sexes, 7–12 wk of age, were used. WT: C57BL/10ScSn, 129/Sv, and BALB/c. NMRI and mutant mice: TLR4-deficient (C57BL/10ScN [references 15, 61] and BALB/c lps/lps [reference 62]), IL-12Rβ2–deficient (C57BL/10ScSn [reference 24]), TLR4/IL-12Rβ2–deficient (C57BL/10ScCr [references 24, 61]), TLR4-transgenic, TLR4/IL-12Rβ2–deficient (TCr-1 [reference 16]), IL-12p40−/− (BALB/c; provided by M. Kopf, Eidgenössische Technische Hochschule Zürich, Switzerland), IL-12p35/p40−/− (129/Sv [reference 61]), and STAT4−/− (BALB/c [reference 63]).

Germ-free IL-12Rβ2–deficient and TLR4/IL-12Rβ2–deficient mice were generated by embryo transfer into germ-free NMRI pseudopregnant recipient females under germ-free conditions. NMRI, and the deficient mice after weaning, were randomly bred at the Max Planck Institute animal facility in plastic isolators on a gamma-irradiated commercial diet and autoclaved water ad lib. Feces samples were routinely cultured in Standard I nutrient broth and examined for indications of contamination. The absence of bacterial skin flora was confirmed by culturing abdominal skin homogenates of germ-free mice under aerobic and anaerobic conditions. 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 Max Planck Institute and the University Medical Center Freiburg.

Chemicals and reagents. TNBS, FITC, and oxazolone were obtained from Sigma-Aldrich, TNCB from VeZerf Laborsynthesen GmbH, and synthetic CpG-ODN 1668 (TLRgrade) and control ODN 1720 (1668/GC; TLRgrade) from Qbiogene. All haptens, CpG-ODN, and control ODN were LPS-free as tested by Limulus amebocyte assay (Endogen). LPS of *Salmonellosis abortus equi* was obtained from Alexis Biochemicals. Antibodies for FACS analysis to CD40 (3/23), CD86 (GL1), CD11c (HL3), and I-A^b (AF6-120.1) were obtained from BD Biosciences, to TLR4/MD-2 (MTS510) from Qbiogene, and to CCR7 (4B12) from eBioscience and were used as FITC, PE, or Biotin conjugates. Streptavidin-Cy-Chrome was obtained from BD Biosciences. Anti–IFN-γ (R4-6A2 and XMG1.2) for ELISA was obtained from BD Biosciences. The HA inhibitor Pep-1 and the control peptide RP have been described previously (32) and were obtained from Iris Biotech GmbH.

Generation of BM-derived DC. BM-derived DC (BM-DC) were prepared as previously described (26).

In vitro stimulation of DC. BM-DC were harvested from cultures on day 7. Stimulation of 5×10^5 DC was done in 2 ml RP-10 for 24 h with 1 μg/ml LPS, 3 μg/ml CpG-ODN or control ODN, or different concentrations of TNCB dissolved in acetone in 24-well plates (BD Biosciences). Unless stated otherwise, the TNCB concentration was 0.1 mM. As a control, DC were treated with the highest concentration of acetone used (0.1 and 1 vol %). This treatment had no effect on cell viability or the outcome of the experiments. Stimulation with TNBS was done in triplicate at a concentration of 0.75 mM TNBS in PBS. As a control, DC were treated with an equal volume of PBS. Results were similar to those obtained with TNCB. For induction of IL-23 mRNA, 10^6 DC/ml culture medium were stimulated in 6-well plates for 3 h.

In vitro priming of naive T cells with DC. Cultures were set up in 96-well plates using a mixture of 4×10^5 spleen/lymph node cells per well and 5×10^3 unmodified or TNBS-modified BM-DC. IFN-γ was measured by ELISA in supernatants from cultures after 5 d.

Immunization and induction of CHS. For induction of CHS, 100 μl of 7% TNCB/acetone or acetone as vehicle control on day 0 was painted on the shaved abdomen for sensitization, followed by epicutaneous application of 20 μl of 1% TNCB on the dorsum of both ears of all mice on day 5 for elicitation. Alternatively, mice were sensitized by i.c. injection of 3×10^5 unmodified or TNBS-modified DC into two sites of the shaved abdomen in 2×50 μl PBS as previously described (26), followed by elicitation as for the mice on day 5. Ear measurement was done before and 24 h after ear challenge using an engineer's micrometer (Mitutoyo). CHS to oxazolone and FITC (Sigma-Aldrich) was induced by sensitization on day 0 with 150 μl of 3% oxazolone in ethanol (EtOH) or EtOH (vehicle control) or 400 μl of 0.2% FITC in 1:1 Ac/DBP (acetone/dibutylphthalate; Sigma-Aldrich) or 1:1 acetone/Ac/DBP (vehicle control) on the shaved abdominal skin and elicitation with 20 μl 1% oxazolone/EtOH or 0.2% FITC/Ac/DBP on both ears on day 6. The 24-h swelling reactions were measured.

Adoptive CHS. Mice of the various donor strains were sensitized by painting the shaved abdominal skin with 100 μl of 7% TNCB and both ears with 20 μl of 1% TNCB. Auricular, axillary, maxillary, and superficial inguinal lymph nodes were harvested 5 d later. Single cell suspensions were prepared and 2×10^7 lymph node cells were injected i.v. into naive WT recipient mice. Thickness of both ears of the recipients was measured 24 h after adoptive cell transfer before painting with 1% TNCB and the swelling was measured 24 h after painting.

Histology. Ears from vehicle or allergen-sensitized mice were removed 24 h after allergen ear challenge and fixed in 4% buffered formaldehyde. 5-μm organ slices were prepared and stained with hematoxylin and eosin.

In vitro stimulation of T cells after CHS. Auricular, maxillary, and axillary lymph nodes were pooled and a single cell suspension was prepared. Erythrocytes were lysed and 4×10^5 cells per well were cocultivated with 3×10^5 irradiated (3,000 rad) C57BL/6 spleen cells modified with 3 mM TNBS for 7 min at 37°C as previously described (26, 27). Cultivation was done in 96-well U-bottom plates (Corning).

In vivo DC migration. Mice were painted on both sides of each ear with 50 μl of 0.2% FITC (Sigma-Aldrich) in Ac/DBP (1:1) or vehicle alone. Auricular lymph nodes were removed 24 h later and live FITC-positive DC were measured in a CD11c/MHC II I-A^b high gate. The positive cells were quantified in comparison to the negative fraction in vehicle-treated mice.

Cytokine detection. ELISA for IFN-γ and IL-4, IL-6, IFN-αβ, IL-12p40, or IL-12p70 was performed as previously described (26, 27). IFN-α/β was detected as previously described using a bioassay (64).

Quantification of IL-23 mRNA levels with reverse transcription and real-time PCR. Total RNA was isolated from 1.8×10^7 DC cells by a guanidinium isothiocyanate-phenol-chloroform-isoamyl alcohol procedure (65) as described previously (66). To exclude DNA contamination, RNA samples were treated with RNase free DNase I (Fermentas). cDNA was prepared using Expand reverse transcription (Roche) and oligo-dT. Real-time PCR quantification of RNA expression was done using the LightCycler II system (Roche) and the Quantitect SYBR Green PCR kit (QIAGEN) according to the manufacturer's instructions. Arbitrary units of relative expression were generated by dividing the value obtained for IL-23 by the value of β-actin. The following primers were used for β-actin real-time PCR: TGG AAT CCT GTG GCA TCC ATG AAA and TAA AAC GCA GCT CAG TAA CAG TCC G. For the measurement of IL-23 mRNAs, gene-specific primers from (Mm_Il23a_SG QuantiTect Primer Assay; QIAGEN) were used.

Flow cytometry. Flow cytometry was done as described previously (26) using antibodies obtained from BD Biosciences and a FACScan instrument (BD Biosciences) with CellQuest Software (BD Biosciences).

Statistics and statistical analysis. Statistical analysis was conducted using one-way analysis of variance with pairwise comparisons according to the Student Newman Keuls method using SigmaStat software (version 3.5; Systat Software, Inc.). Cytokine data were analyzed using Student's *t* test. Data are shown as means ± SD. Differences between groups as marked by asterisks were statistically significant at $P < 0.05$.

Online Supplemental Materials. Fig. S1 shows the CHS in TLR4-deficient BALB/c lps/lps mice. Fig. S2 shows the IFN-γ production by lymph node cells of mice sensitized with oxazolone or FITC. Fig. S3 shows the immunohistology of skin lesions after repeated exposure to FITC. Fig. S4 shows that TNCB-induced CHS in C57BL/10 mice is mediated by Tc1 cells. Fig. S5 shows the restoration of the sensitizing capacity of TLR4/IL-12Rβ2−/− DC-TNP by pretreatment with CpG-ODN. Fig. S6 shows that TNBS fails to induce cytokine production in BMDC from IL-12Rβ2−/− mice. Fig. S7 shows that only WT DC-TNP induce CHS in germ-free IL-12Rβ2−/− mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20070509/DC1.

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