

The HGF Receptor/Met Tyrosine Kinase Is a Key Regulator of Dendritic Cell Migration in Skin Immunity

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The Met tyrosine kinase has a pivotal role in embryonic development and tissue regeneration, and deregulated Met signaling contributes to tumorigenesis. After binding of its cognate ligand hepatocyte growth factor, Met signaling confers mitogenic, morphogenic, and motogenic activity to various cells. Met expression in the hematopoietic compartment is limited to progenitor cells and their Ag-presenting progeny, including dendritic cells (DCs). In this study, we demonstrate that Met signaling in skin-resident DCs is essential for their emigration toward draining lymph nodes upon inflammation-induced activation. By using a conditional Met-deficient mouse model (*Met^{flox/flox}*), we show that Met acts on the initial step of DC release from skin tissue. Met-deficient DCs fail to reach skin-draining lymph nodes upon activation while exhibiting an activated phenotype. Contact hypersensitivity reactions in response to various contact allergens is strongly impaired in Met-deficient mice. Inhibition of Met signaling by single-dose epicutaneous administration of the Met kinase-specific inhibitor SU11274 also suppressed contact hypersensitivity in wild-type mice. Additionally, we found that Met signaling regulates matrix metalloproteinase MMP2 and MMP9 activity, which is important for DC migration through extracellular matrix. These data unveil Met signaling in DCs as a critical determinant for the maintenance of normal immune function and suggest Met as a potential target for treatment of autoimmune skin diseases. *The Journal of Immunology*, 2012, 189: 1699–1707.

Antigen-presenting dendritic cells (DCs) play a crucial role in the induction of primary immune responses (1). DCs are scattered in lymphoid and nonlymphoid organs throughout the body and act as sentinels of the immune surveillance system. In skin, dermal DCs (dDCs) and Langerhans cells (LCs), the epidermal contingent of DCs, constitute the first immune barrier for invading pathogens and contact allergens. Additionally, dDCs and LCs have been implicated in the induction and maintenance of tolerance (2, 3). DC precursors populate and functionally integrate as immature DCs into peripheral tissues, such as skin. Following Ag capture, DCs are activated and migrate via lymphatic vessels to the T cell areas of lymphoid organs where they encounter T cells to present processed Ags to induce specific immune responses. Thus, during the DC life cycle, migration and homing are tightly regulated (e.g., by specific expression of chemokines and chemokine receptors, adhesion molecules, and homing receptors) to ensure the selective positioning of DCs at

specific sites of function (4, 5). Before entering afferent lymphatics to reach draining lymph nodes (LNs), migration of activated tissue-resident DCs requires an initial sequence of steps, including mobilization and detachment from surrounding tissues and transmigration through interstitial space. In skin, proinflammatory cytokines, such as TNF- α and IL-1 β , have been recognized as important mediators of DC mobilization after activation, leading to the emigration from peripheral tissues (4). Similarly, hepatocyte growth factor (HGF; also known as scatter factor) induces emigration of LCs from mouse epidermis in skin biopsy assays (6). Dermal fibroblasts were identified as a major source of HGF in skin upon infection or stimulation with proinflammatory cytokines, such as IL-1 α , IL-1 β , TNF- α (7), or IL-6 (8). More recently, keratinocytes were also reported to produce HGF upon skin injury and inflammation (9, 10).

The proto-oncogene *Met* encodes the high-affinity transmembrane tyrosine kinase receptor for HGF. Met signaling after ligand binding confers mitogenic, morphogenic, and motogenic activity to various cells and is essential for embryogenesis and tissue regeneration. Aberrant Met signaling contributes to invasive growth and metastasis of tumors (11, 12). Met is predominantly expressed on epithelial cells but has also been implicated in hematopoiesis (13). Expression of Met in the hematopoietic compartment was found in progenitor cells, macrophages, B cells, and DCs. Met signaling has been proposed in monocyte-macrophage development (14) and B cell homing to LNs (15). Met expression was also found on splenic DCs (16, 17), bone marrow (BM)-derived DCs, and LCs (6). Like Met, its ligand HGF is indispensable during embryonic development, as shown by the lethal phenotype in utero of conventional Met- or HGF-*null* mutations (18–20). HGF is a pleiotropic factor that promotes cell survival, tissue protection, and regeneration and, hence, exerts anti-inflammatory activities (21). Moreover, HGF was found to regulate various functions of immune cells, including cytokine production, migration, and adhesion (6, 16, 17, 22–26). However, the mechanisms by which HGF impacts on immunological responses have remained elusive.

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Received for publication March 6, 2012. Accepted for publication June 17, 2012.

This work was supported in part by grants from the Deutsche Forschungsgemeinschaft (to M.Z.) and the Interdisciplinary Center for Clinical Research within the Faculty of Medicine at Rheinisch-Westfälische Technische Hochschule Aachen (VV B112-a to T.H. and TV M7 to M.Z.). T.H. was funded by the START program of the Faculty of Medicine at Rheinisch-Westfälische Technische Hochschule Aachen.

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The online version of this article contains supplemental material.

Abbreviations used in this article: BM, bone marrow; CHS, contact hypersensitivity; DC, dendritic cell; dDC, dermal dendritic cell; DNFB, 2,4-dinitrofluorobenzene; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; HGF, hepatocyte growth factor; KO, knockout; LC, Langerhans cell; LN, lymph node; MMP, matrix metalloproteinase; pI:PC, polyinosinic/polycytidylic acid; WT, wild-type.

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In this article, we describe the impact of Met signaling for DC/LC mobilization from skin and homing into peripheral tissues. By using a conditional Met-deficient mouse model (*Met^{flox/flox}*), we demonstrate that Met signaling in skin-resident DCs is essential for their detachment and emigration from surrounding tissue upon activation. As a consequence, Met signaling-deficient skin DCs/LCs fail to reach draining LNs. Met kinase inactivation, either genetically or pharmacologically, resulted in strongly impaired contact hypersensitivity (CHS) reactions in response to contact allergens. We found that Met signaling in DCs modulates their matrix metalloproteinase (MMP)2 and MMP9 activity required for transmigration through extracellular matrix (ECM). Our findings unveil Met signaling in DCs as a critical determinant for the maintenance of the immune surveillance system and suggest Met as a potential target for the treatment of autoimmune skin diseases, such as psoriasis.

Materials and Methods

Animal experiments

C57BL/6, CD45.1 (B6.SJL-*Ptprca*^{Pepe^b}/BoyJ), OT-II (C57BL/6-g (Tcr α Tcr β)425Cbn/J), *Met^{flox/flox}* (C57BL/6), and mice heterozygous for the Met knockout allele (*Met^{+/-}*) and crossed to transgenic mice expressing Cre under the control of the IFN-inducible *Mx* promoter (*Mx-cre* $\times$ *Met^{+/-}*) were maintained under specific pathogen-free conditions in the central animal facility of the Rheinisch-Westfälische Technische Hochschule University Hospital Aachen. All animal experiments were approved by local authorities in compliance with the German animal protection law. *Met^{flox/flox}* mice were interbred with *Mx-cre* $\times$ *Met^{+/-}* mice, and offspring (6–8 wk) received five injections of 300 μ g polyinosinic/polycytidylic acid (pI:pC) i.p. at 2-d intervals for excision of floxed exon 15 of *Met* to generate Met-deficient *Met^{-/-}* mice (Met knockout [KO]) or control (wild-type [WT]) *Met^{+/+}* mice (Met WT). Further functional studies in Met KO and Met WT mice were performed $\geq$ 4 wk after the last pI:pC injection.

To generate LC chimeric mice, 2×10^6 BM cells from CD45.2⁺ Met WT or Met KO mice were transplanted into CD45.1⁺ recipient mice, as previously described (27). Donor chimerism in BM was evaluated by flow cytometry and was $\geq$ 95% for donor cells 7 wk after transplantation. Then mice were exposed to UV light (254 nm) for 30 min (total dose 16.8 kJ) to induce ear skin inflammation and LC chimerism in epidermis (28). The presence of donor- and host-derived LCs was investigated 9 wk after UV irradiation by immunofluorescence microscopy and flow cytometry. Ears of BM chimeric mice were subjected to ex vivo migration assays.

Local skin inflammation was induced by applying 50 μ l acetone/dibutyl phthalate solution (1:1, v/v) on ear skin for 24 h. A systemic inflammatory response was induced by a single-dose injection of 50 ng LPS i.v. into the tail vein, and Met expression on DCs was analyzed 24 h later.

Skin DC migration toward LNs was analyzed by flow cytometry 24–72 h after painting with 50 μ l 0.5% FITC in acetone/dibutyl phthalate solution (1:1, v/v) on the shaved abdomen.

To induce CHS, mice were sensitized by applying 400 μ l acetone/dibutyl phthalate/0.5% FITC solution or 20 μ l 0.5% 2,4-dinitrofluorobenzene (DNFB; (Sigma-Aldrich) in acetone/olive oil (4:1, v/v) on the shaved abdomen. Immunization with DNFB was repeated after 24 h. In some experiments, the Met-specific kinase inhibitor SU11274 (Merck) dissolved in DMSO (200 μ M, final concentration) or DMSO alone was applied 30 min before immunization with DNFB. Basal ear thickness was measured 5 d after immunization with a digital caliper (Hogetex) before applying 20 μ l 0.5% FITC or 0.2% DNFB solutions onto the right ears. Left ears received solvents only as control. CHS responses were determined by gauging the ear swelling 24 h later.

Flow cytometry

Flow cytometry analysis was performed on FACSCalibur and FACSCanto (BD Biosciences) devices, as previously described (6), using fluorochrome-conjugated Abs to stain for Met (clone eBioclone 7), langerin (929F3), CD8 α (53-6.7), CD11b (M1/70), CD11c (N418), CD40 (1C10), CD83 (Michel-17), CD86 (GL1), MHC class II (I-A/I-E; clone 2G9), panCD45 (30-F11), CD45.1 (A20), CD45.2 (104), CD103 (M290), and CD184 (2B11), as well as biotin-conjugated Abs for CD80 (1G10), CD209 (5H10), and CD205 (NLDC-145). For detection of langerin, cells were fixed with PBS/4% paraformaldehyde and permeabilized with PBS/1%

saponin (Sigma-Aldrich). Detection of CCR7/CD197 was done with purified chimeric CCL19-human Fc fusion protein (29). Data were analyzed using FlowJo software (Tree Star).

LC/dDC analyses in skin

Ears from mice were split into dorsal and ventral halves and incubated in PBS/0.02 M EDTA (Sigma-Aldrich) for 90 min at 37°C to separate epidermis from dermis. Dermal and epidermal cell suspensions were obtained by digestion with type V collagenase (1 mg/ml; Sigma-Aldrich) at 37°C for 30 min, passed through a 21-gauge syringe needle and a cell strainer, and assessed by flow cytometry.

Ex vivo migration assay

Dorsal and ventral ear halves were cultivated split-side down for 3 d at 37°C in RPMI 1640 medium containing 10% FCS and GM-CSF (200 U/ml), plus HGF (40 ng/ml; eBiosciences), TNF- α (100 ng/ml; PeproTech), LPS (1 μ g/ml); or no factor. Epidermis was isolated, fixed in acetone, and subjected to immunofluorescence microscopy. Cells that had migrated into culture medium were stained for CD11c, langerin, and Met and analyzed by flow cytometry. Numbers of migrated cells were normalized using Dynabeads (15 μ m diameter; Dynal Polymers), as previously described (6). Ear halves of mice that received UV irradiation were cultivated with or without LPS (1 μ g/ml) for 24 h. Epidermal cell suspensions and cells that had migrated into culture medium were stained for panCD45, CD45.1, CD45.2, CD11c, CD11b, MHC class II, and langerin and analyzed by flow cytometry.

Immunofluorescence microscopy

Cryosections of LNs (5 μ m thickness) and epidermal sheets were fixed in 100% ice-cold acetone for 20 min, rinsed in PBS, and blocked with PBS/3% BSA (Sigma-Aldrich) for 30 min at room temperature before staining. Samples were stained with fluorochrome-conjugated Abs for detection of Met, langerin, and MHC class II and counterstained with DAPI (Vector Laboratories). Images were acquired using an Axiovert 200 fluorescence microscope (Zeiss) and a digital CCD camera (Roper Scientific) operated with IPlab software. Image processing was done with Adobe Photoshop software.

RNA isolation and RT-PCR

To isolate RNA from mouse ear skin, mouse ear tissues were dissociated into single-cell suspensions using the gentleMACS Dissociator (Miltenyi Biotec). Total RNA was isolated using the NucleoSpin RNA II kit (MACHERY-NAGEL) with on-column DNase digestion, according to the manufacturer's instructions, and mRNA was reverse-transcribed with a High Capacity cDNA RT Kit (Applied Biosystems). A total of 50 ng cDNA was used as a template for PCR reactions using gene-specific primers and conditions. Five-microliter aliquots of PCR reactions were subjected to 1% agarose gel containing ethidium bromide, and signals were recorded with GelDocX gel documentation system (Bio-Rad). Expression of *Gapdh* mRNA was used as control. Primers used were as follows: *Gapdh*, forward 5'-ggggTgAggCcgTgCTgATAT-3', reverse 5'-CATT-gggggTaggAACACgAgAgg-3'; *Met*, forward 5'-CATTCTggTgCTgcTg-TTgTCC-3', reverse 5'-TgTCTTTCCAAACCCCTCCTg-3'; *Hgf*, forward 5'-CATTCAAggCCAAGgAgAgg-3', reverse 5'-AACTCggATgTTTggg-TCAg-3'; *Tnfr*, forward 5'-ggCaggTCTACTTTggAgTCATTgC-3', reverse 5'-ACATTCgAggCTCCAgTgAATTCgg-3'; *Mmp2*, forward 5'-CC-CCTATCTACACCTACACCAAGAAC-3', reverse 5'-CATTCCAggAg-TCTgCgATgAgC-3'; and *Mmp9*, forward 5'-AGTTTGGTGTGCGGAG-CAC-3', and reverse 5'-TACATgAgCgCTTCCggCAC-3'.

Functional analyses of Met in DCs

Immature DCs were generated from mouse BM using a two-step amplification and differentiation protocol, as previously described (27). In some experiments, 0.1 μ g/ml LPS (Sigma-Aldrich) was added 24 h prior to treatment with HGF (40 ng/ml) to induce DC maturation.

Ag-specific T cell activation was performed with CD4⁺ T cells from OT-II mice expressing a transgenic TCR that recognizes OVA_{323–339} peptide (ISQAVHAHAHAEINEAGR). Splenic CD4⁺ T cells were prepared by immunomagnetic bead purification using MACS anti-CD4 MicroBeads (Miltenyi Biotec), according to the manufacturer's instructions. Immature and LPS-matured DCs from Met WT and Met KO mice, stimulated with 40 ng/ml HGF for 16 h or left untreated, were pulsed with 0.1 μ M OVA_{323–339} peptide for 30 min and then cocultured at various ratios with isolated OT-II CD4⁺ T cells in 96-well microtiter plates. At day 4 of coculture, 0.75 μ Ci/well [³H]thymidine (GE Healthcare) was added per well. Cells were har-

vested 5 h later, and [^3H]thymidine incorporation was measured with a scintillation counter (MicroBeta TriLux; Wallac). Experiments were done with triplicate samples.

Immunoblotting was carried out essentially as described before (30). Total and phosphorylated p44/42 MAPK were identified using rabbit anti-p44/42 MAPK and anti-p-p44/42 MAPK Ab (Erk1/2, clone 137F5, and pErk1/2, clone D13.14.4E; Cell Signaling Technology), respectively. MMP-2 and MMP-9 were detected using polyclonal rabbit Abs from Cell Signaling Technology. Detection of β -actin (AC-74; Sigma-Aldrich) was used as loading control.

Chemotaxis of DCs in response to CCL19 or CXCL12 chemokines (100 ng/ml each; PeproTech) was performed essentially as described previously (27), with minor modifications. Transwell inserts (5 mm pore size; Costar) were coated with Matrigel (BD Biosciences) at 37°C for 16 h or left uncoated. LPS-activated DCs were stimulated with HGF 30 min prior to the assay start. Transmigration of DCs through uncoated Transwell inserts and through Matrigel matrix was analyzed by flow cytometry after 90 min and 5 h, respectively. The numbers of transmigrated cells were normalized using Dynabeads, as done in *ex vivo* migration assays.

Gel zymography was used to analyze membrane-bound MMP-2 and -9 activities. Native protein lysates of DCs were mixed with 2 $\times$ sample buffer (125 mM Tris-HCl, 20% glycerol, 4% SDS, 0.005% bromophenol blue) for 10 min before gel electrophoresis. After electrophoresis, gels were renatured with 2.5% Triton X-100 for 30 min and incubated with 50 mM Tris base/0.2 M NaCl/5 mM CaCl_2 overnight at 37°C. Degradation of gelatin was visualized by Coomassie Blue R-250 staining.

Statistics

Statistical analyses were performed using the Student *t* test. A *p* value < 0.05 was considered statistically significant.

Results

Activation-dependent Met expression on DCs

DC homeostasis is differentially regulated in steady state and inflammation (31, 32); thus, we analyzed various DC subtypes in mice for Met expression in lymphoid tissues and in skin before and after DC activation. We found Met expression on all DC subtypes analyzed, albeit at different levels according to subtype, location, and activation status (Fig. 1A, 1C–H, Supplemental Figs. 1, 2D). DC subsets in the skin, identified as langerin $^+$ LCs in the epidermis and langerin $^-$ CD103 $^-$ and langerin $^+$ CD103 $^+$ in the dermis (33–36), expressed Met, with the majority of cells showing low expression in the steady state (Fig. 1A, 1C, 1D, 1H). By applying a skin-irritating acetone/dibutyl phthalate solution on ear skin, we induced local inflammation, as evidenced by the increased expression of the proinflammatory cytokine TNF- α (Fig. 1B). Under such conditions, increased expression of Met and its ligand HGF was also observed (Fig. 1B). Met expression on skin DC subsets in skin and in skin-draining LNs was analyzed under such inflammatory conditions and after LPS injection 24 h later. An increased expression of Met on skin DC subsets was found under both local and systemic inflammatory conditions (Fig. 1C, 1D, 1F, 1H). The upregulated expression of Met was more pronounced in epidermal LCs after skin irritation, whereas it was greater in dDC subsets after LPS injection (Fig. 1C, 1D, 1H). In LNs, the DC compartment comprises LN-resident DC subsets and tissue-derived DCs immigrating from peripheral tissues. In the steady state, migratory DCs constitute only a minor population of all DCs in LNs (37), but skin irritation results in an increased influx of skin DCs into draining LNs. Under such inflammatory conditions, the greatest Met expression was found on CD8 α^- CD11b $^+$ MHC class II-high migratory DCs compared with LN-resident CD8 α^+ CD11b $^-$ MHC class II-intermediate DCs (Fig. 1G). The predominant migratory DC subsets in skin-draining LNs, which have transported Ags from peripheral tissues, are langerin $^+$ LCs and langerin $^-$ dDCs (37, 38); both subsets exhibited elevated Met expression compared with steady-state expression in skin (Fig. 1C, 1D, 1F, 1H).

LCs and dDCs were shown to migrate with different kinetics to draining LNs upon activation, with dDCs preceding LCs (38, 39). To study migration kinetics and Met expression of LCs and dDCs, DCs in skin were labeled *in vivo* by applying 0.5% FITC on the shaved abdomen of mice (38). Met expression was analyzed on FITC $^+$ LCs and FITC $^+$ dDCs that had migrated to draining inguinal LNs (Fig. 2). Most of the faster migrating DCs (24 h) showed the greatest Met expression, whereas after 48 h more DCs with intermediate levels of Met were found; numbers of FITC $^+$ DCs started to decline at later time points (Fig. 2A). LCs and dDCs differentially express endocytic receptors DEC205/CD205 and DC-SIGN/CD209 (38, 40). LCs are CD209 $^-$ and upregulate the expression of CD205 upon activation, whereas dDCs express CD209 and intermediate levels of CD205. This expression pattern was used in this study to discriminate FITC $^+$ LCs and FITC $^+$ dDCs upon migration to draining LNs (Fig. 2B) (38, 40). Met expression levels were lower at all time points in FITC $^+$ LCs than in FITC $^+$ dDCs, coinciding with the different migration kinetics of these DC subtypes (Fig. 2). Taken together, these data show that Met expression on peripheral skin DCs is upregulated upon activation, suggesting a functional role of Met for DC migration toward draining LNs.

Met is essential for emigration of skin DCs from residential tissue

Met-signaling deficient mouse models are embryonically lethal; therefore, we used a floxed Met transgenic mouse model (*Met*^{fllox/fllox}) (41) to investigate Met function in skin DCs. *Met*^{fllox/fllox} mice were crossed with mice heterozygous for the Met knockout allele that express Cre under the control of the IFN-inducible *Mx* promoter (*Mx-cre/Met*^{+/-}, Fig. 3A). Repeated injections of pI:pC (1:1; w/w) into animals with an *Mx-cre/Met*^{fllox/-} genotype induced efficient excision of the single floxed Met allele, resulting in a Met-deficient (Met KO) phenotype. *Mx-cre/Met*^{fllox/+} littermates retain the WT phenotype (Met WT) after pI:pC injections and were used as controls. Met KO and Met WT mice show the same number of LCs in epidermis (Supplemental Fig. 2A).

DC emigration from skin upon activation was quantified in skin explant assays (Fig. 3B, 3C, Supplemental Fig. 2) (6). Ear sheets from Met KO and Met WT mice were cultured in the presence or absence of LPS, TNF- α , or HGF (Supplemental Fig. 2B). All factors significantly enhanced emigration of DCs from skin tissue of Met WT mice into culture medium (Fig. 3B). In contrast, none of the stimuli was able to enhance the release of DCs from skin of Met KO mice (Fig. 3B). Immunofluorescence microscopy and flow cytometry analyses revealed that LCs and dDCs remained in the skin of Met KO mice (Fig. 3C, 3D, Supplemental Fig. 2C). The stationary Met KO DCs showed an activated cell surface marker profile after LPS stimulation of the ear sheets identical to that of emigrated DCs from Met WT ear skin (Fig. 3D).

To investigate whether the role of Met signaling for emigration of skin DCs is cell intrinsic, we performed BM transplantation of CD45.2 Met KO and Met WT BM mice into CD45.1 Met WT recipients. We first analyzed LC repopulation in the epidermis of CD45.2 Met KO $\rightarrow$ CD45.1 Met WT BM chimeric mice (Fig. 4A). LCs develop during embryogenesis from a skin-seeding LC precursor (42, 43) and are resistant to radiation-induced ablation. However, UV irradiation causes skin inflammation and results in loss of LCs that are then replaced by circulating BM-derived precursors (28). LC chimerism in skin epidermis of recipient mice was analyzed by fluorescence microscopy 9 wk after UV irradiation. We found no difference in the number of donor-derived LCs in the epidermis of Met KO versus Met WT BM chimeric mice and a comparable high degree of donor LC chi-

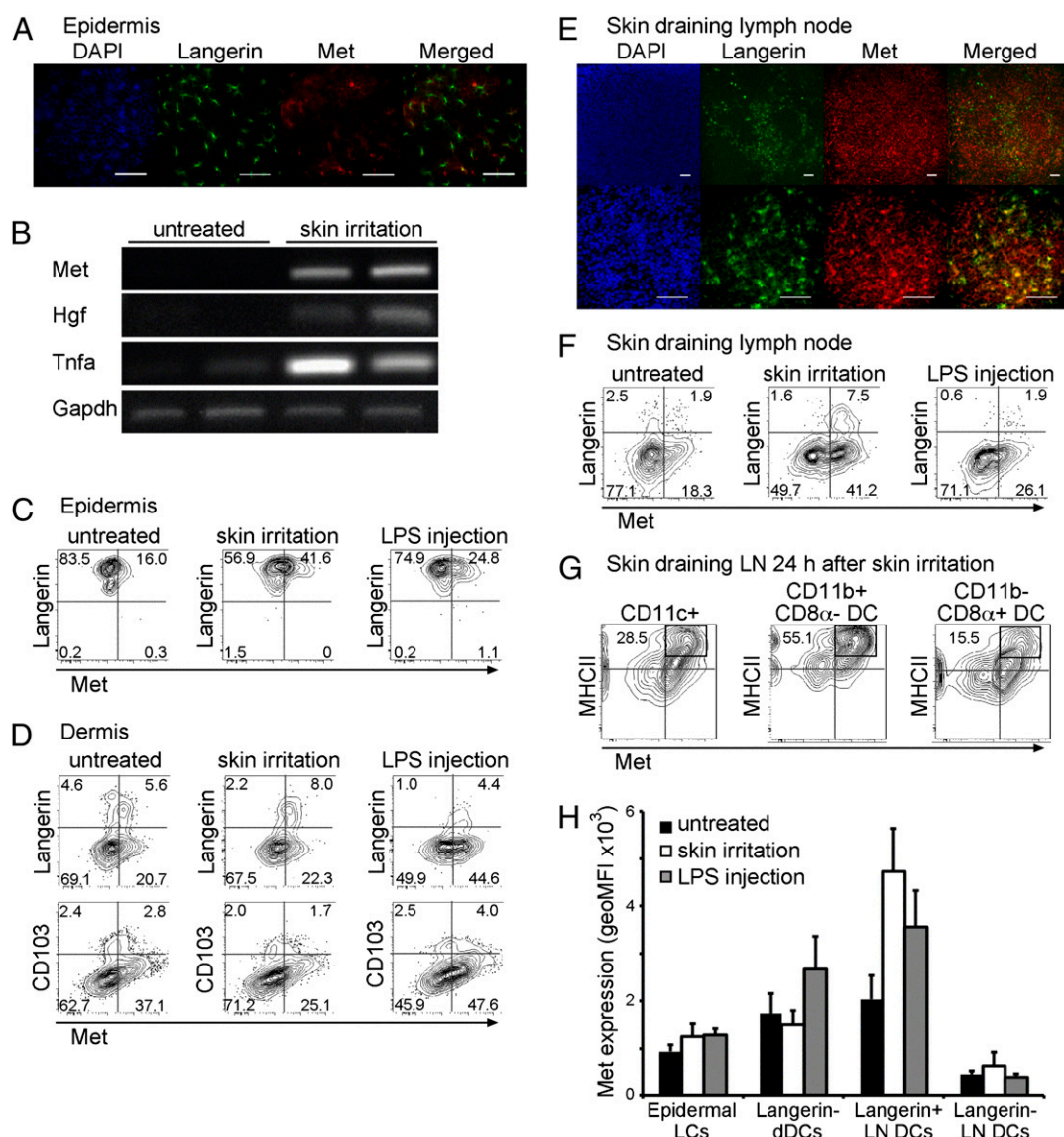


FIGURE 1. Met expression in DCs. **(A)** Immunofluorescence staining of skin epidermis for langerin (green) and Met expression (red); nuclear staining, DAPI (blue). Scale bars, 50 μ m. **(B)** Expression of *Met*, *Hgf*, and *Tnfa* was analyzed by RT-PCR in untreated ear skin tissue and 24 h after skin irritation. Expression of *gapdh* was used as control. Two independent experiments for each condition are shown. **(C and D)** Met expression on LCs/dDCs was analyzed by flow cytometry of untreated skin and 24 h after skin irritation or LPS injection. For detection of LCs and dDCs, cells from epidermal (C) and dermal sheets (D) were stained for CD45, CD11c, langerin, CD103, and Met and gated for CD45 and CD11c expression. **(E)** Immunofluorescence staining of inguinal LN 24 h after skin irritation for langerin (green) and Met expression (red); nuclear staining, DAPI (blue). Scale bars, 50 μ m. *Lower panels* represent higher-power views of sections shown in the *upper panels*. **(F and G)** Met expression on LN DCs was analyzed by flow cytometry of untreated mice and 24 h after skin irritation or LPS injection. **(F)** LN DCs were stained for CD45, CD11c, langerin, and Met and gated for CD45 and CD11c expression. **(G)** CD11b⁻ and CD8 α ⁺ DCs represent LN residential DCs, and CD11b⁺CD8 α ⁻ MHC class II^{high} cells are migratory DCs. LN DCs were analyzed 24 h after skin irritation by staining for CD45, CD11c, CD11b, CD8 α , MHC II, and Met. **(H)** Relative Met expression intensities on skin LC/dDCs and LN DC subpopulations. Data show geometric mean fluorescence intensity (geoMFI) values $\pm$ SEM ($n = 4$).

merism (Fig. 4B, 4C). These results show that Met signaling is dispensable for LC precursor immigration into skin.

In a second set of experiments using CD45.2 Met KO $\rightarrow$ CD45.1 Met WT BM chimeric mice, emigration of Met KO LCs from skin was analyzed in skin explant assays 9 wk after UV irradiation using flow cytometry (Fig. 4A, 4D–F). Again, no differences in LC chimerism between Met KO and Met WT BM chimeric mice were observed (Fig. 4D). Ear sheets from BM chimeric mice were cultured in the presence or absence of LPS for 24 h, and emigration of donor-derived LCs into culture supernatant was analyzed. Emigration of Met WT LCs from BM chimeric ear skin explants was significantly induced by LPS stimulation after 24 h

(Fig. 4E). Concomitantly, the number of Met WT LCs in the epidermis diminished (Fig. 4F). In contrast, Met KO LCs from BM chimeric mice were severely impaired in their ability to emigrate from skin into culture supernatant after LPS stimulation and were retained in epidermis (Fig. 4E, 4F). These data confirm and further extend our results from skin explant assays in conditional Met KO and Met WT mice and demonstrate the cell-intrinsic function of Met in LCs.

Next, migration kinetics of skin DCs from Met KO and Met WT mice toward draining LNs were analyzed in vivo after labeling with FITC. Brachial LNs are distant from the site of FITC painting; therefore, direct labeling of LN-resident DCs by passively trans-

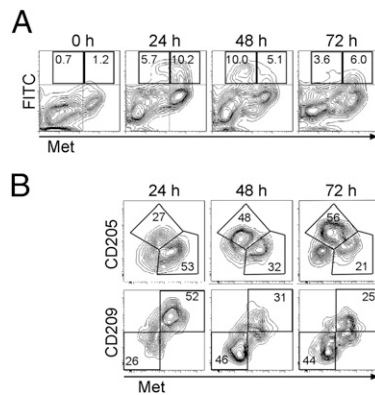


FIGURE 2. Migration kinetics of Met⁺ LCs and dDCs. Inguinal LNs were analyzed by flow cytometry at the indicated time points after FITC painting. **(A)** LN DCs were stained for CD11c, MHC class II, and Met. **(B)** LC (CD205^{high}, CD209[−]) and dDC (CD205^{low}, CD209⁺) subtypes of FITC⁺ LN DCs were analyzed for Met expression. Representative results from three to eight independent experiments are shown.

ported FITC through lymphatics is minimized (5, 38). Brachial LNs and nondraining mesenteric LNs were analyzed for FITC⁺ DCs. In Met WT mice, a rapid and sustained migration of FITC⁺

MHC class II^{high} DCs to the draining LNs was observed (Fig. 5A, 5B, Supplemental Fig. 3). In contrast, the presence of FITC⁺ DCs was severely diminished in Met KO mice.

Met-deficient mice were then examined for their ability to establish CHS. Met KO and Met WT mice were sensitized using the chemical haptens FITC (Fig. 5C) or DNFB (Fig. 5D, 5E). In Met WT mice, both DNFB and FITC elicited a strong CHS response, whereas in Met KO mice the CHS response was substantially attenuated (Fig. 5C, 5D). Next, we addressed whether inhibition of Met signaling by pharmacological means could repress CHS. The shaved abdominal skin of WT mice was treated with a single dose of the Met inhibitor SU11274 for 30 min before applying DNFB. CHS responses were elicited and analyzed as before; they revealed that pharmacological inhibition of Met signaling significantly diminished ear swelling (Fig. 5E).

Met regulates transmigration through ECM barriers and MMP activities in DCs

To investigate molecular mechanisms of Met signaling in DCs, homogenous DC populations from BM of Met KO and Met WT mice were generated that exhibit a phenotype similar to skin DCs (Fig. 6A) (27). We found no differences in the surface marker profile of immature DCs from both Met KO and Met WT mice and of mature DCs after stimulation with LPS (Fig. 6A) or TNF- α (Supplemental Fig. 4A). The marker profile of mature Met KO and Met WT DCs was also virtually identical to that of emigrated and retained DCs of Met KO and Met WT mice in skin explant assays (Figs. 3D, 6A). Activation of Met KO and Met WT DCs caused efficient upregulation of MHC class II and costimulatory molecules; accordingly, no differences in their capacity to activate Ag-specific naive T cells were observed (Fig. 6B, Supplemental Fig. 4B). It is noteworthy that Met KO DCs still express Met on their cell surface, because Cre-mediated excision of floxed exon 15 affects only the intracellular ATP-binding site, similar to the classical Met KO (18, 41). Met-signaling deficiency in Met KO DCs was confirmed by analyzing MAPK activation in response to HGF stimulation, which showed a rapid and sustained ERK1/2 activation, particularly of ERK1/p44 MAPK (Fig. 6C). In contrast, Met KO DCs were Met-signaling incompetent, because no ERK1/2 activation was observed in response to HGF (Fig. 6C).

Next, the migratory functions of HGF-stimulated DCs from Met WT and Met KO mice were analyzed. DCs express CCR7 and CXCR4 chemokine receptors and respond to their cognate chemokines CCL19 (also known as MIP3- β /ELC) and CXCL12 (also known as SDF-1), respectively (4). Chemotaxis responses to CCL19 and CXCL12, using uncoated transmembrane inserts in Transwell-chamber assays, showed no differences in the migratory activity of both Met KO and Met WT DCs (Fig. 6D). This is in line with the observed expression profiles of CCR7 in BM and skin-derived DCs from Met KO and Met WT mice (Figs. 3D, 6A).

Then, transmembrane inserts were coated with an ECM compound that requires partial degradation by DCs for transmigration toward a chemokine gradient (44). Transmigration of DCs from Met KO and Met WT mice through ECM in response to CCL19 and CXCL12 revealed a deficiency of Met KO DCs to pass the ECM barrier (Fig. 6E). Mature DCs express proteolytic enzymes to overcome ECM and basement membrane barriers (e.g., in skin) (4). Particularly, maturing skin DCs upregulate the expression of MMP-2 and MMP-9 (44, 45), and both MMPs were expressed in Met KO and Met WT DCs (Fig. 6F, 6G). However, we found no *Mmp2* and *Mmp9* gene expression regulation after HGF stimulation within the time period of DC migration through the ECM barrier in transmigration assays (Fig. 6F). Therefore, we next addressed regulation of proteolytic activities in DCs by LPS and

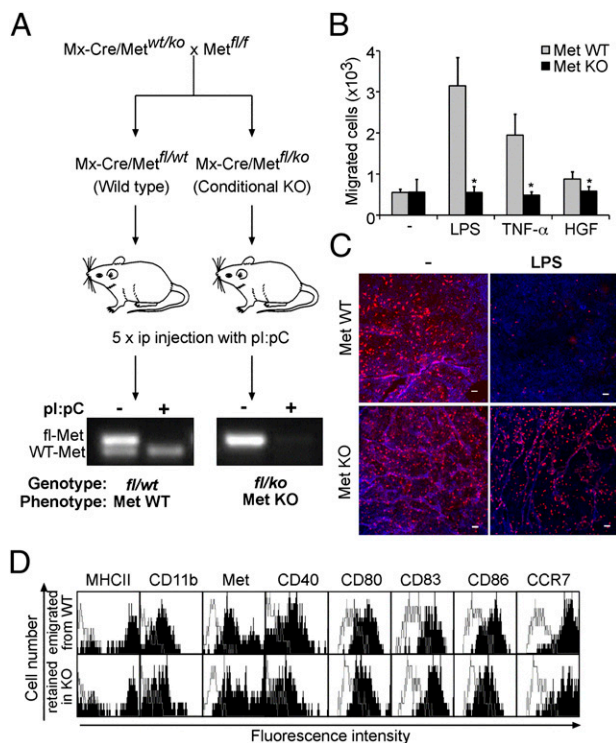


FIGURE 3. Skin DCs from Met KO mice fail to emigrate. **(A)** Breeding strategy to obtain conditional Met-deficient (Met KO) mice and Met WT littermates. Representative genotyping by PCR of BM before and after pl:pC injection. **(B)** and **(C)** Emigration of DCs from Met KO and Met WT ear sheet skin explants after 72 h in culture. **(B)** Bar graph shows numbers of CD11c⁺ DCs in culture supernatants. Data are the mean $\pm$ SEM (*n* = 5). **(C)** Immunofluorescence microscopy of epidermal sheets stained for MHC class II (red); DAPI (blue) shows nuclear staining. Scale bars, 50 μ m. **(D)** Phenotype of skin DCs from Met KO and Met WT mice were analyzed by flow cytometry. Ear skin explants were cultivated for 3 d with LPS. Single-cell suspensions from skin tissue or emigrated DCs in culture supernatants were examined by flow cytometry for surface Ag expression (filled histograms). Open histograms, isotype controls. Representative results from five independent experiments are shown. **p* < 0.05.

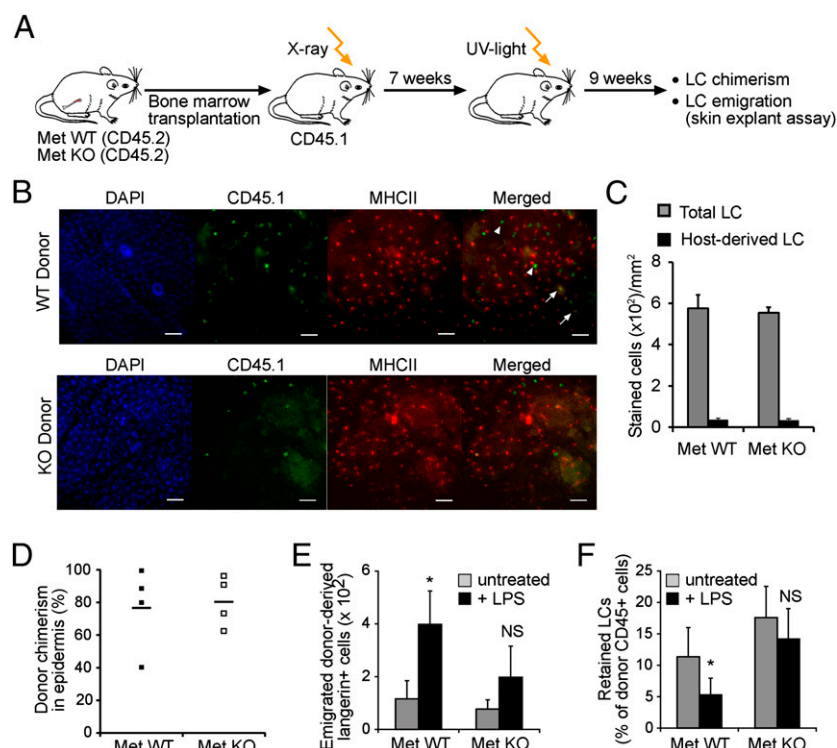
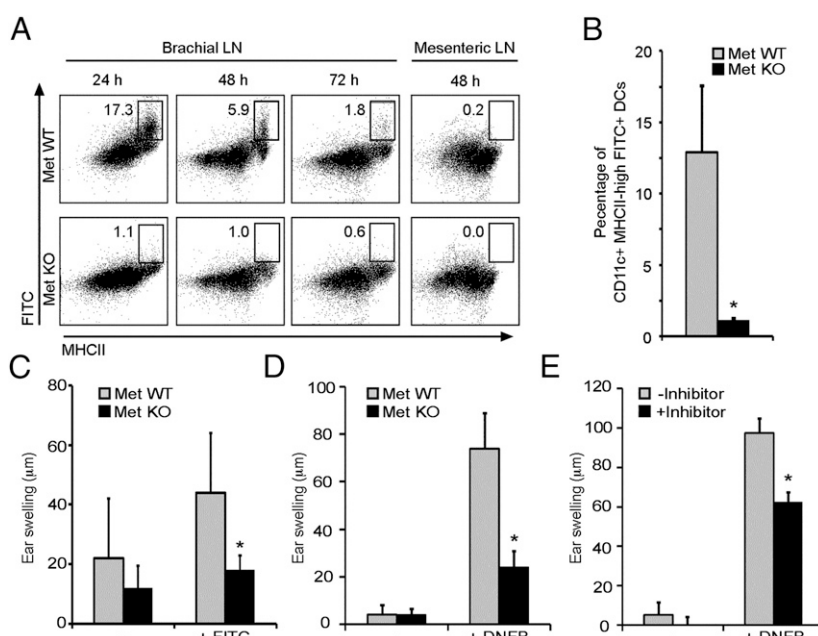


FIGURE 4. LC repopulation of skin epidermis is Met independent. **(A)** Schematic diagram of experimental outline to determine LC repopulation by BM-derived Met KO and Met WT precursors (CD45.2⁺) and their emigration from CD45.1⁺ WT tissue. **(B)** Repopulation of LCs in epidermal sheets after UV irradiation was examined by fluorescence microscopy. Epidermal sheets from CD45.1⁺ recipient animals transplanted with Met KO or Met WT BM were stained for MHC class II (red) and CD45.1 (green) expression, as well as with DAPI (blue). Representative images are shown. Scale bars, 50 μ m. **(C)** Bar graph represents the number of LCs/mm² in epidermal sheets 9 wk after UV exposure; five areas were counted/ear sheet (10 areas/mouse). Data are the mean $\pm$ SEM of three animals/group. **(D–F)** Emigration of Met KO and Met WT DCs from WT ear skin was analyzed by flow cytometry of skin explants after 24 h in culture. For detection of donor-derived LCs, single-cell suspensions were stained for CD45.2, CD11c, CD11b, MHC II, and langerin. **(D)** Total CD45.2⁺ donor cell chimerism in epidermis of CD45.1⁺ recipients. Data points represent frequencies of CD45.2⁺ cells in individual mice; horizontal lines denote mean. **(E)** Bar graph shows numbers of emigrated donor-derived LCs (CD45.2⁺, CD11c⁺, CD11b⁺, MHC II⁺, and langerin⁺) into culture supernatants. Data are the mean $\pm$ SEM ($n = 4$). **(F)** Epidermal sheets were analyzed for retained LCs. Frequencies of CD45.2⁺ LCs (mean $\pm$ SEM, $n = 4$). * $p < 0.05$.

HGF stimulation using gel zymography. Met WT DCs showed high MMP-2 and low MMP-9 activities of membrane-bound forms after LPS activation, which correlated with protein ex-

pression levels (Fig. 6G). HGF stimulation of LPS-matured Met WT DCs enhanced MMP-9 activity, whereas MMP-2 activity was abrogated (Fig. 6G). In contrast, Met KO DCs showed complete

FIGURE 5. Met deficiency impairs DC emigration from skin and ensuing immune function. Met KO phenotype was induced by pI:pC treatment 4 wk prior to initial hapten painting, as described in *Materials and Methods*. **(A and B)** Migration of skin DCs toward LNs were analyzed by flow cytometry 24–72 h after FITC painting of abdominal skin. Nondraining mesenteric LNs were analyzed as control. Numbers in **(A)** indicate frequencies of gated CD11c⁺ MHC class II⁺ FITC⁺ DCs. **(B)** Data of migrated DCs after 24 h (mean $\pm$ SEM, $n = 3$). **(C–E)** CHS reactions in response to haptens 24 h after challenge of right ear skin. Left ears received solvent only. Ear swelling in Met KO and Met WT mice in response to FITC **(C)** or DNFB **(D)**. **(E)** Ear swelling in WT mice pretreated with Met kinase inhibitor SU11274. Data are mean $\pm$ SEM ($n = 5$). * $p < 0.05$.



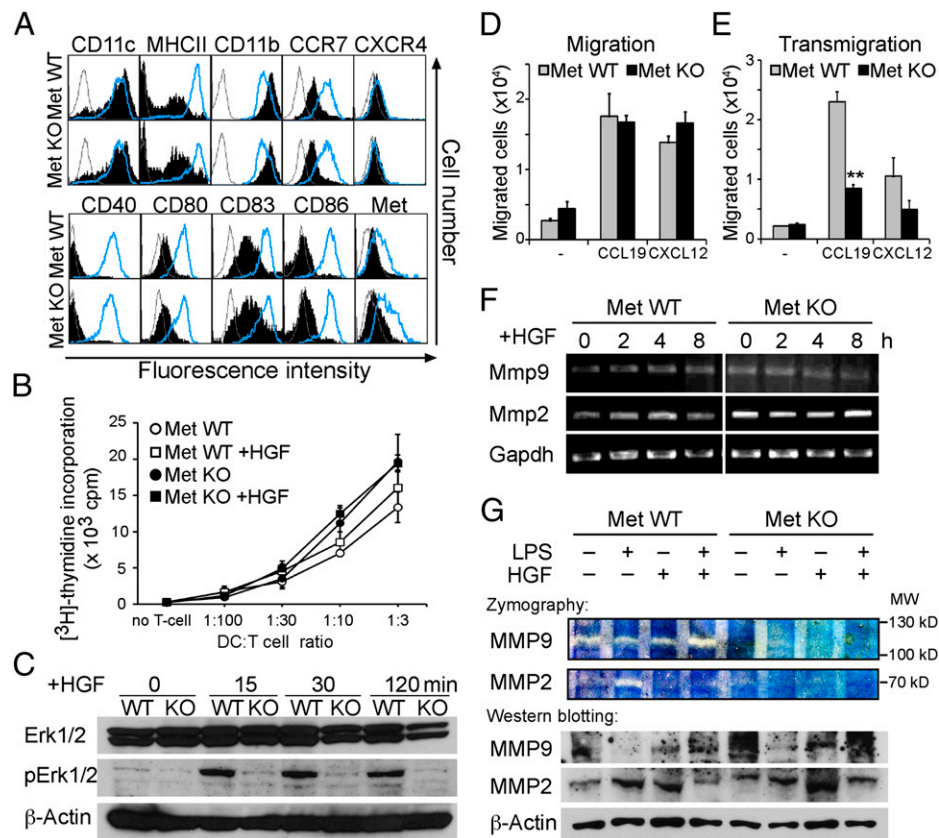


FIGURE 6. Met regulates MMP activities in DCs for transmigration through ECM barriers. **(A)** Flow cytometry of DCs from BM of Met KO and Met WT mice for surface Ag expression (filled graphs). Open blue graphs represent expression after LPS stimulation; open gray graphs represent isotype controls. **(B)** Ag-specific activation of CD4⁺ T cells from OT-II mice was analyzed by [³H]thymidine incorporation in DC/T cell cocultures. OVA peptide-pulsed mature DCs from Met KO and Met WT mice were stimulated with HGF for 24 h or left untreated. Results (mean triplicate values $\pm$ SD) from one representative of three individual experiments are shown. **(C)** Western blot analysis of HGF-stimulated Met KO and Met WT DCs for total and phosphorylated ERK1/2 expression. Detection of β -actin was used as loading control. Number of migrated DCs in Transwell chemotaxis assays in the absence **(D)** or presence **(E)** of Transwell membrane coating. Data are the mean $\pm$ SEM ($n = 3$). **(F)** Expression of *Mmp9* and *Mmp2* in DCs was analyzed by RT-PCR after the indicated time periods of HGF stimulation. Expression of *gapdh* mRNA was used as control. **(G)** Zymography (upper panel) and Western blotting (lower panel) were performed with lysates of DCs stimulated with LPS and HGF for 24 h. Zymograms were stained with Coomassie Blue, and white bands reveal gelatin degradation. Detection of β -actin was used as loading control in Western blot analysis. ** $p < 0.01$.

absence of membrane-bound MMP-2 and MMP-9 activities, whereas protein expression levels were similar to those in Met WT DCs (Fig. 6G). Taken together, these findings show that Met signaling regulates MMP activities in DCs and suggests a potential role of the Met–MMP pathway in the emigration of LCs and dDCs from peripheral skin tissue.

Discussion

The important role of the receptor tyrosine kinase Met and its cognate ligand HGF in embryonic development has been well recognized. In adults, Met signaling is involved in tissue repair and invasive tumor growth; thus, it has received special attention as a potential target in cancer therapy (46). Expression of Met in B cells, monocytes, and DCs, but not T cells, has been described, yet Met function on the immune system is not understood. Moreover, results from recent studies that focused on a potential role for Met in modulating immune responses are highly controversial (17, 47). We demonstrate in this study the impact of Met signaling on DC emigration from peripheral skin tissue toward lymphoid organs, which is in line with the motogenic activity of Met signaling in other cells, including monocytes (22, 23).

The Met ligand HGF is produced by various mesenchymal cell types and acts as a paracrine factor on Met-expressing cells located in close proximity (48). Stromal fibroblasts, vascular endothelial

and smooth muscle cells, neutrophils, and mast cells are major sources of HGF after activation by tissue injury or inflammatory cytokines (49). Thus, we propose a model where, upon skin injury and/or inflammation, HGF is produced (e.g., by fibroblasts), which, in a paracrine fashion, acts on and mobilizes skin DCs. In contrast, anti-inflammatory factors, such as glucocorticoids (50), 1,25-dihydroxyvitamin D₃ (51), and TGF- β (52), inhibit HGF production.

Fibroblast-like stromal cells from human lymphoid tissues were found to constitutively produce HGF that is modulated by activated T cells (53). Thus, HGF might play additional roles within lymphoid organs on Met-expressing cells, such as regulation of cell survival (54) and cytokine production (16, 17, 24–26), thereby influencing immune responses.

Met interaction with other cell surface receptors important for cellular migration was described, including integrin $\alpha 6 \beta 4$, plexin-B1, CD44, and Mif receptor (54, 55). Plexin-A1 was recently found to regulate DC entry into lymphatics (56), which opens the possibility that this occurs through interaction with Met. Additionally, a similar role in DC migration was described for plexin-C1 (57), and the expression of plexin-B2 on DCs was recently described (58). Plexins and Met share a highly homologous Sema domain that allows physical interactions between Met and plexins (59). Consequently, semaphorin binding to plexins can lead to

HGF-independent Met transactivation that might account for some Met-dependent activities (60). Such versatile interactions are expected to allow integration of various extracellular stimuli supplied at different tissue environments to the signaling and locomotive machinery of Met-expressing cells, including DCs.

In many biological systems, Met signaling leads to the transient conversion of cells from an epithelial to a mesenchymal phenotype (epithelial-to-mesenchymal transition [EMT]), followed by well-controlled cell migration (frequently referred to as invasive growth) (11, 12). In epithelial cells, physical contact with neighboring cells is mediated by adherens junctions, and various mechanisms have been described that result in adherens junction destabilization by Met signaling, including activation of MMPs (55, 61). Met signaling in tumor cells can stimulate the proteolytic activity of MMPs, thus facilitating their dissociation and invasive scattering (55, 61). Interstitial DCs express E-cadherin and tight junction proteins (4, 32, 62) and, thus, exhibit features common to epithelial cells, which might be required for their integration into the skin epithelial cell layer. The impact of E-cadherin on DC function has been well recognized (63). Thus, Met signaling in DCs appears to recapitulate properties of EMT, including detachment from the tissue of residence, adherence to and migration through ECM, and crossing tissue boundaries. The important roles for MMPs, particularly MMP-2 and MMP-9, in interstitial DC migration have been well recognized (4, 44, 45). Our data provide evidence that regulation of MMP activities by Met in DCs, as shown in this study, is involved in this process.

In tumor cells, Met regulates MMP activities by both enhancing the transcriptional levels of various MMPs and by stimulating conversion of the inactive precursor forms into active enzymes (61). We found no evidence for immediate regulation of MMP-2 and MMP-9 activities on the transcriptional level by HGF stimulation in DCs, suggesting a posttranscriptional control mechanism. Met-deficient LCs and dDCs remained in their residential tissue, indicating that Met signaling in DCs acts at the initial step of the EMT program (i.e., disruption of the intercellular network junctions). Regulation of MMP activities in DCs by Met is likely not sufficient to account for all Met-induced activities. Thus, additional Met-dependent mechanisms that control this program in DCs remain to be identified.

The findings reported in this article also suggest a role for Met signaling in trafficking of interstitial DCs in other peripheral tissues, such as lung and gut; further studies are needed to scrutinize this notion. Hence, the question arises whether treatment of cancer with Met inhibitors interferes with immune functions, leading to not-yet-recognized side effects. In addition, the proposed model emphasizes the importance of the tissue environment as a source of cytokines and mediators that modulate DC functions. Finally, the discovery of Met as a fundamental regulator of DC emigration from peripheral tissues, such as skin, makes it an attractive target for the treatment of autoimmune skin diseases or other autoimmune disorders and in transplantation.

Acknowledgments

We thank S. Rose-John (University of Kiel, Kiel, Germany) for rIL-6/IL-6R fusion protein, S. Krautwald (University of Kiel) for purified chimeric CCL19/ELC-human Fc fusion protein, Z. Guhe (Rheinisch-Westfälische Technische Hochschule) for technical assistance, A. Teubner (Rheinisch-Westfälische Technische Hochschule) for supporting animal experiments, and S. Diebold (King's College London, London, U.K.) for critical reading of the manuscript.

Disclosures

The authors have no financial conflicts of interest.

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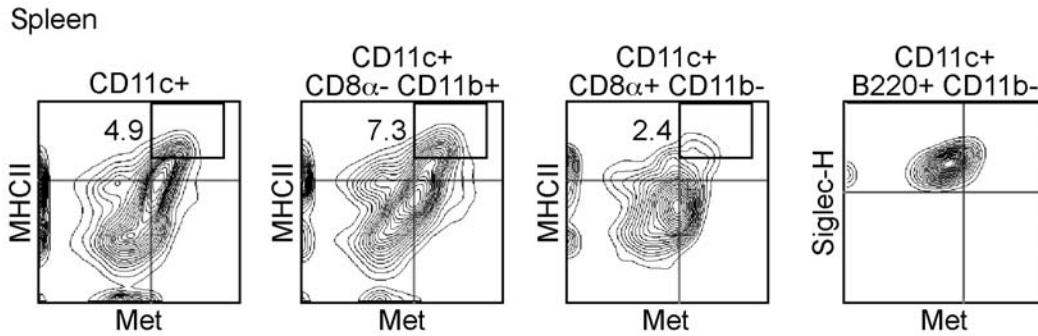
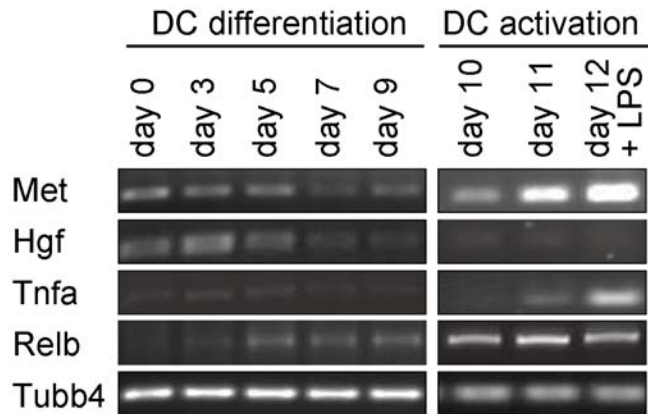
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Supplemental Material

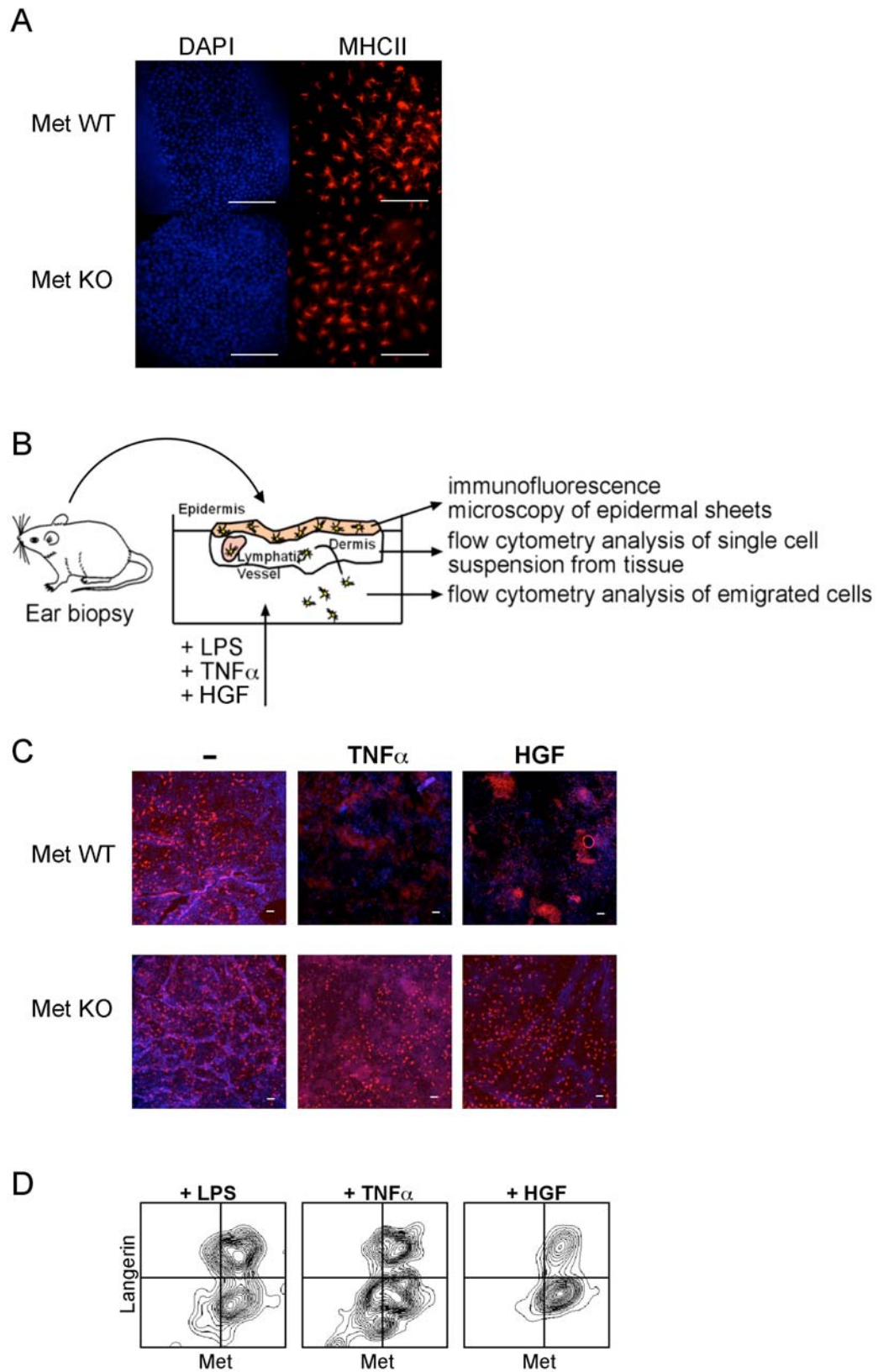
The HGF receptor/Met tyrosine kinase is a key regulator of dendritic cell migration in skin immunity

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A**B****Supplemental Figure 1. Met expression and regulation in DCs.**

(A) Met expression on lymphoid tissue DCs in steady state was analyzed by flow cytometry. To determine Met expression on cDC subsets, single-cell suspensions of spleen DCs were stained for CD45, CD11c, CD11b, CD8 α , MHC class II (MHCII) and Met. For analysis of pDCs, cells were stained for CD45, CD11c, CD11b, B220, Siglec-H and Met. CD45⁺ cells were gated for cDCs and pDCs as indicated. Numbers give the frequencies of gated MHC class II⁺ cDC populations with highest Met and MHC class II expression.

(B) Expression of *Met*, *Hgf*, *Tnfa*, and *Relb* mRNA during differentiation of DCs from BM-derived Flt3⁺ progenitor cells (day 0) and after LPS activation. Transcriptional regulation was analyzed by reverse transcription PCR. Expression of tubulin (*Tubb4*) was used as control. Additional primer sequences used: *Tubb4*, forward 5'-GATCGGTGCTAAGTCCTGGGA-3', reverse 5'-CACAGAGATTCCGGTCCAGCTG-3'; *Relb*, forward 5'-GGTGACG-GCGTGCCTGGTGTG-3' and reverse 5'-ACGGCCCGCTCTCCTTGTTGATTC-3'.



Supplemental Figure 2. Skin DCs in Met KO mice fail to emigrate.

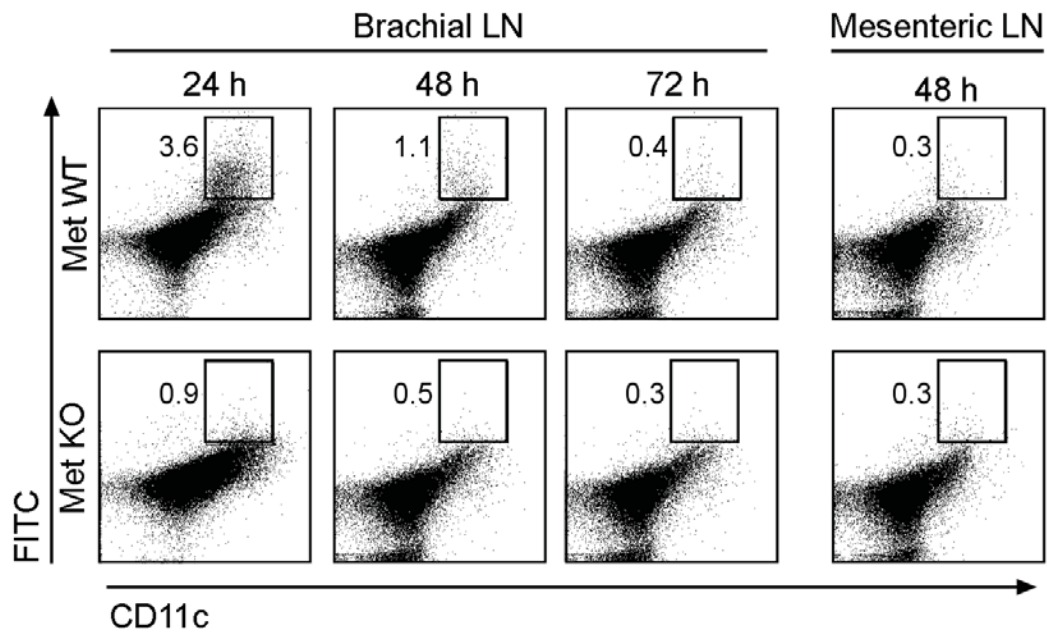
Supplemental Figure 2. Skin DCs in Met KO mice fail to emigrate.

(A) Immunofluorescence microscopy of freshly isolated skin epidermis from Met KO and Met WT mice. Epidermal sheet were stained for MHC class II (MHCII; red) and reveal comparable numbers and morphology of LCs for Met KO and Met WT mice. DAPI (blue) was used for nuclear staining. Scale bars, 50 μ m.

(B) Schematic representation of ear skin explant assays. Dorsal and ventral halves of Met KO and Met WT mouse ears were cultivated split-side down in the presence of LPS, TNF α , HGF or without factor. After three days in culture, cells in culture supernatant and single cell suspensions from tissue were analyzed by flow cytometry. Epidermal sheets were prepared and subjected to immunofluorescence microscopy for detection of LCs.

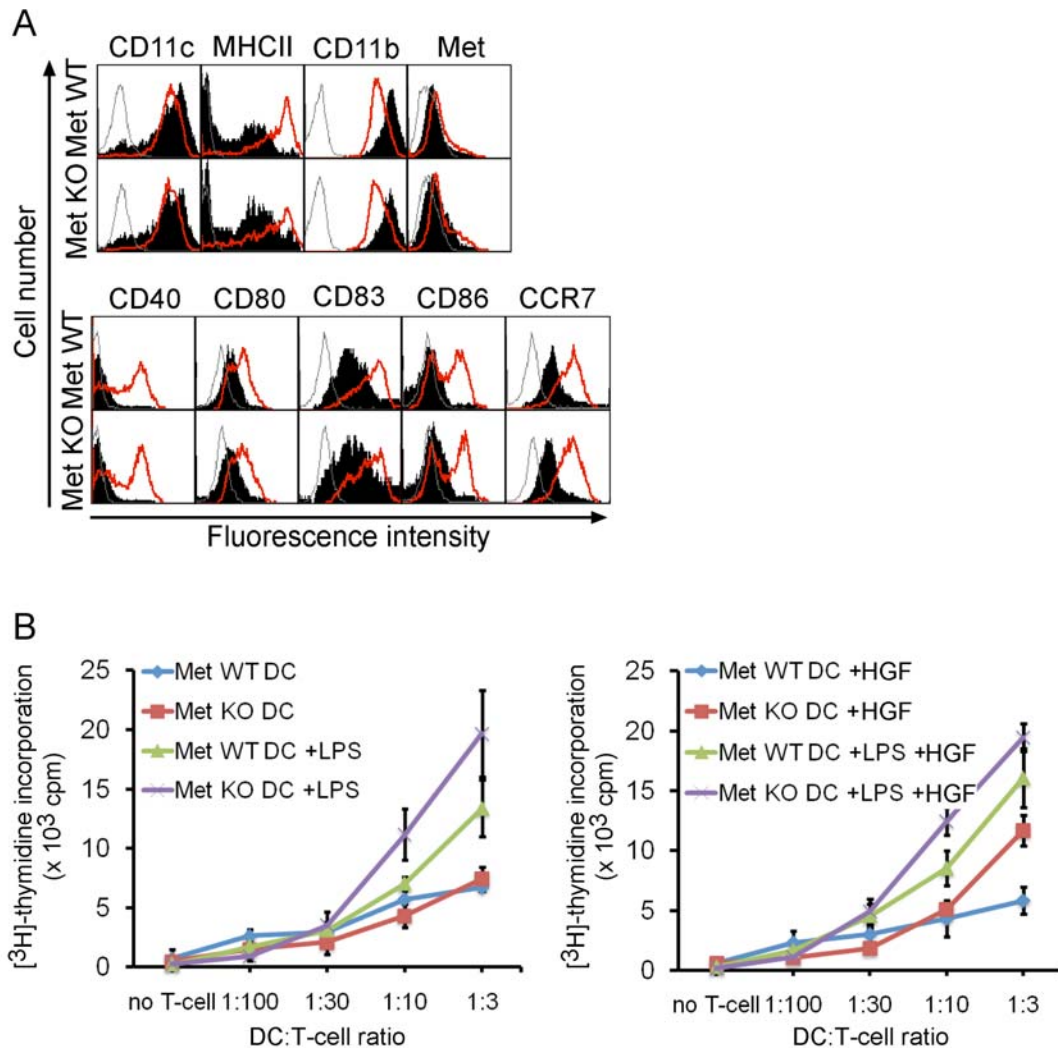
(C) Immunofluorescence microscopy of epidermis from Met KO and Met WT ear skin explants. After three days in culture in the presence or absence of factors as indicated epidermal sheet were prepared and stained for MHC class II (red); DAPI (blue) was used for nuclear staining. Scale bars, 50 μ m.

(D) Met expression on emigrated LCs/dDCs from Met WT mice analyzed by flow cytometry. Dorsal and ventral halves of mouse ear skin explants were cultivated for three days in the presence of LPS, TNF α , HGF, or without factor. Cells in culture supernatants were harvested and stained for CD11c, langerin and Met. Contour plots show langerin and Met expression on CD11c+ gated cells. Representative results from 5 independent experiments are shown.



Supplemental Figure 3. DC migration from skin towards draining LNs is impaired in Met KO mice.

Single-cell suspensions of LNs from Met KO and Met WT mice were analyzed by flow cytometry 24 h, 48 h, and 72 h after FITC-painting of abdominal skin. For detection of FITC+ DCs, cells were stained for CD45, CD11c, and MHC class II and total cells were gated on CD45. Mesenteric LNs were analyzed as non-draining LN control. Numbers in each histogram indicate frequencies of gated FITC+ CD11c+ DCs within total LN lymphocyte (CD45+) population. Representative results from 3 independent experiments are shown.



Supplemental Figure 4. Phenotype and antigen-presenting function of BM-derived DCs from Met KO and Met WT mice.

(A) DCs were differentiated from BM-derived Flt3+ progenitor cells of Met KO and Met WT mice and examined by flow cytometry for surface antigen expression as indicated (filled histograms). Open red histograms show surface antigen expression after TNF α stimulation for 24 h; open grey histograms, isotype controls. Representative results from 3 independent experiments are shown.

(B) CD4+ T cells from OT-II mice were co-cultured with OVA peptide-pulsed BM-derived DCs from Met KO and Met WT mice at various ratios. Antigen-specific activation of CD4+ T cells was analyzed by $[^3\text{H}]$ -thymidine incorporation in DC/T cell co-cultures without HGF stimulation of DCs (left panel) or after stimulation with HGF (40 ng/ml) for 24 h (right panel). Results (mean triplicate values $\pm$ s.d.) from one representative out of three individual experiments are shown.