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A novel role of MMP-13 for murine DC function: its inhibition dampens T-cell activation

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Abstract

Dendritic cells (DCs) have been shown to express matrix metalloproteinase 13 (MMP-13), but little is known about its specific function in DCs and its role in inflammatory conditions. In the present study, we describe a novel role of MMP-13 in regulating the immunostimulatory function of murine DCs through moderating MHC-I surface presentation, endocytosis and cytokine/chemokine secretion. MMP-13 expression was confirmed in bone marrow-derived DCs at both the mRNA and the protein level and, furthermore, at the activity level. Remarkably, LPS treatment strongly enhanced MMP-13 mRNA expression as well as MMP-13 activity, indicating an important role of MMP-13 in inflammatory processes. Functionally, MMP-13 inhibition did not influence the DC migratory capacity, while endocytosis of ovalbumin was significantly decreased. Inhibition of MMP-13 lowered the capability of murine DCs to activate CD8⁺ T cells, apparently through reducing MHC-I surface presentation. Decreased surface expression of CD11c on DCs, as well as changes in the DC cytokine/chemokine profile after MMP-13 inhibition, emphasizes the influence of MMP-13 on DC function. Moreover, T-cell-targeting cytokines such as IL-12, IL-23 and IL-6 were significantly reduced. Collectively, our data reveal a novel involvement of MMP-13 in regulating DC immunobiology through moderating MHC-I surface presentation, endocytosis and cytokine/chemokine secretion. Furthermore, the reduced MHC-I surface presentation by DCs resulted in a poor CD8⁺ T-cell response *in vitro*. This novel finding indicates that MMP-13 might be a promising target for therapeutic intervention in inflammatory diseases.

Keywords: CD8⁺ T-cell activation, cytokine profile, DCs, endocytosis, MHC-I, MMP-13

Introduction

Matrix metalloproteinases (MMPs) belong to the family of zinc endopeptidases that are involved in many different cellular processes (1, 2). Beyond their main activity as enzymes degrading extracellular matrix proteins, recent reports further indicate a role in immunological processes (3–6). MMP-13 is a member of the collagenase subfamily and is mainly expressed in chondrocytes and osteoblasts as well as in a variety of neoplastic cells (7–9). Moreover, recent studies show that MMP-13 is also expressed in murine bone marrow-derived dendritic cells (DCs) (10) as well as in murine pulmonary DCs (11). Nevertheless, very little is known about the specific function of MMP-13 in DCs and its role in inflammatory conditions.

DCs are professional antigen-presenting cells that link innate and adaptive immunity (12, 13). During their life cycle, DC precursors migrate into tissue where they act as sentinel cells against intruders. After antigen uptake and processing, DCs migrate to the draining lymph nodes where they activate T lymphocytes. There are three main pathways to present peptides to T cells. Endogenous proteins are presented on MHC-I to CD8⁺ T cells, whereas exogenous proteins, internalized by endocytosis and degraded in endosomal compartments, are loaded onto MHC-II and activate CD4⁺ T cells (14). The third mechanism called cross-presentation refers to the process where exogenous proteins are also presented on MHC-I and stimulate CD8⁺ T cells. This procedure is thought to be crucial for the defense against viruses and tumor cells (15, 16). Another important feature of DCs is the release of cytokines and chemokines to skew the T-cell response to a specific type (T_h1/T_h2) or to bait other immune cells. During maturation, DCs undergo dramatic morphological changes that enable them to optimally pursue their different functions. As immature cells, DCs strongly express receptors required to capture antigens, whereas mature DCs down-regulate these receptors and up-regulate co-stimulatory and MHC molecules for efficient T-cell activation (17, 18).

For specific peptidases, a participation in diverse processes such as in migration, cytokine regulation and receptor cleavage has been reported (1, 4, 5); however, in DC biology, the function of MMPs, especially of MMP-13, is largely unknown.

Dysregulation of DCs or MMPs is reported in a wide range of diseases such as in graft dysfunction (bronchiolitis obliterans syndrome) and in inflammatory diseases (e.g. multiple sclerosis and arthritis) (19–29). A deeper understanding of DC biology with regard to MMP regulation will facilitate the specific targeting of DCs to possibly countervail DC dysregulation and ameliorate disease pathology. MMP-13 is of specific interest for two reasons: firstly, the availability of MMP-13-specific small molecule inhibitors and secondly, its more DC-restricted expression pattern compared to other MMPs (i.e. MMP-2 and -9), which are widely expressed across all leukocyte subpopulations. Therefore, inhibiting MMP-13 may allow us to target DC dysregulation more precisely leaving other immunological processes largely unaffected. Here, we provide for the first time a comprehensive analysis of MMP-13 involvement in DC biology and the moderation of DC function under specific MMP-13 inhibition, including *in vitro* migration, endocytosis, maturation, antigen presentation and cytokine/chemokine release.

Methods

Mice

Female C57Bl/6 mice were obtained from Jackson ImmunoResearch Laboratories and used at the age of 8–10 weeks. All experiments were conducted according to the guidelines of the Ethics Committee of the Helmholtz-Center Munich and approved by the local government authority (Regierung von Oberbayern, Bavaria, Germany).

Primary cells

DCs were generated as described by Brinker *et al.* (30). with some modification. Briefly, bone marrow cells were collected from C57Bl/6 mice. Then, 0.75×10^6 cells per ml were cultured in DC medium [RPMI 1640 with 10% heat-inactivated fetal bovine serum (FBS)], L-glutamine (500 µg ml^{-1}), 100 U ml^{-1} penicillin–streptomycin, 1 mM sodium pyruvate, 1× non-essential amino acids, 10 mM HEPES (all from PAA Laboratories) and 50 µM 2-mercaptoethanol (Invitrogen, Life Technologies, Carlsbad, CA, USA). Fresh medium with GM-CSF (1 µg ml^{-1}, PreproTech, Hamburg, Germany) was provided every second day. On day 6, non-adherent cells were seeded in new plates and, 24 h later, non-adherent cells were harvested. Cells were analyzed on day 7 for the surface expression of MHC-I, MHC-II, CD80, CD86, CD40 and CD11c (BioLegend, Fell, Germany). The purity of CD11c$^+$ DCs was more than 70% with a homogenous maturation state. If not stated otherwise, the following concentrations were used for the treatment of DCs with LPS (*Escherichia coli* K-235, Sigma-Aldrich, Taufkirchen, Germany), ovalbumin (OVA; grade VI, Sigma-Aldrich), or MMP-13 inhibitor (CL82198, Tocris Bioscience/Biozol, Eching, Germany): LPS (10 µg ml^{-1}), OVA (1 mg ml^{-1}) and CL82198 (10, 50, 100, 200, 500 µM).

Cell lines

The CD8$^+$ T-cell line B3Z was used which is specific for $OVA_{257–264}$-peptide (SIINFEKL) and has β-galactosidase expression controlled by the IL-2 promoter, kindly provided by Nilabh Shastri's group (Department of Molecular and Cell Biology, University of California) (31). Additionally, the CD4$^+$ T-cell line DOBW was used which is specific for $OVA_{323–339}$-peptide (32), kindly provided by Cliff Harding`s group (Department of Pathology, Washington University School of Medicine). Cells were cultured in IMDM with 10% heat-inactivated FBS, L-glutamine and penicillin–streptomycin.

Flow cytometry and surface expression of DCs

If not stated otherwise, cells were washed with PBS supplemented with 2% FBS, blocked with F_c-blocker (1% Gamunex®, Grifolds, Barcelona, Spain) and incubated on ice for 30 min in the dark with the respective antibodies, followed by multiple washing steps. Data were collected on LSR II (BD Biosciences, Heidelberg, Germany) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

To determine the surface expression of CD80 (Pacific blue, #104723), CD86 (PE, #105007), CD40 (PE/Cy7, #124621), CD11c (APC, #117310), H-2k^b MHC-I (PE #116507) and MHC-II (PerCP/Cy5.5, #107626; antibodies all from BioLegend, Fell, Germany), cells were pretreated with MMP-13 inhibitor (CL82198 hydrochloride, Tocris) and supplemented with LPS overnight. In case of the surface expression of H-2k^b MHC-I (PE #116507) and MHC-II (PerCP/Cy5.5, #107626), cells were additionally supplemented with OVA. To determine total H-2k^b MHC-I, cells were permeabilized after CD11c staining using Cytofix/Cytoperm™ according to the intracellular staining protocol of BD Biosciences, followed by H-2k^b MHC-I staining. To determine the presentation of the specific SIINFEKL peptide on MHC-I, DCs, generated from bone marrow, were pretreated with the indicated amounts of CL82198 for 1 h and pulsed with 1 mg ml^{-1} soluble OVA for 3 h, followed by washing and further cultivation for 6 h in DC medium supplemented with CL82198. SIINFEKL peptide (1 µg ml^{-1}) was used as a positive control. Afterward, DCs were washed and stained for H-2K^b combined SIINFEKL (PerCP/Cy5.5 anti-mouse H-2K^b bound to SIINFEKL #141610, BioLegend). For flow cytometry, cells were selected by forward and side scatter, followed by exclusion of cell doublets and live–dead staining with propidium iodide (PI). Afterward, the remaining cells were gated on CD11c$^+$ cells, and further gating was performed as required for the respective experiments.

mRNA isolation and qRT–PCR

Total RNA was extracted using pepGold total RNA kit (PeqLab, Erlangen, Germany), according to the manufacturer's protocol.

Subsequently, reverse transcription was performed using the GeneAMP PCR kit (Invitrogen, Life Technologies) together with random hexamers and 1 µg of isolated mRNA per reaction. qRT–PCR reactions were performed with SYBR Green I Master in a LightCycler® 480II (Roche Diagnostics, Mannheim, Germany) with standard conditions. Target genes were normalized to α-enolase expression. Mouse primer sequences were as follows: MMP-13_Fwd: ATCCCTTGATGCCATTACCA, MMP-13_Rev: AAGAGCTCAGCCTCAACCTG; MMP-2_Fwd: TGATGCTTTTGCTCGGGCCTTA, MMP-2_Rev: TTTACGCGGA CCACTTGTCCTT; MMP-9_Fwd: CGTCGTGATCCCCACTT ACT, MMP-9_Rev: AACACACAGGGTTTGCCTTC; MMP-12_ Fwd: TGATGCAGCTGTCTTTGACC, MMP-12_Rev: GTGGAA ATCAGCTTGGGGTA; TIMP-1_Fwd: GGCATCCTCTTGTTG CTATCACTG, TIMP-1_Rev: GTCATCTTGATCTCATAACGCT GG; TIMP-2_Fwd: GGCGTTTTGCAATGCAGACGTA, TIMP-2 _Rev: ATCTTGCACTCACAGCCCATCT; TIMP-3_Fwd: TTCT GCAACTCCGACATCGTGA, TIMP-3_Rev: CAGGCGTAGTGT TTGGACTGAT; IL-12p35_Fwd: ACTAGAGAGACTTCTTCCA CAACAAGAG, IL-12p35_Rev: GCACAGGGTCATCATCAAA GAC; IL-12p40_Fwd: GGAAGCACGGCAGCAGAATA, IL-12p 40_Rev: AACTTGAGGGAGAAGTAGGAATGG; IL-23_Fwd: CAGCAGCTCTCTCGGATTCTC, IL-23_Rev: TGGATACGGGC CACATTATTTTT; CXCL2_Fwd: TCCAGAGCTTGAGTGTGACG, CXCL2_Rev: TCCAGGTCAGTTAGCCTTGC; α-enolase_Fwd: TTGCTTTGCAGGGATCCTACT, α-enolase_Rev: GATCATCAG CTTGTCAATCTT.

Western blot analysis

DCs were harvested. Cell lysates containing equal amounts of total proteins were mixed with 50 mM Tris–HCl, pH 6.8, 100 mM dithiothreitol, 2% SDS, 1% bromophenol blue and 10% glycerol, separated in a 10% SDS–PAGE and blotted onto a nitrocellulose membrane. After blocking with 5% milk in Tris-buffered saline (TBS) with Tween 20 (0.1% Tween 20/TBS), membranes were incubated with anti-MMP-13 antibody (ab75606, Abcam, Cambridge, UK) overnight, followed by HRP-conjugated secondary antibodies overnight at 4°C. The protein bands were detected by enhanced chemiluminescence.

Zymography assay

Cross-activity of the MMP-13 inhibitor toward MMP-2 and -9 was controlled by zymography. Briefly, substrate-specific zymography for MMP-2 and -9 activities was performed with supernatant of LPS-stimulated DCs that had been treated with different concentrations of MMP-13 inhibitor. Nine microlitres of a 6× concentrated non-reducing loading buffer (0.6 g SDS, 1 mg bromophenol blue, 3 ml 1 M Tris–HCl (pH 6,8), 5 ml glycerol added to 10 ml H_2O) was mixed with 45 µl of supernatant. Proteins were separated by electrophoresis in a 10% SDS–PAGE gel containing 1% gelatin (Invitrogen) at 110 V. Recombinant MMP-2 and -9 proteins were run in parallel with the samples. After electrophoresis, gels were incubated in renaturing buffer containing 1× developing buffer (10× developing buffer: 60.6 g Tris–base [0.5 M, pH 7.5], 117 g NaCl (2 M), 7.4 g $CaCl_2$ (50 mM) and adjust to pH 7.5 by adding H_2O to 1000 ml) with 2.5% Triton X-100 for 1 h. Afterward, gels were washed two times in 1× developing buffer and incubated at 37°C with 1× developing buffer overnight. Gels were heated in PageBlue™ protein staining solution (Invitrogen, Life Technologies), then cooled while shaking. Finally, gels were washed with distilled H_2O, and enzymatic bands were visualized using the ChemiDoc imaging system (BioRad, Hercules, CA, USA)PeproTech.

MMP-13 activity assay

DCs were cultured with CL82198 for 24 h, followed by treatment with LPS. Active MMP-13 in the supernatant of DCs was measured 3 h after LPS treatment using the SensoLyte® Plus 520 specific MMP-13 assay (AnaSpec, Seraing, Belgium). To quantify the efficacy of CL82198 to inhibit murine MMP-13, recombinant murine MMP-13 protein (EMELCA Biosciences, Breda, Netherlands) was activated by 4-aminophenylmercuric acetate for 40 min at 37°C, incubated with CL82198 and quantified by the SensoLyte® 520 MMP-13 assay (AnaSpec) at Ex/Em = 490 nm/520 nm. The amount of recombinant MMP-13 protein used for this assay was 5 ng (100 ng ml^{-1}), which is much more than the amount measured in the supernatants of DCs treated with LPS (20–60 ng ml^{-1}) to ensure that the determined efficacy range of the inhibitor is sufficient to also block the amount of MMP-13 in the experimental samples.

MMP-12 activity assay

Cross-activity of the MMP-13 inhibitor toward MMP-12 was controlled by an MMP-12 activity assay that also detects MMP-3 activity. DCs were cultured with CL82198 for 24 h, followed by treatment with LPS. Active MMP-12 and -3 in the supernatant of DCs was measured 3 h after LPS treatment using the SensoLyte® 520 MMP-12 assay (AnaSpec) according to the manufacturer's instructions (extinction = 490 nm; emission = 520 nm). The SensoLyte® 520 MMP-12 assay is based on the same method as described for the SensoLyte® Plus 520 specific MMP-13 assay but without the usage of the specific anti-MMP antibody.

Cell viability

First, 1×10^6 DCs, 0.5×10^6 B3Z cells or 1×10^6 DOBW cells per ml were treated with the indicated amounts of MMP-13 inhibitor for 24 h. WST-1 reagent and PI/Annexin staining were used to determine the toxic effect of CL82198 for the different cell types. WST-1 (Roche) was used according to the manufacturer's instructions with a 30-min incubation step. For live–dead staining, DCs, B3Z and DOBW cells were treated as described above, washed two times with cold PBS and stained with 5 µl Annexin V-FITC antibody and PI (BD Biosciences) for 15 min at room temperature. Cells were diluted in 400 µl 1× binding buffer (0.1 M HEPES, pH 7.4; 1.4 M NaCl; 25 mM $CaCl_2$) and measured by flow cytometry within 1 h. Cells were analyzed for percentages of dead and apoptotic cells using an LSR II (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Migration assay

Collagen gel was prepared according to the manufacturer's instructions (Biochrom AG/Merck, Grafing, Germany). Each transwell (PC membrane, 5.0 µm pore size, Corning, Thermo Fisher Scientific, Schwerte, Germany) was filled with 50 µl gel, polymerized for 2h at 37°C and placed in 600 µl RPMI/0.5% BSA supplemented with CCL19 (10 µg ml^{-1}, PeproTech, Rocky Hill, NJ, USA). DCs were pretreated with CL82198 for 1h in RPMI/0.5% BSA. Afterward, 2×10^5 DCs were seeded on top of the collagen-filled transwells, treated with LPS or left untreated. Twenty-four hours later, 500 µl of cell suspension from each bottom chamber was harvested and stained for CD11c and MHC-II. CountBright™ absolute counting beads (Invitrogen, Life Technologies) were used without any further washing steps to count the $CD11c^+$ DCs by flow cytometry.

Endocytosis assay

DCs (2×10^5) were pretreated using CL82198 for 1h at 37°C, cooled down on ice and incubated with $OVA_{Alexa\ 488}$ (10 µg ml^{-1}, Invitrogen, Life Technologies) for 1h at 4°C (control) or 37°C. Cells were washed and stained for CD11c and MHC-II. DCs were divided in different analysis groups based on their expression intensity of MHC-II. The intensity of OVA uptake was analyzed by flow cytometry.

T-cell activation assay

$CD8^+$ T-cell activation via MHC-I presentation was detected using the $CD8^+$ T-cell line B3Z. Then, 2×10^5 DCs per 96-well plate were either not treated (group 1) or pretreated with CL82198 for 1h at 37°C (group 2). Subsequently, cells were pulsed with soluble OVA or 1 µg ml^{-1} SIINFEKL peptide for 3h. DCs were washed and cultured with fresh CL82198 overnight in the presence of 1×10^5 B3Z cells. Cell supernatant was harvested, and the remaining cells were incubated for 3h with LacZ buffer [β-mercaptoethanol, 4.5mM $MgCl_2$, 0.065% NP40, 0.046mg ml^{-1} chlorophenol red-β-D-galactopyranoside (Roche) in PBS] at 37°C. Activation of B3Z cells was monitored by measuring IL-2 accumulation in the cells by a colorimetric LacZ assay (absorbance 570/620nm) and in the supernatant by an IL-2 ELISA (R&D Systems; Wiesbaden-Nordenstadt, Germany). To exclude direct influences of CL82198 on B3Z cells, untreated DCs were pulsed with SIINFEKL for 2h, fixed in 1% paraformaldehyde (PFA) for 30min and co-incubated with B3Z cells supplemented with CL82198. Activation of $CD4^+$ T cells through MHC-II presentation pathway was detected using the $CD4^+$ T-cell line DOBW. Experiments were performed as described for B3Z cells with slight variations. Then, 1×10^5 DCs were co-incubated with 2×10^5 DOBW cells. As positive control and to detect the influence of CL82198 on DOBW cells, $OVA_{323–339}$-peptide (AnaSpec) was used. Activation of DOBW cells was monitored using an IL-2 ELISA. Note, the $CD4^+$ T-cell line secreted less IL-2 (mean value: 9 pg ml^{-1}) than the $CD8^+$ T-cell line (mean value: 300 pg ml^{-1}).

Cytokine and chemokine measurement

DCs were cultured with CL82198 for 24h, followed by LPS treatment for 3h. Cytokine and chemokine profiles were analyzed in the supernatant using a luminex bead-based multiplex screening assay (R&D) that screens the following chemokines/cytokines: CCL2/JE/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, CCL20/MIP-3α, CXCL1/KC, CXCL2/MIP-2, CXCL10/IP-10/CRG-2, IFN-γ, IL-1β, IL-6, IL-10, IL-12 p70, IL-13, IL-23 p19, CXCL5/LIX, TNF-α and CXCL12/SDF-1α.

Statistics

Statistical analysis was performed using GraphPad Prism4 (GraphPad software). Data are presented as mean ± SD or median with min. to max. (box plot) using one-way ANOVA with Dunnett's multiple comparison test or one-way ANOVA with Kruskal–Wallis Dunn's comparison test, or one-sample *t*-test.

Results

Expression of MMP-13 in DCs is increased by LPS stimulation

We first examined the expression of MMP-13 and the natural inhibitors [tissue inhibitor of metalloproteinases (TIMPs: TIMP-1, -2, -3)] in untreated DCs derived from bone marrow. MMPs and TIMPs were detected via qRT–PCR (Fig. 1A). Western blot analysis confirmed the presence of the different forms of the MMP-13 protein, proenzyme, active form and cleaved fragments (Fig. 1B). To determine the expression of MMP-13 during inflammatory conditions, DCs were treated with LPS. Treatment with LPS significantly increased the MMP-13 mRNA level (Fig. 1C). Additionally, we investigated whether the activity state of MMP-13 changed during inflammatory conditions. Three hours after LPS treatment, active MMP-13 increased with absolute values of active MMP-13 ranging from 20 to 60ng ml^{-1} (Fig. 1D). Taken together, these results confirmed the expression of MMP-13 under baseline conditions and showed significant up-regulation in response to an inflammatory stimulus.

To clarify the importance of MMP-13 for the phenotype and function of DCs, including migration, endocytosis, cytokine release, antigen presentation and T-cell activation, we used the specific small molecule MMP-13 inhibitor CL82198. First, we verified the efficacy of inhibition for murine MMP-13 using a recombinant murine protein. As demonstrated in Supplementary Figure 1A, available at *International Immunology* Online, MMP-13 inhibitor CL82198 decreased active recombinant murine MMP-13 dose dependently.

To exclude the toxic effects of CL82198 on DCs and T cells (B3Z, DOBW), cells were treated with inhibitor, followed by cell viability assays. CL82198 did not show toxic effects on DCs as revealed by measuring metabolically active cells by the WST assay (Supplementary Figure 1B, available at *International Immunology* Online) and by Annexin/PI staining (Supplementary Figure 1C, available at *International Immunology* Online). More than 80% of cells were still metabolic, active at the highest inhibitor concentration (Supplementary Figure 1B, available at *International Immunology* Online) and no increase in apoptosis could be detected (Supplementary Figure 1C, available at *International Immunology* Online).

The cross-activity of CL82198 toward MMP-2, -9 and -12, which are also expressed in DCs (Supplementary Figure 2A, available at *International Immunology* Online), was controlled. A zymography assay for MMP-2 and -9 activities showed no decrease in MMP-9 activity in DCs treated with the MMP-13 inhibitor (Supplementary Figure 2B, available at *International Immunology* Online). MMP-2 activity was below the detection limit of the assay. A potential effect of the MMP-13 inhibitor towards MMP-12 was evaluated using a fluorescence resonance energy transfer-based activity assay (AnaSpec). In

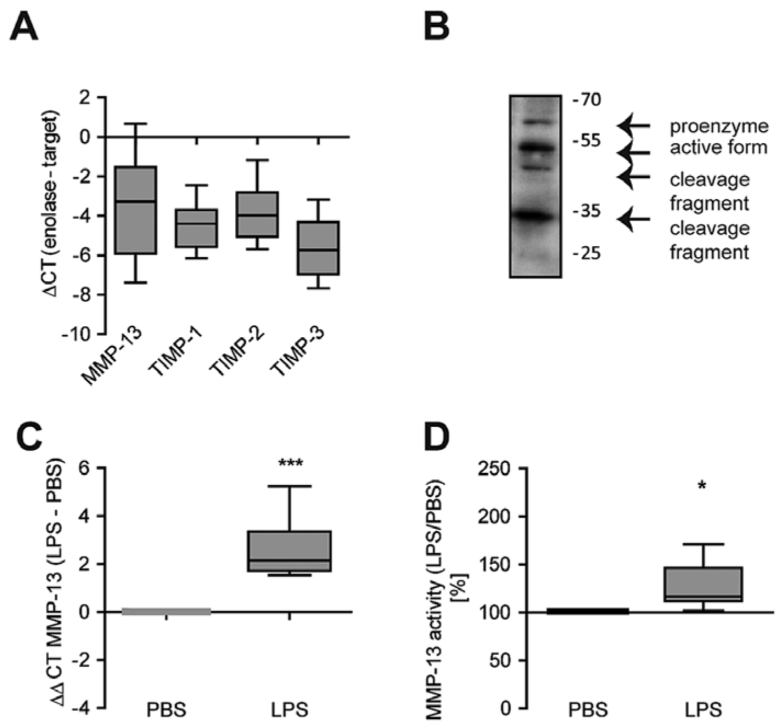

Fig. 1. MMP-13 expression is up-regulated in response to inflammatory stimulus in DCs *in vitro*. (A) Total cell lysates of DCs were analyzed on day 7 by qRT–PCR for the presence of MMP-13 in relation to TIMPs. Data are illustrated in a box plot (min. to max.) as ΔCT values (n = 13). (B) The different forms of MMP-13, proenzyme (60 kDa), active form (54 kDa) and cleaved fragments (48/34 kDa) were validated by western blot. Molecular weights are indicated in kilodaltons. Results are representative of five independent experiments. (C) DCs were cultured with LPS for 3 h. Expression of MMP-13 was analyzed by qRT–PCR. Data are shown as ΔΔCT values of LPS-treated DCs normalized to PBS-treated cells of 11 independent experiments (box plot with min. to max.). (D) Active MMP-13 in the supernatant of DCs was validated 3 h after LPS treatment by SensoLyte® Plus 520 specific MMP-13 assay. Data are displayed as box plot (min. to max.) with relative values of LPS-treated cells normalized to PBS-treated cells in percentages (n = 6). Statistical analysis: one-sample t-test. $^{*}P < 0.05$ and $^{***}P < 0.001$.

addition to MMP-12, this assay also detects MMP-3 activity. As shown in Supplementary Figure 2C, available at *International Immunology* Online, no inhibition of MMP-3/-12 activity was observed in MMP-13 inhibitor-treated DCs.

MMP-13 does not regulate the migratory capacity of DCs

The capability to migrate is a central function of DCs: in the process of host defense, DCs must leave the periphery to reach the area of infection and then move on to the lymph nodes. To investigate whether MMP-13 plays a role in DC migration, we performed a 3D-collagen migration assay with CCL19 as chemoattractant using LPS-stimulated and non-stimulated DCs in response to MMP-13 inhibition. As demonstrated in Fig. 2(A), no migration was detected without the chemoattractant CCL19 in the LPS-stimulated and unstimulated DCs. LPS-stimulated DCs showed a higher percentage of migrated cells compared with unstimulated DCs (Fig. 2A). With regard to MMP-13 inhibition, CL81298 treatment did not inhibit the migration of LPS-stimulated (Fig. 2B) or unstimulated DCs (data not shown). A slight increase in migration was observed at 200 μM inhibitor, which did not reach significance.

MMP-13 is involved in OVA endocytosis by DCs

To determine whether MMP-13 influences antigen uptake by DCs, we exposed DCs to fluorochrome-labeled OVA. As shown in Fig. 3(A), soluble OVA was efficiently captured by CD11c^{+} DCs. After application of CL82198, the mean fluorescence intensity (MFI), which visualizes the amount of captured OVA, decreased dose dependently. The summary of nine independent experiments clearly demonstrates a significant decline in the capability of DCs to endocytose OVA in the presence of CL82198 (Fig. 3B). To further determine whether the observed effect can be correlated to a specific DC phenotype, CD11c^{+} cells were grouped according to their MHC-II surface expression profile and subgroups were compared for their OVA uptake intensity. In line with other studies (33), immature DCs with low MHC-II surface expression showed the strongest capability to capture OVA compared with MHC-II^{high} DCs (data not shown), but the reduction in the OVA uptake intensity was observed in all subgroups.

MMP-13 is required for the activation of CD8^{+} T cells by DCs

Another fundamental ability of DCs is the processing and presentation of antigen on MHC molecules and, thereafter, the activation of the adaptive immune response. DCs treated with soluble OVA can either activate CD4^{+} T cells via the MHC-II pathway or CD8^{+} T cells by cross-presentation via the MHC-I pathway.

To elucidate the role of MMP-13 in CD8^{+} T-cell activation, we used CL82198-pretreated DCs, pulsed them with OVA, followed

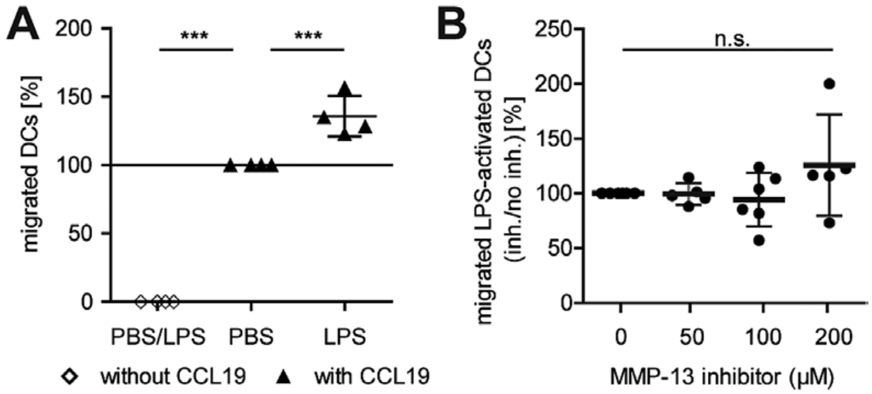

Fig. 2. MMP-13 does not regulate migratory capacity of DCs. LPS-stimulated or unstimulated DCs were seeded overnight on collagen-filled transwells. DCs that migrated into the bottom chamber to CCL19 as chemoattractant were stained for CD11c and MHC-II and counted by flow cytometry. (A) Comparison of DCs without and with CCL19 in the bottom chamber as well as DCs stimulated with LPS and without LPS (PBS). Data are shown as relative values between LPS/PBS-treated groups without CCL19 and with CCL19 as well as between LPS-stimulated and unstimulated groups (both times supplemented with CCL19; mean ± SD; *n* = 4). (B) LPS-stimulated DCs were pretreated with different concentrations of CL82198 or left untreated. Data are shown as relative values between inhibitor-treated (inh.) and untreated (no inh.) groups (mean ± SD; *n* = 5–6). Statistical analysis: ANOVA with Dunnett's multiple comparison test. ***$P < 0.001$; n.s. = non-significant.

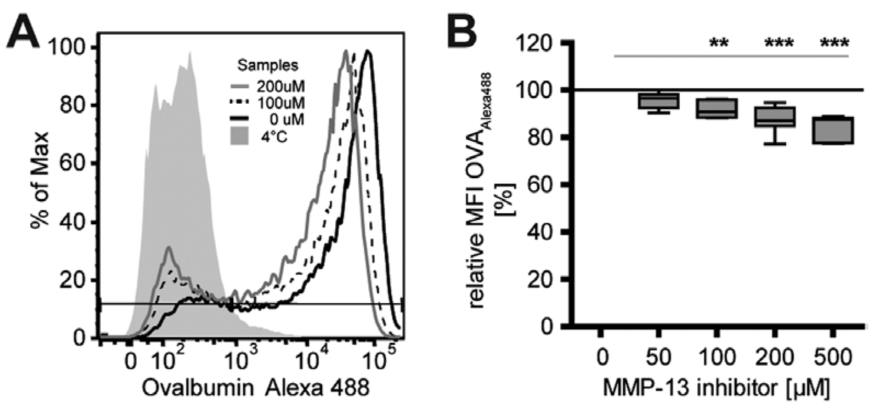

Fig. 3. MMP-13 is involved in endocytosis of soluble OVA by DCs. DCs were pretreated with MMP-13 inhibitor at 37°C or left untreated, cooled down on ice, incubated with $OVA_{Alexa\ 488}$ at 4°C (control) or 37°C, stained for CD11c and MHC-II and analyzed by flow cytometry. Data represent uptake of $OVA_{Alexa\ 488}$ by CD11c$^+$ DCs. (A) Representative result of nine independent experiments. (**grey area** = control on 4°C, black line = no CL82198 at 37°C, dashed lines = 100 µM, **grey line** = 200 µM CL82198 at 37°C). (B) Summary of nine experiments illustrated in a box plot (min. to max.) as relative MFI values between treated (inh.) and untreated (no inh.) groups. Statistical analysis: ANOVA with Dunnett's multiple comparison test. **$P < 0.01$ and ***$P < 0.001$.

by cocultivation with B3Z cells (CD8$^+$ T cells), in the presence of CL82198. As readout for T-cell activation, IL-2 production was measured. Quantifying IL-2 production by ELISA and LacZ assay, we observed a reduced capability of DCs to activate CD8$^+$ T cells in the presence of CL82198 in a dose-dependent manner (Fig. 4Ai and ii). To determine whether the observed effect is due to the reduced endocytic capability shown in Fig. 3, we applied CL82198 exclusively after OVA pulsing. As depicted in Fig. 4(Aiii and iv), a similar reduction in T-cell activation was detected, indicating that the reduction of OVA uptake did not significantly influence B3Z cell activation. To control that the inhibitor does not act directly on the CD8$^+$ T cells and thereby inhibits the IL-2 secretion, we used SIINFEKL-pulsed and fixed DCs without inhibitor pretreatment and co-incubated them with CD8$^+$ T cells in the presence of CL82198. Notably, no reduction in IL-2 secretion was detected (Fig. 4Av and vi), excluding a direct effect of CL82198 on CD8$^+$ T cells.

To further elucidate a potential role of MMP-13 in the classical MHC-II pathway, we performed similar experiments using CD4$^+$ T cells (DOBW cells). We observed a slight reduction in CD4$^+$ T-cell activation by DCs, measuring IL-2 secretion by ELISA (Fig. 4Bi). To exclude a direct effect of CL82198 on CD4$^+$ T cells, we used $OVA_{323-339}$-peptide pulsed and fixed DCs without inhibitor pretreatment and co-incubated them with T cells in the presence of CL82198. We observed a reduction in IL-2 secretion comparable to that seen with DCs that were treated with CL82198, indicating that CL82198 might directly affect CD4$^+$ T cells (Fig. 4Bii). Indeed, low levels of MMP-13 transcript (Fig. 4C) as well as active MMP-13 (data not shown) were detected in DOBW cells.

In summary, these results suggest that MMP-13 plays a crucial role in CD8$^+$ T-cell activation in murine DCs.

MMP-13 is involved in MHC-I surface expression

To further investigate the mechanism leading to reduced CD8$^+$ T-cell activation, we considered the possibility of reduced peptide presentation by DCs. To analyze this, DCs

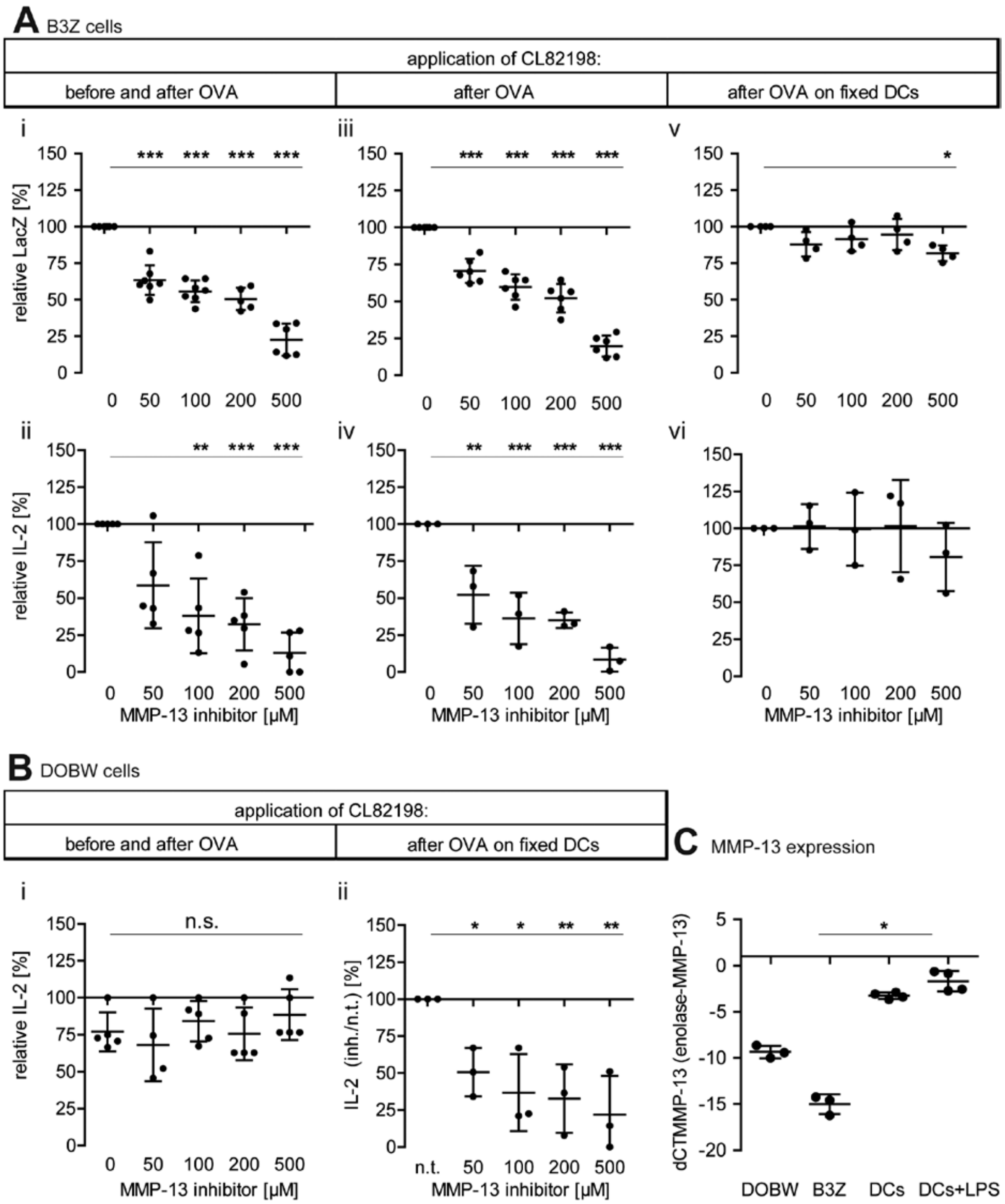

Fig. 4. MMP-13 inhibition decreases the capacity of DCs to activate CD8+ T cells via MHC-I molecules. (Ai and ii) 2×10^5 DCs were pretreated with the indicated amounts of CL82198 or left untreated, followed by protein pulsing with OVA or PBS (as control), washed and cultured with the indicated amounts of CL82198 overnight in the presence of 1×10^5 CD8+ T cells (B3Z cells). (Ai) Activation of B3Z cells was monitored by measuring IL-2 accumulation in a colorimetric LacZ assay ($n = 6$) or (Aii) IL-2 secretion in the supernatant by an ELISA ($n = 5$). (Aiii and iv) The experiment was performed as described above with only a slight variation: DCs were treated after the protein pulsing with OVA was already completed (Aiii: $n = 6$, Aiv: $n = 3$). (Av and vi) To exclude influences of CL82198 on B3Z cells, untreated DCs were pulsed with SIINFEKL, washed, fixed in 1% PFA, washed and co-incubated with the indicated amounts of MMP-13 inhibitor and B3Z cells. (Av: $n = 4$, Avi: $n = 3$). (B) Activation of CD4+ T cells via MHC-II molecules was detected by IL-2 ELISA, performing similar experiments as described in (A) with slight variations: (Bi) 1×10^5 DCs were pretreated with the indicated amounts of MMP-13 inhibitor, followed by protein pulsing with OVA or PBS and cultured with the indicated amounts of CL82198 overnight in the presence of 2×10^5 CD4+ T cells (DOBW; $n = 5$). (Bii) To exclude influences of CL82198 on DOBW cells, untreated DCs were pulsed with $OVA_{323–339}$-peptide, washed, fixed in 1% PFA, washed and co-incubated with the indicated amounts of MMP-13 inhibitor and DOBW cells ($n = 3$). Data are illustrated as relative values between CL82198-treated (inh.) and -untreated (no inh.) groups (mean ± SD). (C) qRT–PCR analyses of MMP-13 expression in B3Z, DOBW and DCs treated with LPS or left untreated. Data are shown as $\Delta CT_{(\alpha\text{-enolase–MMP-13})}$ values. Statistical analysis: ANOVA with Dunnett's multiple comparison test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ and n.s. = non-significant.

were treated with CL82198 and OVA overnight, followed by staining for CD11c and SIINFEKL bound to H-2K^b of MHC-I. As depicted in Fig. 5(A), SIINFEKL presentation on MHC-I was slightly reduced in the presence of CL82198 at the highest inhibitor concentration. To further clarify whether this observation was due to a general reduction of MHC-I surface expression or an OVA-peptide-specific effect, we performed surface stainings for MHC-I and MHC-II molecules.

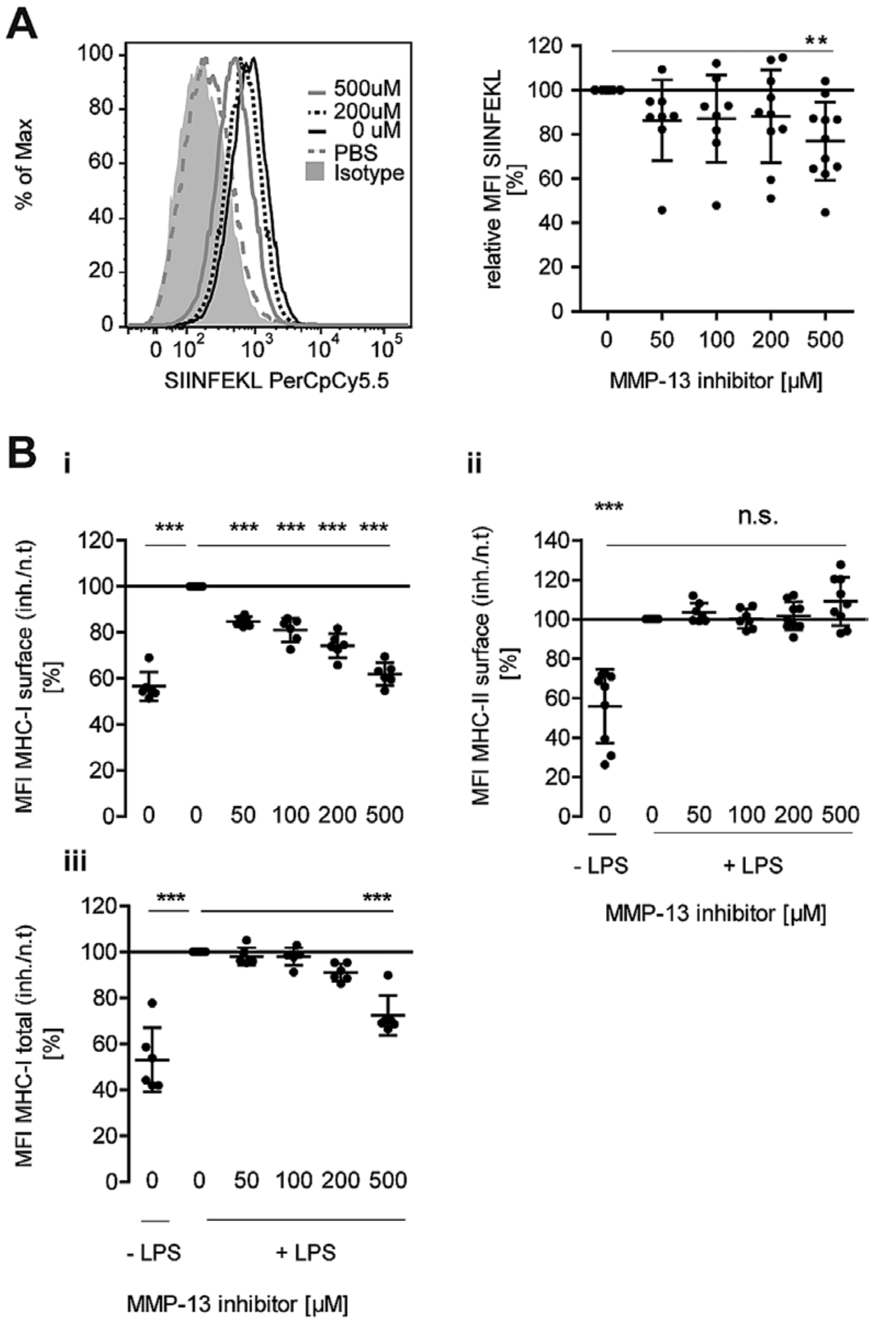

Fig. 5. MHC-I but not MHC-II is decreased on the surface of DCs after MMP-13 inhibition. (A) DCs were pretreated with the indicated amounts of CL82198, followed by treatment with OVA or PBS (control), washed and cultured for 5 h in the presence of CL82198. Cells were washed and stained for CD11c and SIINFEKL bound to H-2K^b of MHC-I. (Left) Representative result of 10 independent experiments, (grey area = isotype, grey dashed lines =PBS, black line = without CL82198, black dotted line = 200 µM, grey line = 500 µM CL82198). (Right) Data summary is illustrated as relative MFI values between inhibitor-treated (inh.) and -untreated (no inh.) DCs (mean ± SD). (B) DCs were pretreated with the indicated amounts of CL82198, followed by treatment with OVA and cultivation overnight. Cells were washed and stained for CD11c, followed by staining of (i) surface MHC-I, (ii) surface MHC-II and (iii) total MHC-I using permeabilized DCs. Expression was analyzed by flow cytometry (n = 4, MHC-II: n = 9). Data are shown as relative MFI values between inhibitor treated (inh.) and untreated (no inh.) OVA-stimulated CD11c$^+$ DCs and between unstimulated (no OVA) and untreated (no inh.) OVA-stimulated DCs (mean ± SD). Statistical analysis: ANOVA with Dunnett's multiple comparison test. **P < 0.01 and ***P < 0.001, n.s. = non-significant.

In line with the T-cell activation assay, surface expression of MHC-I was significantly reduced on DCs treated with inhibitor (Fig. 5Bi), whereas MHC-II was not significantly changed (Fig. 5Bii).

To gain insight into the mechanism that leads to the reduced MHC-I surface expression, the total amount of MHC-I was determined by staining permeabilized cells. As shown in Fig. 5(Biii), in contrast to surface MHC-I (Fig. 5Bi), total MHC-I was largely unchanged with a moderate decrease only at the highest inhibitor concentration. As the strong decline in the MHC-I surface expression after MMP-13 inhibition is not recapitulated at the total MHC-I protein level, one might speculate that the surface presence is reduced due to altered MHC-I trafficking or MHC-I cycling.

MMP-13 inhibition decreases CD11c while preserving DCs' maturation profile

T cells are activated by DCs through the recognition of specific peptides on MHCs together with the help of co-stimulatory molecules, such as CD40, CD80, and CD86, which are up-regulated during DC maturation. To determine whether MMP-13 has an influence on the maturation profile of DCs, we next analyzed CL82198-treated DCs after LPS stimulation. As expected, CD40, CD80 and CD86 were strongly up-regulated by DCs during LPS treatment compared with the untreated controls. This up-regulation occurred similarly when MMP-13 was inhibited (Fig. 6Ai–iii). This implies that MMP-13 does not alter this process of DC maturation in murine DCs.

Next, we analyzed the surface expression of the α-integrin CD11c. Previous reports suggested that CD11c might be involved in antigen presentation by DCs (34). Remarkably, MMP-13 inhibition diminished the intensity of CD11c surface expression dose dependently (Fig. 6B).

MMP-13 maintains the cytokine profile of mature DCs

A fundamental characteristic of DCs is the ability to release cytokines that determine the polarization of the innate and adaptive immune response. Therefore, we next addressed the role of MMP-13 in regulating the cytokine profile of DCs, especially with regard to cytokines that influence T-cell-mediated immune responses. We measured the release of 18 different cytokines/chemokines in response to the inflammatory stimulus LPS. Consistent with the literature (35), all detectable cytokines/chemokines were increased upon inflammatory stimulus (Fig. 7; Supplementary Figure 2C, available at *International Immunology* Online). IL-13, CCL20, CXCL12, IL-10, IL-1β and IFN-γ were below the detection limit. Therefore, the effect of MMP-13 inhibition could not be determined. IL-12p70, IL-23p19 and IL-6, which are known to moderate the polarization of T-cell response, were significantly decreased by MMP-13 inhibition in a dose-dependent manner (Fig. 7Ai–iii). Especially IL-12p70 (Fig. 7Ai) and IL-23p19 (Fig. 7Aii) were strongly decreased to around 50% compared with untreated cells. On the contrary, CXCL10, a T-cell-targeting chemokine, was increased after MMP-13 inhibition (Fig. 7Aiv), while chemokines CCL5, CXCL1, CXCL5, CCL3, CCL4 and CXCL2, which are mainly chemoattractants and activators of neutrophils, basophils and eosinophils, remained unchanged after MMP-13 inhibition (Fig. 7Bi–vi).

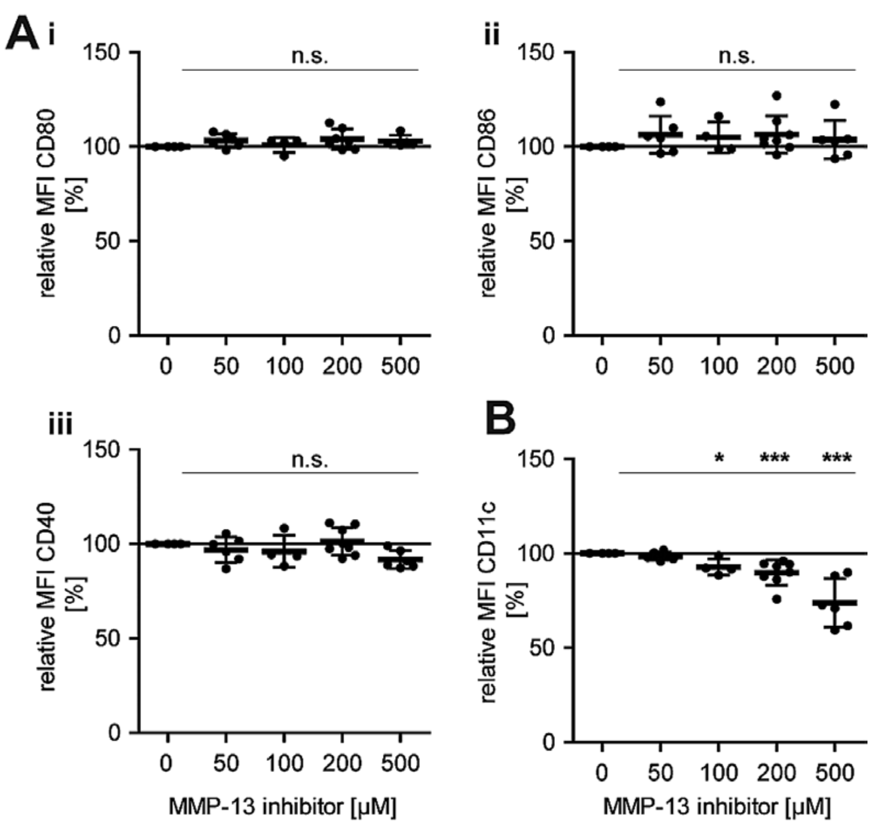

Fig. 6. MMP-13 does not regulate the maturation process of DCs, while MMP-13 inhibition decreases CD11c surface expression. DCs were pretreated with the indicated amounts of CL82198, followed by stimulation with LPS and cultivation overnight. Cells were stained for CD11c, MHC-II, CD80, CD86 and CD40. Data are illustrated as relative MFI values between inhibitor treated (inh.) and untreated (no inh.) CD11c+ DCs. (Ai–Aiii) Maturation markers. (B) CD11c. Statistical analysis: ANOVA with Dunnett's multiple comparison test (mean ± SD). $^{*}P < 0.05$, $^{***}P < 0.001$, n.s. = non-significant.

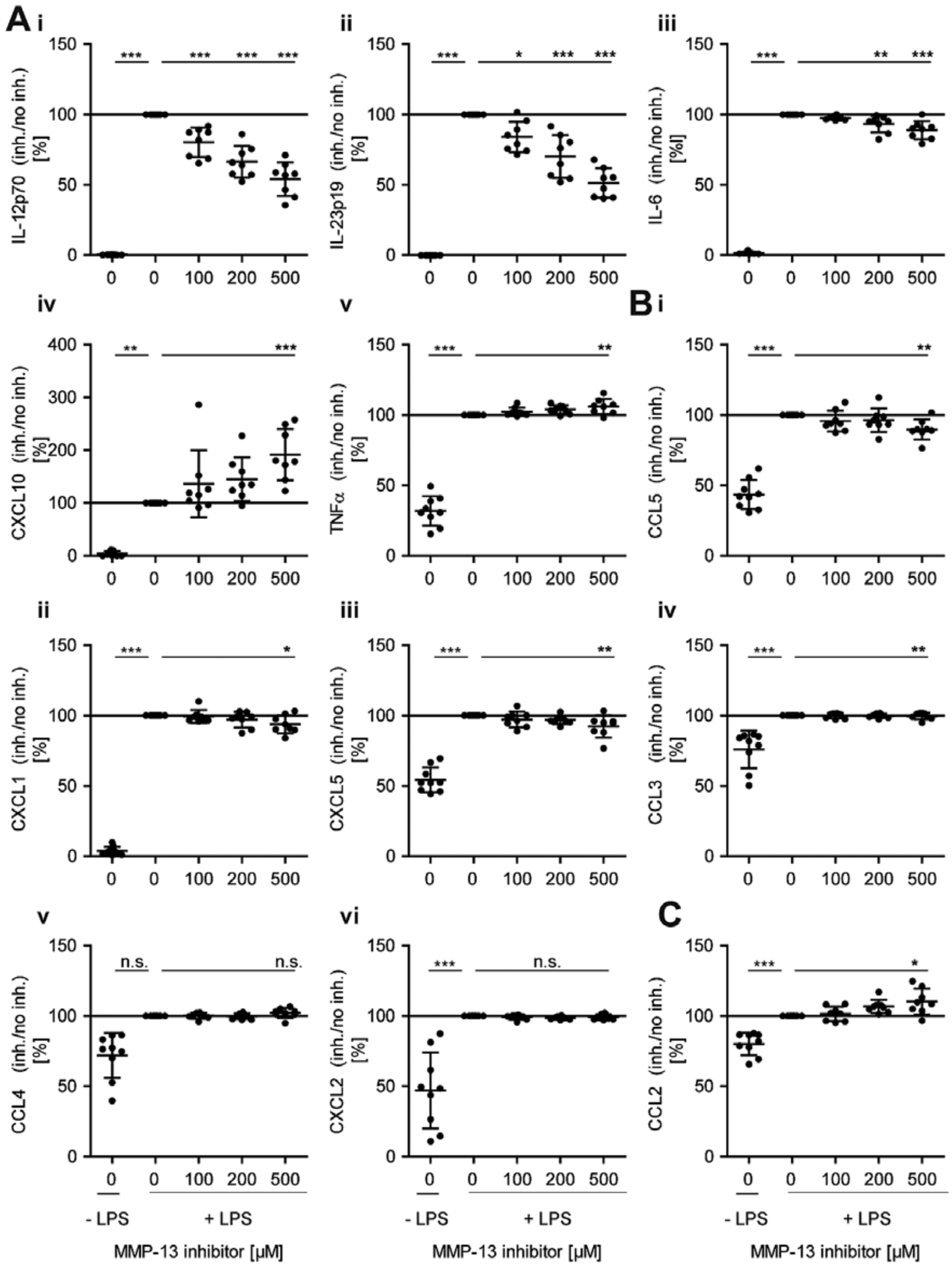

Fig. 7. Inhibition of MMP-13 in LPS-stimulated DCs leads to a different cytokine profile of typical T-cell affecting cytokines, showing decreased amounts of IL-12, IL-23 and IL-6 and increased CXCL10. DCs were cultured with CL82198 for 24 h, followed by treatment with LPS or PBS as control. The supernatant of DCs was harvested 3 h after LPS stimulation. The following cytokines and chemokines were analyzed using Luminex screening assay (R&D): CCL2/JE/MCP-1, CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES, CCL20/MIP-3α, CXCL1/KC, CXCL2/MIP-2, CXCL10/IP-10/CRG-2, IFN-γ, IL-1β, IL-6, IL-10, IL-12 p70, IL-13, IL-23 p19, CXCL5/LIX, TNF-α and CXCL12/SDF-1α. LPS-stimulated DCs treated with different concentrations of CL82198 or DCs treated with PBS instead of LPS (PBS) as unstimulated control are illustrated as relative values normalized to DCs treated with LPS without inhibitor ($n = 9$). (Ai–Av) IL-12p70, IL-23p19, IL-6, CXCL10 and TNF-α are typical cytokines/chemokines that target T cells. (Bi) CCL5 functioned as a chemoattractant for T cells as well as for basophils, neutrophils, monocytes, macrophages and immature DC cells. (Bii–vi) CXCL1, CXCL5, CCL3, CCL4 and CXCL2 are cytokines/chemokines that target neutrophils, subpopulations of T cells, monocytes and macrophages. (C) CCL2 belongs to the chemokines that recruits monocytes, memory T cells and DCs. Statistical analysis: ANOVA with Dunnett's multiple comparison test (mean ± SD). $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$, n.s. = non-significant.

CCL2, a chemokine that recruits monocytes, memory T cells and DCs to areas of infection, was also unchanged by MMP-13 inhibition (Fig. 7C).

These results suggest that MMP-13 plays a crucial role in shaping the cytokine profile of DCs.

To define whether the reduction in the amount of secreted cytokine was due to reduced transcription, qRT–PCR of selected genes was performed. As shown in Fig. 8, transcript levels of the two cytokines IL-12p35 and IL-23, whose secreted levels were dramatically reduced by MMP-13

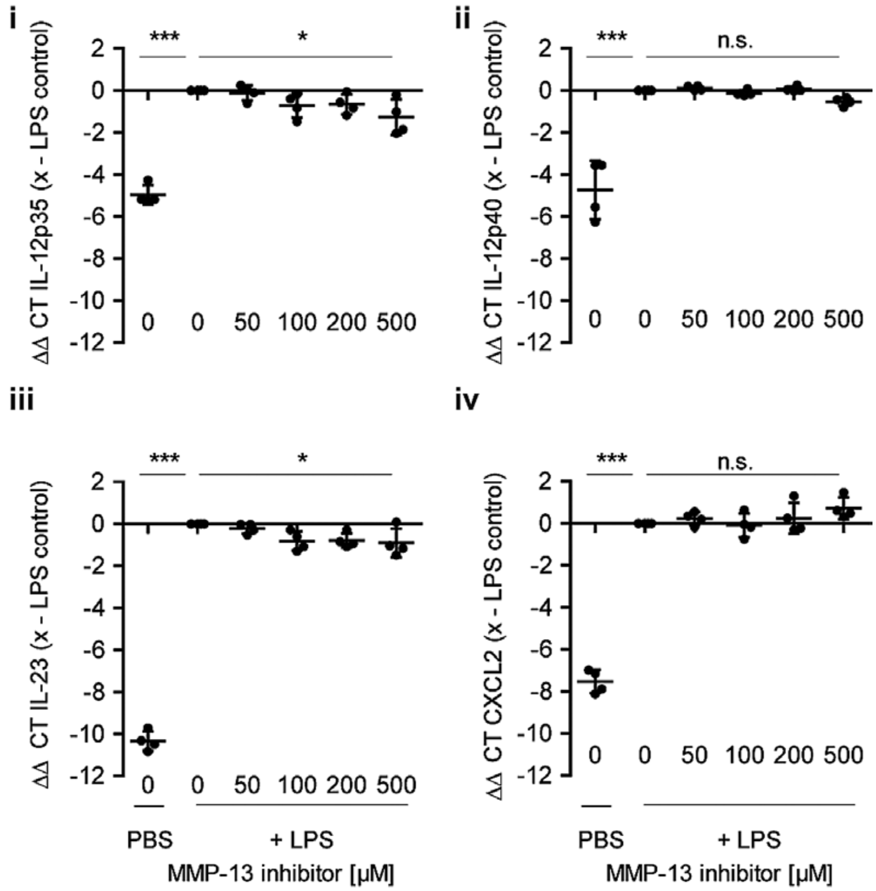

Fig. 8. Inhibition of MMP-13 in LPS-stimulated DCs reveals no or only minor alterations of IL-12p35, IL-12p40, IL-23 and CXCL2 on the transcriptional level. DCs were cultured with CL82198 for 24 h, followed by treatment with LPS or PBS as control. Cells were harvested 3 h after LPS stimulation and total cell lysates of DCs were analyzed by qRT–PCR for alterations in IL-12p35, IL-12p40, IL-23 and CXCL2. Data are illustrated as $\Delta\Delta CT_{(x - \text{LPS control})}$ values with *x* being DCs treated with PBS or LPS in combination with indicated inhibitor concentrations. Statistical analysis: ANOVA with Dunnett's multiple comparison test (mean ± SD). **P* < 0.05, ****P* < 0.001 and n.s. = non-significant.

inhibition, were largely unaltered with a moderate decline only seen at the highest inhibitor concentration (Fig. 8i and iii). Transcript levels of IL-12p40, the subunit shared by IL-12 and IL-23, were unaltered as was the transcript level of the chemokine CXCL2, which was also not affected on the level of secretion (Fig. 8ii and iv). These results indicate that MMP-13 acts more dominantly on processes of protein secretion than on transcription. The observation that MMP-13 inhibition manipulates mainly T-cell-targeting cytokines further suggests that MMP-13 can change the polarization of the adaptive immune response through modulating the capacity of DCs to secrete specific cytokines.

Discussion

In the present study, we analyzed the expression of MMP-13 in DCs during inflammatory conditions and evaluated the role of MMP-13 in essential immunological functions of DCs. We reported a novel role of MMP-13 in OVA endocytosis, MHC-I presentation and T-cell targeting cytokine release of DCs. Moreover, we discovered that MMP-13 inhibition diminishes the DCs' capacity of T-cell activation via the MHC-I pathway.

Recently, it has been demonstrated that LPS-treated DCs exhibit increased expression of MMP-13 at the mRNA level (10). We confirmed this result and, moreover, demonstrate that active MMP-13 increased in response to LPS treatment (Fig. 1). These results suggest that, under inflammatory conditions, DCs need MMP-13 for specific functions to fully act as antigen-presenting cells. To substantiate this hypothesis, we performed various functional assays in the presence of the specific MMP-13 inhibitor CL82198. We analyzed the involvement of MMP-13 in migration, endocytosis, antigen presentation, cytokine release and T-lymphocyte activation and, thereby, identified novel roles of MMP-13 in DC biology.

For the functional assays, we used a specific MMP-13 inhibitor (CL82198). This inhibitor was developed for human MMP-13 but was demonstrated in the current study to also react with murine MMP-13, albeit with lower activity. Cross-species application has been described for other human MMP inhibitors, such as the MMP-12 inhibitor MMP408, again with the requirement to use higher concentrations. For the MMP-13 inhibitor CL82198, we tested a concentration range between 50 and 500 µM in the murine system and could confirm efficient inhibition of murine MMP-13 using this concentration range (Supplementary Figure 1, available at

International Immunology Online). Notably, we tested the efficacy of inhibition against a high concentration (100 ng ml^{-1}) of murine recombinant MMP-13, which is much higher than the concentration of MMP-13 secreted by DCs after LPS stimulation. Thus, the determined effective range of the inhibitor should well suffice to inhibit MMP-13 in our assay systems. Using zymography and a MMP-3/-12 activity assay, it was determined that the MMP-13 inhibitor did not cross-inhibit MMP-9 or MMP-3/-12, respectively, in the concentration as applied (Supplementary Figure 2, available at *International Immunology* Online). The observation that MMP-13 inhibition does not cross-inhibit MMP-9 can be further deduced from the observed lack of inhibition of DC migration, which is known to involve MMP-9 (36).

Therefore, these assays provided evidence that the MMP-13 inhibitor CL82198 does not exhibit overt cross-activity against two of the highest expressed MMPs (MMP-9 and -12) in murine DCs. At the highest inhibitor concentration, a slight increase of MMP-3/-12 was detected (Supplementary Figure 2C, available at *International Immunology* Online), which might be a compensation effect due to the inhibition of active MMP-13 protein.

Several previous studies delineated an involvement of MMPs in cell migration and their contribution to various diseases such as cancer, myocardial infarction and hypertension (36–42). MMP-13 has already been intensively studied in keratinocyte (7) and fibroblast migration (43, 44). In DC biology, different groups reported that MMP-9 is crucial for migration, studied in murine Langerhans cells (45) and DCs (36), as well as in human DCs (39), by performing MMP-9 blocking studies. In the present study, we observed that MMP-13 inhibition does not alter the migration behaviour of DCs through collagen I (Fig. 2).

To date, nothing is reported about the involvement of MMPs in endocytosis. We observed that MMP-13 inhibition decreased the capability of DCs to capture soluble OVA (Fig. 3). The uptake of large amounts of soluble OVA (MFI^{high}) in DCs is mainly executed by the mannose receptor that mediates cross-presentation of OVA peptides via MHC-I, whereas the uptake of small amounts of OVA (MFI^{low}) via macropinocytosis drives OVA into the classical MHC-II pathway (33, 46–50). We observed a significant reduction in the OVA uptake, but how exactly MMP-13 is involved in antigen uptake remains to be clarified. No downstream effects of the reduced endocytosis on T cells could be substantiated, since no cumulative effect was seen when DCs were treated with CL82198 before plus after the OVA uptake (Fig. 4A). Obviously, despite reduced uptake through MMP-13 inhibition, enough OVA can still be captured by DCs to provide sufficient antigen presentation to OVA-specific T cells. As this is the first time showing an involvement of MMPs in the process of endocytosis, it will be of interest in the future to determine the relevance of MMP-13 for other antigens, including those with low expression levels.

While the downstream effects of MMP-13-regulating endocytosis in the OVA system remain to be determined, our further findings clearly demonstrate that CD8$^+$ T-cell activation was reduced when DCs were treated with MMP-13 inhibitor in a manner that was obviously independent of endocytosis (Fig. 4A).

To identify why CD8$^+$ T-cell activation was decreased, we investigated whether MMP-13 inhibition influenced the amount of H-2k^b MHC-I surface molecules. We detected decreased H-2k^b MHC-I surface expression on inhibitor-treated DCs (Fig. 5B). The immunological synapse between T lymphocytes and DCs is critical for successful T-cell activation. As synapse formation requires the recognition of the peptide–MHC complex by the specific T-cell receptor (51), the observed reduced MHC-I surface expression could be sufficient to impair the formation of a fully functional immunological synapse that may explain the decreased T-cell activation. The reduction in H-2k^b MHC-I surface molecules could be due to alteration in the MHC-I trafficking and cycling process since we observed that the total amount of MHC-I (surface plus intracellular amount) was largely the same in untreated and inhibitor-treated DCs (Fig. 5Biii). Hence, apparently, an involvement of MMP-13 in transcription or protein synthesis of MHC-I molecules cannot or cannot fully explain the observed reduction of surface MHC-I. The reduced surface presence of MHC-I might be due to retention of MHC-I on the way to the cell surface or enhanced removal from the cell surface because of recycling. Cleavage from the cell surface is unlikely since this would reduce the surface as well as the total amount of MHC-I. Trafficking to the cell surface is only permitted for fully assembled MHC-I/ß2-microglobulin/peptide complexes. This assembly occurs in the endoplasmic reticulum (ER) and is regulated by the so-called loading complex. This multiprotein complex consists at least of TAP, tapasin, ERp57 and calreticulin, (52) of which the latter is known as a target substrate of MMP-2 (53). Therefore, MMPs might control MHC-I assembly by interfering with the MHC-I loading complex. However, in our assay, we used an antibody that detects only the fully assembled H-2K^b molecule. Thus, an involvement of MMP-13 in the MHC-I assembly process is unlikely, leaving the post-ER trafficking and recycling as sites of MMP-13 intervention in MHC-I surface expression.

As B3Z cells need neither co-stimulatory molecules nor cytokine stimulation by DCs, the reduced T-cell activation in our system is likely caused by the reduced MHC-I presentation.

Co-stimulatory molecules, such as CD80, CD86 and CD40, which are required for efficient T-cell activation by DCs under physiological conditions (17, 18) were not changed by MMP-13 inhibition (Fig. 6A). In contrast, a clear decrease in the surface expression of CD11c was observed (Fig. 6B). It is known that CD11c binds to complement fragment (iC3b), adhesion molecules and matrix proteins and might, therefore, be involved in antigen presentation and inflammation (54). As a strong CD11c reduction could only be detected with high inhibitor concentrations, it is unlikely to affect antigen presentation in our system.

Beside MMP-13 regulating MHC-I and CD11c surface expression, we observed that MMP-13 inhibition changed the cytokine profile of DCs which could *in vivo* lead to altered T-cell polarization. Using a Luminex screening assay for 18 different targets, cytokines that regulate T_h1/T_h17 cell polarization such as IL-12p70, IL-23p19 and and IL-6 were strongly decreased by MMP-13 inhibition, whereas cytokines that affect mainly neutrophils remained unaffected (Fig. 7). In particular, IL-12 is known as a pivotal pro-inflammatory cytokine that serves as a critical mediator of CD8+ T-cell activation by driving the necessary help of CD4+ T cells toward a T_h1 phenotype (55–58). Moreover, IL-12 is reported to increase the expansion and survival of effector/memory T-cell populations by reducing apoptosis in CD8+ T cells (59). IL-23, a potent pro-inflammatory cytokine, was similarly reduced due to MMP-13 inhibition. Although IL-23 is closely related to IL-12, the dominant role of IL-23 is to stimulate a unique T-cell subset to produce IL-17. Therefore, IL-23 plays an important role in memory T-cell responses and in autoimmune diseases (60, 61). IL-23p19 and IL-12p70 share the same p40 subunit, which they require for secretion (56). Along this line, IL-6, another pro-inflammatory cytokine homologous to IL-12p35/IL-23p19 subunits and a downstream factor of IL-23, was also significantly reduced. The homology of these cytokines might explain their concerted reduction upon MMP-13 inhibition as they might exhibit a joint processing site. Notably, previous reports have linked IL-12 and IL-23 to specific MMPs. One study demonstrated an indirect effect of MMP-2 on IL-12p70 via degradation of the type-I IFN receptor that inhibited STAT1 phosphorylation and thereby reduced IL-12p35 production (62). In line with this, another study reported MMP-9 as an indirect regulator of IL-23 via membrane stem cell factor and receptor tyrosine kinase c-kit ligation (63). To gain more insight into the mechanism that led to the reduced cytokine detection of IL-12p70 and IL-23p19 in the current study, transcript levels of selected genes were analyzed. In contrast to the strong inhibition of secreted protein, marginal inhibition only at the highest inhibitor concentration was observed (Fig. 8), suggesting that MMP-13 moderates the amount of secreted cytokines/chemokines at the protein level. This suggestion is further supported, as the alterations in the cytokine secretion occurred very quickly within 3 h after the LPS-induced DC activation.

Considering the observed increase of CXCL10 (Fig. 7), an inhibitor for neovascularization and hematopoietic progenitor cells, through MMP-13 inhibition, one might speculate an ensuing weakening of the immune response by reducing vascularization and immune cell supply (64, 65). As CXCL10 is also reported to recruit T and NK cells, (66) the downstream effect of increased CXCL10 by MMP-13 inhibition has to be investigated in future.

In summary, our data reveal a new role of MMP-13 in MHC-I presentation, endocytosis and cytokine/chemokine release of DCs. While the reduction of MHC-I molecules led to a reduced T-cell activation, the downstream effects of the diminished endocytosis remain to be identified. Furthermore, we could exclude a participation of MMP-13 in the migration process of DCs as well as in their maturation process. The altered cytokine profile gives rise to interesting future research, denoted toward elucidating the exact downstream effect on T cells in a more physiological system.

Regarding therapeutic intervention, inhibition of MMP-13 is particularly interesting, as it allows a more specific targeting of DCs compared with the inhibition of other MMPs that are more broadly expressed in the immune system. The data shown here provide evidence that the targeting of MMP-13 with a small molecular inhibitor profoundly affects diverse DC functions with consequences for memory T cells, NK cells, T_h cells and cytotoxic T lymphocytes through the reduced MHC-I presentation capability and through changes in the cytokine profiles of DCs. In particular, the alteration in pro-inflammatory T-cell addressing cytokines could be an interesting aspect of therapeutic intervention in diseases with pathogenic T_h1/T_h17 inflammation. These could be autoinflammatory diseases or graft dysfunction after transplantation, such as in bronchiolitis obliterans, where the immune system must be attenuated.

Considering future clinical application, we have intentionally chosen the inhibitor approach and avoided the utilization of DCs from knockout mice to investigate the role of MMP-13 and its effect on DC function. In a clinical setting, small molecules inhibitors are attractive. Thereby, MMP-13 activity will be reduced but never completely blocked as found in MMP-13 knockout cells. Another argument against using knockout cells is that these cells might exhibit compensatory changes that alter their phenotype and function. Indeed, we observed alterations in the phenotype of MMP-12 knockout cells compared with wild type (data not shown).

Unfortunately, CL82198 was the only specific MMP-13 inhibitor that was soluble in water or saline; all other inhibitors were either not specific for only MMP-13 or required DMSO, which itself had an impact on cross-presentation and thus could not be used in our experiments. We acknowledge the limitations of our study due to utilization of only one MMP-13 inhibitor. Nevertheless, based on our observations, new therapeutic approaches to counteract pathogenic T_h1/T_h17 inflammation represent logical extensions of our study.

With the intention to move toward *in vivo* models, we performed our analysis with murine bone marrow-derived DCs. Indeed, using a bronchiolitis obliterans mouse model, we obtained preliminary evidence that MMP-13 might be involved in the progression of obliterative bronchiolitis and that inhibition of MMP-13 might attenuate the bronchiolitis obliterans phenotype (data not shown). While these observations have to be substantiated with larger mouse numbers, they do provide first evidence that MMP-13 inhibitors could be promising new therapeutics in bronchiolitis obliterans.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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