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NLRP12 controls dendritic and myeloid cell migration to affect contact hypersensitivity[1]

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Abstract

Nucleotide-binding domain leucine rich repeat (NLR) proteins have emerged as fundamental regulators of inflammation and immunity. Although first described eight years, a physiologic role for NLRP12 has remained elusive until now. Here we describe a novel role for NLRs in inflammation by regulating immune cell migration. We find that murine *Nlrp12*, an NLR linked to atopic dermatitis and hereditary periodic fever in humans, is prominently expressed in dendritic cells (DCs) and neutrophils. *Nlrp12*-deficient mice exhibit attenuated inflammatory responses in two models of contact hypersensitivity that exhibit features of allergic dermatitis. This cannot be attributed to defective antigen processing/presentation, inflammasome activation or measurable changes in other inflammatory cytokines. Rather, $Nlrp12^{-/-}$ DCs display a significantly reduced capacity to migrate to draining lymph nodes. Both DCs and neutrophils fail to respond to chemokines *in vitro*. These findings indicate that NLRP12 is important in maintaining neutrophils and peripheral DCs in a migration competent state.

Keywords

NLR; skin contact hypersensitivity; cell migration

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Introduction

NLRs constitute a large family of mammalian genes that are homologous to innate immune defense genes extending back to the plant kingdom. In animals, NLRs function as important components of inflammation and immunity. To date, at least five NLR proteins are predicted to form a large complex termed the inflammasome, which serves as a scaffold for caspase-1 activation and subsequent release of bioactive IL-1β and related cytokines. Other NLR proteins such as NOD1, NOD2 and NLRX1 have been shown to function as regulators of inflammatory cytokines, chemokines and antimicrobial peptides (1, 2); however, the in *vivo* functions of the majority of NLR proteins remain to be elucidated.

We have previously shown that human *NLRP12* is expressed by monocytes/macrophages and granulocytes and inhibits the activation of noncanonical NF-κB by associating with and inducing proteasome-mediated degradation of NF-κB inducing kinase (NIK) (3, 4). More recently, mutations that result in a truncated form of NLRP12 are linked to hereditary periodic fevers that manifest with recurrent fevers, joint pain, and skin urticaria (5). In addition, a single nucleotide polymorphism in Intron 9 of *NLRP12* is loosely associated with atopic dermatitis (6). Here we present the first-identified *in vivo* role for NLRP12 by demonstrating its role in the migration of DCs and neutrophils. This impacts contact hypersensitivity (CHS), but cannot be attributed to impaired IL-1β production, and hence is distinct from the inflammasome function.

Materials and Methods

Mice

Nlrp12$^{-/-}$ and *Nlrp3*$^{-/-}$ mice were generated by homologous recombination and backcrossed for 9 generations to C57BL/6 (Jackson Laboratories) and maintained in specific pathogen-free housing. OT-II mice, which express the OVA 323-339-specific TCR transgene on the C57BL/6 background, were kindly provided by M. Croft (La Jolla Institute of Allergy and Immunology). Experiments were performed with 6–12 week old age- and sex- matched mice. All studies were conducted in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals* and the Institutional Animal Care and Use Committee guidelines of UNC-CH.

Nlrp12$^{-/-}$ Genotyping

Genomic tail DNA was amplified with: F1 5’-CCCACAAAGTGATGTTGGACTG-3’, F2 5’-GCAGCGCATCGCCTTCTATC-3’, R1 5’-GAAGCAACCTCCGAATCAGAC-3’.

Expression analysis of *Nlrp12*

cDNA was synthesized from total RNA using moloney murine leukemia virus (MMLV) reverse transcriptase (Invitrogen). *Nlrp12* intron-spanning primers: F 5’-GTCCAGACTCAGTCCACATA, R 5’-GTATAAGGCCAGCTCGATCA. *GAPDH*: F 5’-TGAAGCAGGCATCTGAGGG, R 5’-CGAAGGTGGAAGAGTGGGAG. Cell populations were isolated as described: T cells and B cells by negative selection; neutrophils from bone marrow (BM) (7); DCs (8), macrophages (9), mast cells (10) and osteoclasts (11) from BM precursors; Raw264.7 macrophages from American Type Culture Collection (ATCC); Resident peritoneal macrophages by lavage with PBS and overnight adherence.

Generation of DCs and isolation of neutrophils

DCs were generated from BM precursors as described (8). Neutrophils were purified from BM using a discontinuous Percoll (Sigma) gradient as described (7). Purity was 80–90% by differential staining of cytospins and flow cytometry. Viability was >95%.

Contact hypersensitivity (12)

Mice were sensitized by topical application of hapten to the footpads and depilated abdomen: either 200μl of 3% oxazolone (Sigma-Aldrich) in ethanol, or 200μl of 0.5% FITC (Sigma-Aldrich) in 1:1 acetone:dibutyl phthalate (Sigma-Aldrich). Five days later, 20μl of 1% oxazolone in ethanol or 20μl of 0.5% FITC in acetone:dibutyl phthalate was topically applied to one ear. The opposite ear was mock-treated with solvent, and control mice were treated with solvent on both ears. After 24 hours, mice were euthanized and 8mm circular samples of ear tissue were excised and weighed, then the weight of the carrier-treated ear was subtracted from that of the hapten-treated ear. Ear tissue was frozen on dry ice (see below) or fixed in formalin, paraffin embedded, sectioned, and stained with H&E. Immune cell infiltration was quantified as average pixel density ($\times 10^4$) using ImageJ software from 4 fields per ear.

Ear tissue homogenates

Individual ears were manually homogenized in T-PER reagent (Thermo Scientific) using RNase/DNase-free plastic pestles (Kontes) then sonicated. Total protein concentration was determined by Bradford assay (Bio-Rad). IL-1β, TNFα, and MPO were measured by ELISA (IL-1β and TNFα, BD Biosciences; MPO, Hycult Biotechnology).

FITC-induced in vivo migration (13)

Twenty μl of 0.5% FITC in 1:1 acetone:dibutyl phthalate was applied topically to both ears. After 24 or 48 hours, draining (auricular and cervical) and non-draining lymph nodes (LNs) were removed. Cells were stained with anti-CD11c-APC (HL3, BD Biosciences) and analyzed by flow cytometry using a CyAn ADP flow cytometer (Beckman Coulter) and FlowJo software (Tree Star Inc.). Ear epidermal sheets (14) were stained with biotin-labeled anti-I-A^b (AF6-120.1; BD Biosciences), plus streptavidin-Alexa Fluor 595 (Invitrogen), then visualized by fluorescent microscopy. DCs per 400× field are the mean of 4 fields per sample, counted by a blinded reader.

Ova-induced in vivo migration (15)

Mice were injected s.c. into one foodpad with 20μl of Alexa Fluor 647 labeled Ova (2 mg/ml in PBS; Invitrogen) emulsified in CFA. After 24 hours, draining (popliteal) and non-draining LNs were removed and analyzed by flow cytometry as above.

In vitro migration

Bone marrow DCs (BMDC) were seeded at 2×10^5 per upper well of 96-well transwell plates with 5μm pores (ChemoTx System; NeuroProbe), over chemokines (Peprotech) in serum-free RPMI, and incubated at 37°C for 3 hours. Migrated cells were quantified using XTT (Sigma). For neutrophil migration: 3μm pores, cultured 40 minutes at 37°C, migrated cells quantified using ToxiLight Bioassay Kit (Lonza).

Statistics

Central tendencies are presented as mean ± SEM. Pairwise comparisons were made using two-tailed tests, all $\alpha = 0.05$: Mann Whitney U (*in vivo* Fig. 1B–E, 3B–D); Student's t test (*in vitro* Fig. 2F–G, 4I); Wilcoxon matched pairs (paired data Fig. 3E); One-sample Wilcoxon signed-rank, hypothesized median = 100% (Fig. 3F). Outliers by Grubb's test, statistics computed with Prism 4 (GraphPad).

Results and Discussion

Nlrp12$^{-/-}$ mice were generated by replacing a region of exon 3 containing the Walker A and Walker B sequences with the neomycin resistance gene (Fig. 1A–B). *Nlrp12*$^{-/-}$ mice displayed no gross abnormalities, and there were no identifiable abnormalities in the cellularity of the peripheral blood, BM, spleen or LNs (Fig. S1). Similar to human *NLRP12,* murine *Nlrp12* was expressed in the BM and spleen, and at the cellular level in neutrophils and DCs (Fig. 1C). Unlike human *NLRP12*, however, murine *Nlrp12* was not highly expressed in transformed Raw264.7 macrophages, macrophages differentiated from bone-marrow precursors, or macrophages resident within the peritoneum.

NLRP12 is not required for IL-1β secretion in response to TLR +ATP stimulation

Given the critical role of other NLR proteins in IL-1β processing, we tested the ability of *Nlrp12*$^{-/-}$ cells to produce IL-1β after stimulation with a variety of elicitors. No significant difference in IL-1β production was detected in *Nlrp12*$^{-/-}$ vs. WT BM cells or BMDCs stimulated with LPS, LPS + ATP, other TLR ligands, or TNFα (Fig. 1D). As a positive control, *Nlrp3*$^{-/-}$ cells failed to produce IL-1β when properly stimulated (Fig 1D). *Nlrp12* did not affect the production of IL-12p40, IL-6 and TNFα (Fig. S2), nor did it affect survival or liver and kidney function in models of LPS-induced endotoxic shock (Figure S3). Thus *Nlrp12* in murine DCs did not significantly affect cytokine production in response to TLR stimulation.

NLRP12 promotes hapten induced contact hypersensitivity

The importance of *NLRP12* in cutaneous inflammation has been suggested through its recent linkage to human hereditary periodic fevers with skin urticaria (5). We evaluated the role of NLRP12 in cutaneous inflammation by assessing CHS, a mouse model of allergic dermatitis. WT and *Nlrp12*$^{-/-}$ mice were sensitized topically on the abdomen with hapten – either oxazolone or FITC and elicited 5 days later on the ear. Compared to controls, *Nlrp12*$^{-/-}$ mice displayed a weaker response to both haptens, as indicated by significantly reduced swelling (Fig. 2A–C) and reduced cellular accumulation at the site of elicitation (Fig. 2D). The partial effect of *Nlrp12* deletion is typical of other NLR responses in mice (16). During a CHS response, neutrophils migrate out of the bloodstream and into the affected skin tissue (17). In *Nlrp12*$^{-/-}$ mice, the accumulation of myeloperoxidase-positive (MPO) neutrophils in the skin was significantly reduced in hapten-treated ears compared to WT mice (Fig 2E).

Others have shown that CHS is attenuated in *Nlrp3*$^{-/-}$ mice due to decreased IL-1β (18). Ear tissues from *Nlrp12*$^{-/-}$ mice did not show reduced IL-1β (Fig. 2F) nor reduced TNFα (Fig. 2G). These data suggest that *Nlrp12* does not affect inflammasome function nor TNFα in CHS.

NLRP12 potentiates DC migration from the periphery to the draining lymph nodes

Given the critical role of DCs in the CHS model (19) coupled with the high expression of *Nlrp12* in these cells, we determined if DC function is dependent upon NLRP12. Expression of MHC class II and co-stimulatory factors CD80, CD86 and CD40 was not affected by *Nlrp12* during BMDC maturation (Fig S4). To determine if *Nlrp12* affected antigen processing and presentation, WT and *Nlrp12*$^{-/-}$ BMDCs were pulsed with OVA and then co-cultured with CFSE-labeled OT-II splenocytes specific for the OVA peptide 323–339. WT and *Nlrp12*$^{-/-}$ BMDCs induced equivalent antigen-dependent T cell proliferation, indicating that NLRP12 was not required for antigen presentation by DCs (Fig. S5).

A key function of DCs in the CHS model is to collect peripheral antigens and migrate to draining LNs. To evaluate DC migration in vivo, FITC was applied topically to the ears of WT and *Nlrp12*$^{-/-}$ mice. Draining LNs were removed 24 and 48 hours later, and the presence of FITC^{+} CD11c^{+} DCs was assessed by flow cytometry. FITC^{+} DCs in the draining LNs of *Nlrp12*$^{-/-}$ mice were significantly reduced compared to WT mice 24 and 48 hours after FITC application (Fig. 3A–C). These DCs did not traffic to other immune organs, as FITC^{+} CD11c^{+} DCs were not detected in non-draining LNs, BM or spleen (Fig. 3A and S6). In contrast, *Nlrp3*$^{-/-}$ DC migration to draining LNs was comparable to WT DCs (Fig. 3D). Quantification of DCs in untreated skin revealed that *Nlrp12* did not affect resting DC numbers (Fig. 3E). However, following FITC treatment the number of WT skin DCs decreased by over 40% while the number of *Nlrp12*$^{-/-}$ skin DCs changed by less than 10%, indicating that *Nlrp12* is required for DCs egress from the skin (Fig. 3E).

To further examine the migratory capacity of *Nlrp12*$^{-/-}$ DCs, we measured migration of DCs to the draining LNs in response to s.c. OVA antigen. The absence of *Nlrp12* reduced OVA^{+} DCs in the draining LNs by nearly 75% (Fig. 3F). These results support migratory defects in *Nlrp12*$^{-/-}$ DCs.

Nlrp12$^{-/-}$ DCs and neutrophils fail to respond to chemokines *in vitro*

DCs migrate to draining LNs in response to chemokine engagement of CCR7 and CXCR4 on DCs (20, 21). *Nlrp12* did not affect the expression of CCR7 and CXCR4 on BMDCs (table S1). However *Nlrp12*$^{-/-}$ BMDCs demonstrated significantly reduced migration toward CCR7 and CXCR4 ligands CCL19, CCL21, and CXCL12 in an *in vitro* transwell assay (Fig. 4A–C and table S2). In contrast, *Nlrp3*$^{-/-}$ BMDCs exhibited normal migration toward these chemokines (Fig. 4D–F). As a negative control, BMDC from all genotypes failed to migrate toward CCL5 (Fig. 4G–H), a chemokine for immature DCs (22). The migration of *Nlrp12*$^{-/-}$ neutrophils to the neutrophil attracting chemokine CXCL1 was also reduced by approximately 50% when compared to WT neutrophils (Fig. 4I).

NLR proteins intersect various pathways that are integral to inflammation and immunity (1). Based upon our previous observation of inappropriate noncanonical NF-κB activation in human monocytic cell lines with reduced NLRP12, we predicted a pro-inflammatory phenotype for *Nlrp12*-deficient mice. Surprisingly, pro-inflammatory cytokine production was unaffected by the absence of NLRP12, and the CHS response mounted in these mice was significantly attenuated relative to WT mice. *Nlrp12* did not affect BMDC maturation nor antigen presenting functions *in vitro*. Instead, *Nlrp12* affected DC migration from the periphery to the draining LNs during CHS and subcutaneous immunization and migration to LN homing chemokines. *Nlrp12* also affected neutrophil response to chemotactic stimuli, indicating a central role for NLRP12 in licensing cellular migration. Hereditary periodic fever is associated with two mutations in human *NLRP12* (5), and it is tantalizing to speculate that altered neutrophil migration or retention of activated DCs in the periphery may lead to the recurring cutaneous inflammation experienced by these patients.

We did not find an *in vivo* effect of NLRP12 on pro-inflammatory cytokine production. In contrast to these results, a previous study showed that silencing of *NLRP12* with small hairpin (sh) RNA leads to increased production of IL-6 and TNFα in human cells of monocytic lineage (4). This implies that NLRP12 may exhibit distinct functions in DCs and monocytes/macrophages; however, since mouse macrophages do not express detectable *Nlrp12*, its deletion is not predicted to affect macrophage function in mice. We also were unable to find an effect of *Nlrp12* deletion on IL-1β production, which is a well-established role for other NLRs. Human NLRP12 can co-localize with inflammasome components and promote IL-1β secretion when overexpressed (23). It is possible that the proper agonist/ligand, once identified, can activate NLRP12 inflammasome function. In agreement with

previous studies from our group and others (4, 24), we found that NLRP12 was not required for IL-1β secretion in response to TNFα or TLR ligands +/− ATP.

In summary we reveal a novel regulatory role for NLRP12 in licensing cellular migration that affected a DC-dependent model of cutaneous inflammation. Unlike *Nlrp3*$^{-/-}$ mice, this is not correlated with a defect in inflammasome activation and IL-1β production. Considering that NLRs appear to cluster by their functional properties, it is likely that other NLRs will be found to similarly affect physiological processes by controlling cellular migration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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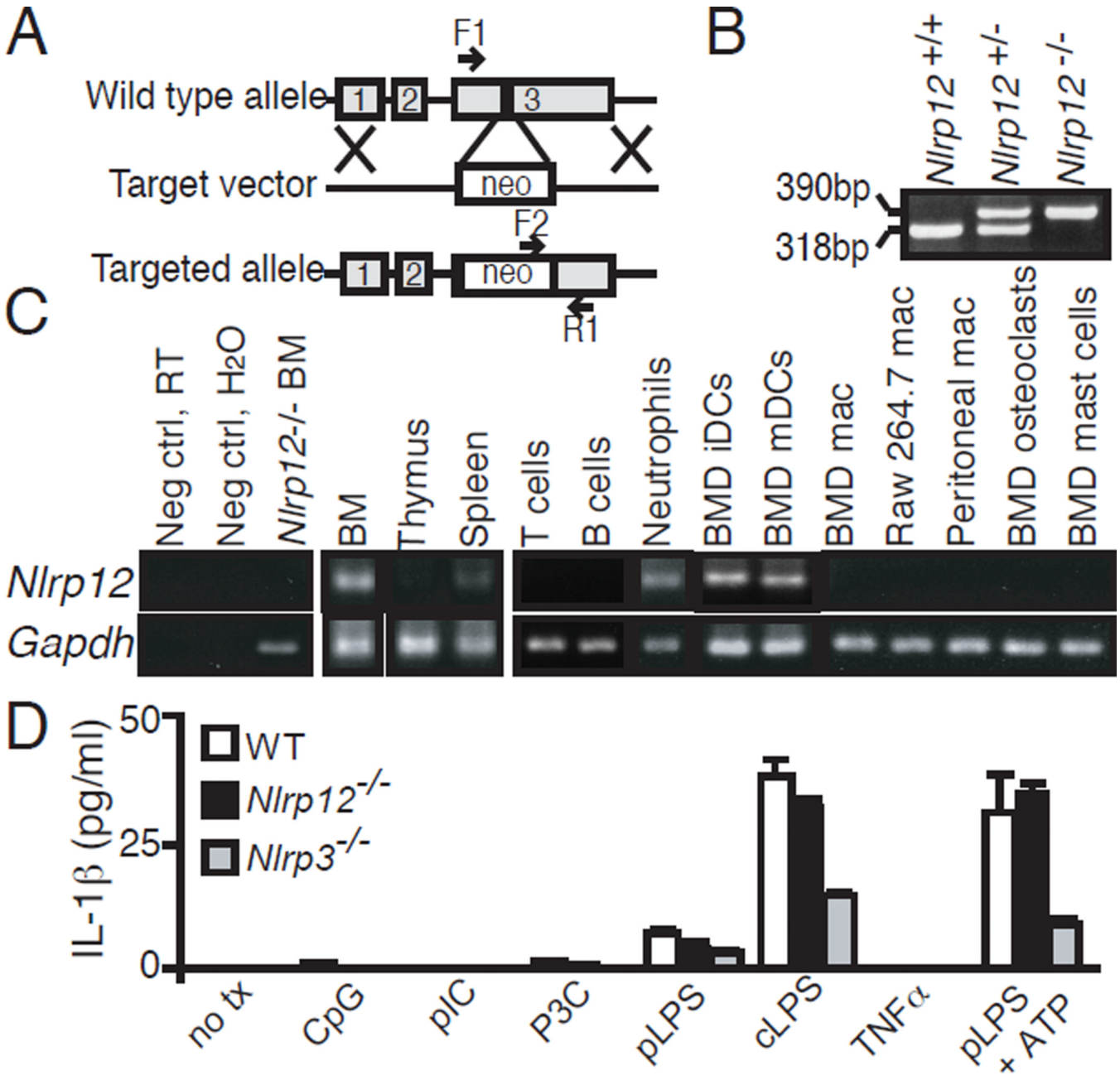

Fig. 1.

(A) Targeted disruption of the *Nlrp12* gene. (B) PCR genotyping from *Nlrp12*$^{+/-}$ crosses. (C) Expression analysis of *Nlrp12* by RT-PCR. BMD, bone marrow derived; iDC, immature DCs before maturation stimulus; mDC, DCs after TNFα maturation; mac, macrophage. (D) IL-1β production from BMDC. No tx, no treatment; pIC, polyinosine-polycytidylic acid; pLPS, phenol purified LPS; cLPS, commercial LPS.

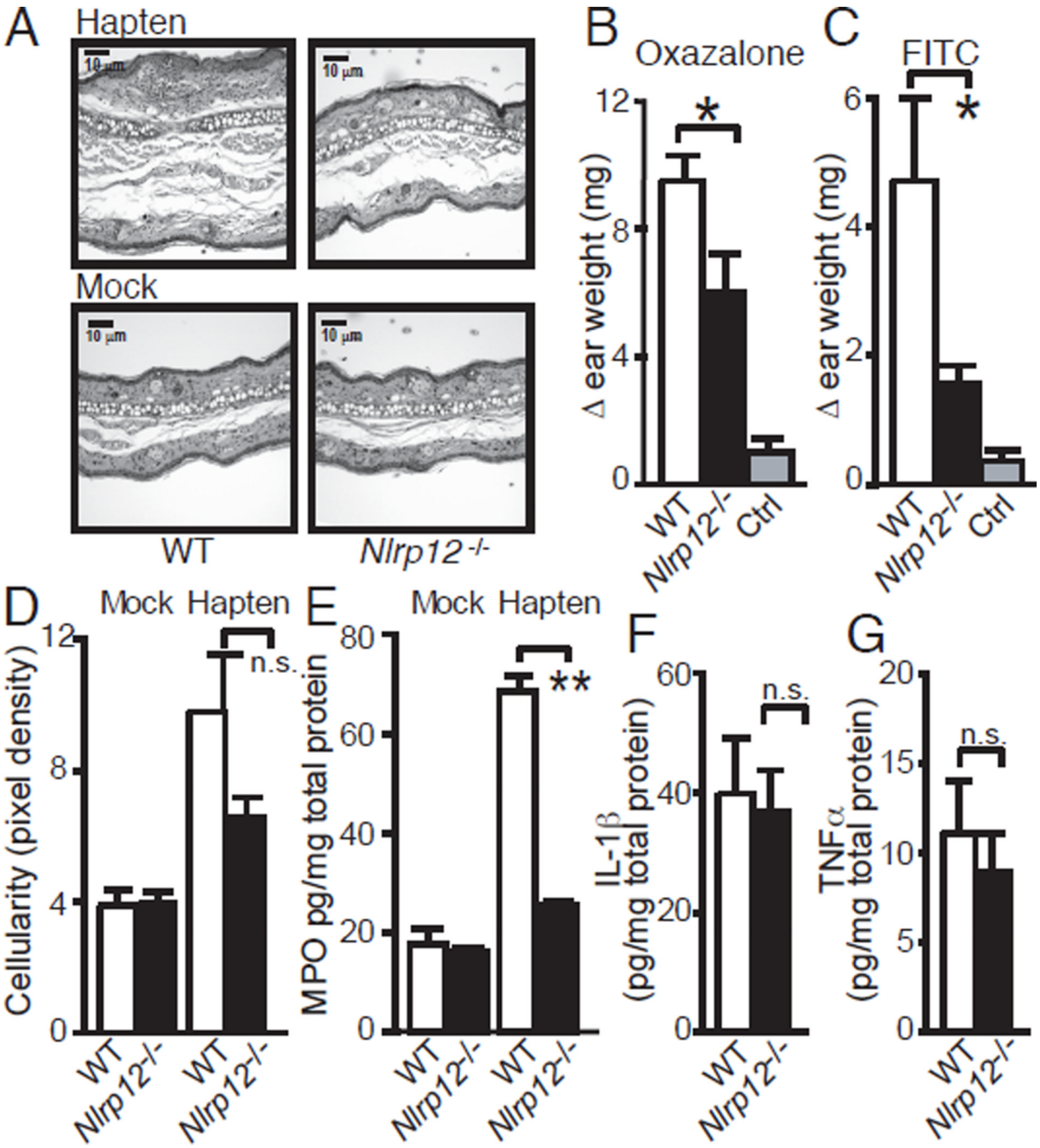

Fig. 2.

Nlrp12$^{-/-}$ mice fail to mount a robust CHS response. (A–C) CHS-induced ear swelling in response to oxazalone (A–B, WT n=12, *Nlrp12*$^{-/-}$ n=13, Ctrl n=6) and FITC (C), WT n=7, *Nlrp12*$^{-/-}$ n=8, Ctrl n=3). (D) Cellularity of CHS ear tissue (WT n=8, *Nlrp12*$^{-/-}$ n=7). (E) Quantification of MPO+ neutrophils in CHS ear tissue (WT n=8, *Nlrp12*$^{-/-}$ n=8). (F) IL-1β and (G) TNFα in CHS ear tissue (WT n=5, *Nlrp12*$^{-/-}$ n=5). All experiments were repeated 2–3 times, all data are presented as mean ± SEM. * $P<0.05$, ** $P<0.01$.

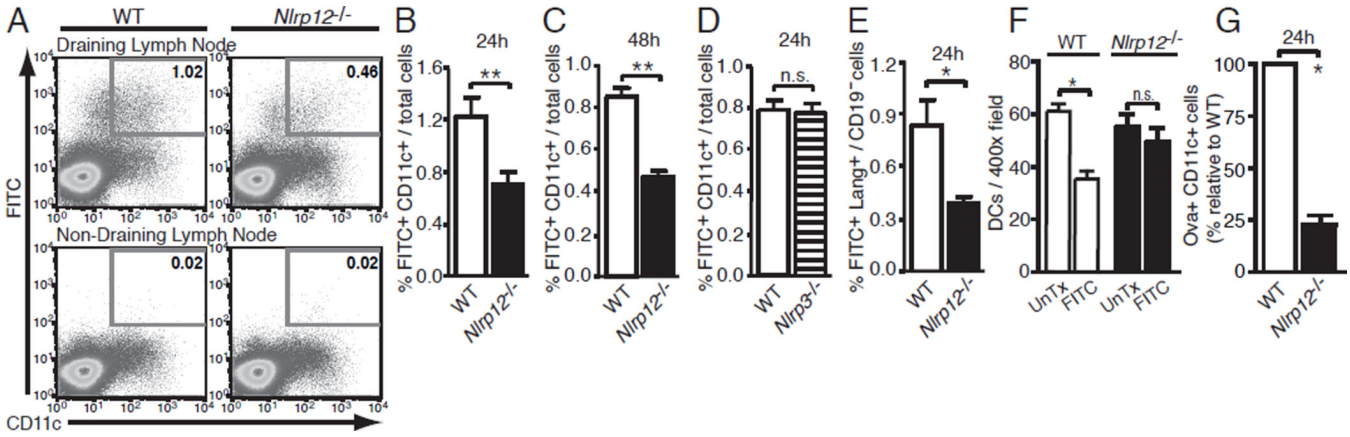

Fig. 3.

DC migration to draining LNs is significantly impaired in *Nlrp12*$^{-/-}$ mice (A–C) FITC^{+} CD11c^{+} cells in the draining LN 24 h (A–B) and 48 h (C) after topical application of FITC (WT n=5, *Nlrp12*$^{-/-}$ n=5). (D) FITC^{+} CD11c^{+} cells in the draining LN of WT (n=5) and *Nlrp3*$^{-/-}$ (n=5) DCs 24 h after topical application of FITC. (E) Quantification of I-Ab^{+} skin DCs in ear epidermal sheets, untreated (Untx) or FITC-treated (FITC) for 24 h (WT n=4, *Nlrp12*$^{-/-}$ n=5). (F) Ova^{+} I-Ab^{+} CD11c^{+} cells in the draining LN 24 h following s.c. injection of fluorescent Ova in CFA. *Nlrp12*$^{-/-}$ values were normalized to WT values set at 100% (WT n=6, *Nlrp12*$^{-/-}$ n=5). All experiments were repeated 2–3 times, all data are presented as mean ± SEM. * P<0.05, ** P<0.01.

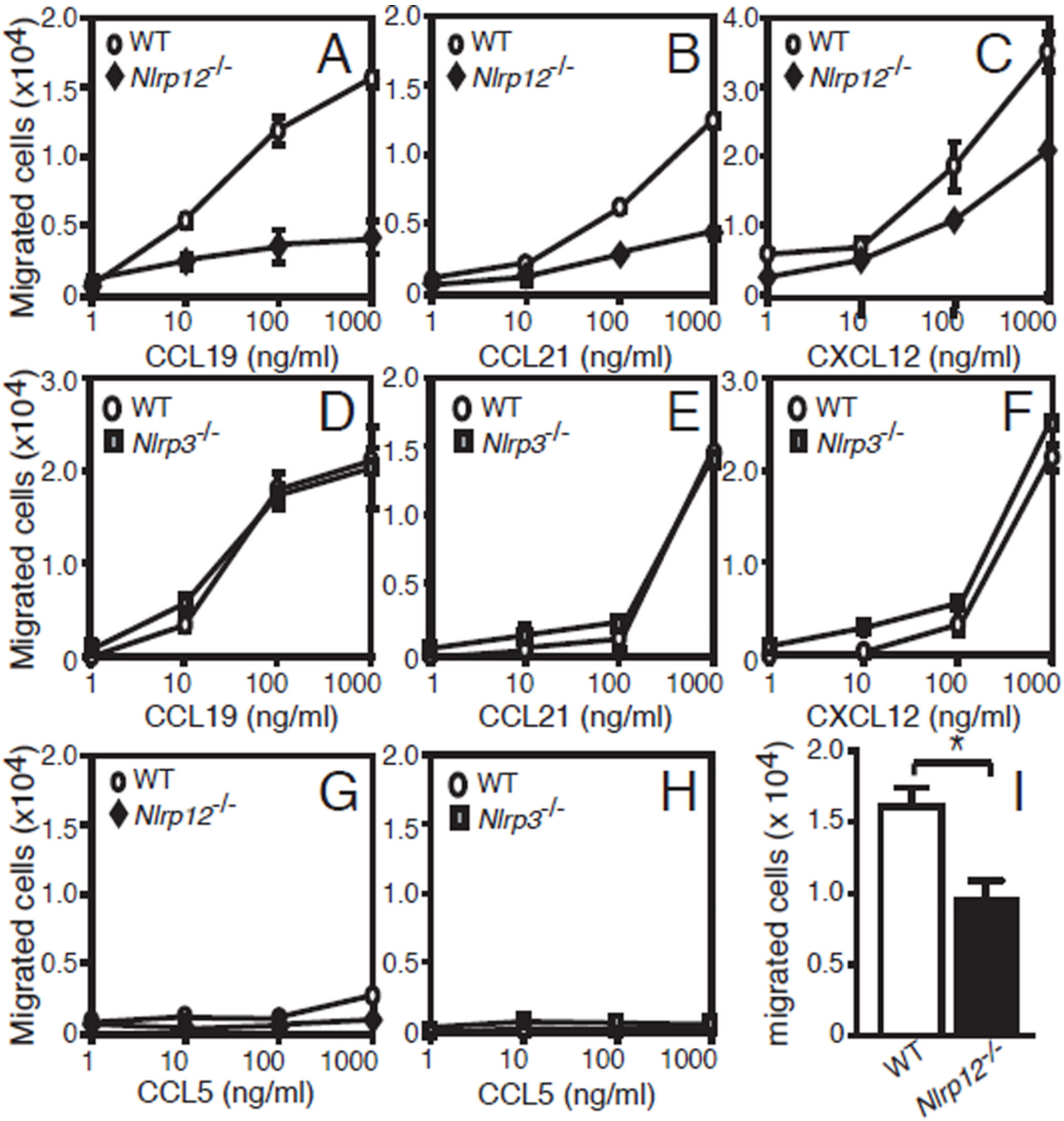

Fig. 4.

Deletion of *Nlrp12* impairs cell migration *in vitro*. Migration of (**A–C, G**) WT (○) and *Nlrp12*$^{-/-}$ (◆) BMDCs, and of (**D–F, H**) WT (○) and *Nlrp3*$^{-/-}$ (■) BMDCs to the indicated chemokine. Data are representative of 3–5 experiments and are presented as mean ± SEM of one experiment. Data from all experiments with associated pairwise comparison statistics are presented in table S4. (**I**) Migration of WT and *Nlrp12*$^{-/-}$ neutrophils to CXCL1. Data are comprised of three independent experiments, presented as mean ± SEM, and pairwise comparisons were made using two-tailed Student's t test, $\alpha = 0.05$. * $P<0.05$.