MATERIALS AND METHODS

1. Bacterial strains and growth conditions
Mycobacterial strains were grown in Middlebrook 7H9 broth (Difco) supplemented with 0.2% glycerol and albumin-dextrose complex (ADC, BD Sciences) (consisting of 0.5% bovine serum albumin (BSA), fraction V, 0.085% NaCl, and 0.2% glucose) to the final concentration of 10%. When required, the media was added with 20μg/mL kanamycin for MtbecD3ko, MtbΔppe25-pe19 and Mtbv1794ko or 20μg/mL kanamycin and 50μg/mL hygromycin for Mtbv1794ko-C.

Bacteria were harvested during the logarithmic growth phase, centrifuged at 4230xg for 15 min and subsequently washed twice by centrifugation at 4230xg for 15 min in phosphate buffered saline (PBS). To eliminate clumps of bacteria, after washing, cells were shaken with glass beads for 20 min and then resuspended in PBS at OD590 of 3 (3x10^8 colony-forming units (CFU)/ml). Aliquots were kept frozen at –80°C until use.

For each experiment an aliquot of the bacteria was thawed, diluted in RPMI 1640 to a final OD590 of 0.1 and immediately before use was passed through a 29 gauge syringe to obtain a predominantly single-bacterial-cell suspension. The number of microorganisms was assessed by plating 10-fold dilutions of the bacterial suspension, in duplicate, on Middlebrook 7H11 agar (BD Sciences) supplemented with 0.5% glycerol and oleic albumin-dextrose complex (OADC, BD Sciences) (consisting of 0.06% oleic acid, 5% BSA, 2% dextrose and 0.0003% catalase) to the final concentration of 10%, and CFU were counted after 3 weeks of incubation at 37°C.

Killed bacteria were obtained by exposing bacteria to +80°C for 1h in water-bath.
2. **Cell populations**

Heparinized blood was obtained from healthy volunteers. Informed consent was obtained, and the protocol was approved by the local ethics committee. Blood was diluted in PBS containing 10% (vol/vol) sodium citrate and layered on a standard density gradient (Lymphoprep, Cederlane, Canada). After centrifugation at 160xg for 20 min at room temperature, supernatants were removed, without disturbing the lymphocyte layer at the interface, to eliminate platelets. The gradient was further centrifuged at 800xg for 20 min, and peripheral blood mononuclear cells (PBMC) were collected from the interface. Cells were washed three times with PBS containing 0.5% (wt/vol) BSA and 10% sodium citrate and enriched for monocytes via a magnetic cell sorter by using the Monocyte Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s protocol. The negatively isolated fraction, containing untouched monocytes, was collected and purity was assessed by fluorescence-activated cell sorter analysis. The purified cell population contained at least 80% CD14+ CD3-CD56- cells.

Purified monocytes were resuspended in RPMI 1640 supplemented with 2 mM L-glutamine, 10% heat-inactivated autologous serum and were seeded in 24-well plates at a density of 1 x 10^6 cells/well.

MoDCs were obtained by incubation of the monocytes with 500U/mL recombinant human (rh) Granulocyte Macrophage-Colony-Stimulating Factor (GM-CSF) and 1000U/mL rhIL-4 (Miltenyi Biotec,) while, two macrophage subsets with distinct functional properties, Mϕ1 (pro-inflammatory) and Mϕ2 (regulatory), were obtained by incubation of the cells with 50U/ml rhGM-CSF or 50U/ml rh Macrophage-Colony-Stimulating Factor (M-CSF) (Miltenyi Biotec,), respectively. After 6 days of culture, the cells were analysed for the expression of surface markers associated with MoDCs as well as macrophage differentiation. The resulting immature MoDCs population was expressing CD209 but not CD14, while the Mϕ1 were expressing CD14 very low CD163 and Mϕ2 were expressing high levels of CD163 and CD14.
3. Infection
Following 6 days of culture, Mφ1, Mφ2 and MoDCs were harvested, washed and resuspended in RPMI 1640 supplemented with 2mM L-glutamine and 10% heat-inactivated autologous serum, and seeded in 48-well plates at a density of 5x10^5 cells/well.
Cells were then infected with bacteria at a multiplicity of infection (MOI) of 2 bacteria per cell and incubated for 24h at 37°C in humidified air containing 5% CO2. Where appropriate, 40mM potassium chloride (KCl) (Sigma-Aldrich), 10µM CA-074-methyl ester (cathepsin B inhibitor, Enzo Life Sciences), 10µM of Pepstatin A (cathepsin D inhibitor, Enzo Life Sciences), 10µM of Z-Phe-Tyr(tBu)-diazomethylketone (cathepsin L inhibitor, Enzo Life Sciences), or 50µM Ac-YVAD–CMK (caspase-1 inhibitor, Enzo Life Sciences) were added to the culture.

4. Immunofluorescence staining for surface markers
Following 24h of infection, MoDCs were collected, washed, resuspended in PBS and incubated with saturating amounts of antibodies for 30 min at 4°C. The following monoclonal antibodies were used for staining: fluorescein isothiocyanate (FITC)-conjugated anti-HLA-DR (eBiosciences) anti-CD209 (Miltenyi Biotec,); phycoerythrin (PE)-conjugated anti-CD83 and CD-14 (Miltenyi Biotec,); peridinin chlorophyll-cyanin 5.5 (PerCP-Cy5)-conjugated anti-CD80 and anti-CD86 (BD Pharmingen); and isotype-matched mouse immunoglobulin G (IgG) (as negative controls) (Miltenyi Biotec,). Stained cells were fixed with 1% paraformaldehyde in PBS for 24h at +4°C to kill any remaining mycobacteria and 15000 events were acquired ungated in a FACSort flow cytometer (BD Biosciences). For analyses, all viable cells were selected by a widely set gate on a two parameter plot of side-scatter versus forward-angle scatter. CellQuest software (BD Biosciences) was used for computer-assisted analyses. The levels of surface marker expression were evaluated by mean fluorescence intensity (MFI) analyses.
5. **Determination of cytokines in culture supernatants**

After 24h of infection culture supernatants were collected, passed through a 0.22µm filter, and stored at –80°C until use. The quantity of the cytokines (IL-1β, IL-6, IL-10, IL-18, IL-23, TNF-α) was determinated by a flow cytometer based multibead capture assay (FlowCytomix) (Bender MedSystems). Briefly, samples were incubated with a mixture of beads characterized by different size and red (FL3) fluorescence intensity each-set coated by an antibody specific for a given cytokine. Next, a biotin-conjugated specific antibody mix, followed by a streptavidin-PE solution, was added to the beads to quantify the captured analytes (sandwich assay). Samples were run on a FACsort flow cytometer (BD Biosciences), analyzed with FlowCytomix Pro 2.2 Software (Bender MedSystems), and referred to a standard curve obtained by using a known quantity standard for each cytokine tested. Results were expressed as pg or ng/ml.

6. **mRNA purification and Real Time-PCR**

After 4h or 24h of infection, supernatants were discarded and Mϕ1 were collected by using RNA protect Cell Reagent (Qiagen), to stabilize the cellular RNA and preserve the gene expression profile. Total mRNA was extracted from Mϕ1 by using the RNAeasy protect cell mini kit (Qiagen) according to the manufacturer’s instructions. Briefly, the cells in RNAprotect Cell Reagent were centrifuged, and the resulting cell pellet was lysed and homogenized in RNA lysis buffer (Buffer RLT Plus). The lysate was then passed through a gDNA Eliminator spin column, to remove genomic DNA. Ethanol (70% v/v) was added to the lysate, which was then applied to an RNA easy spin column. After centrifugation contaminants were washed away and total RNA was eluted in 40µL of RNase-free water.

Isolated RNA was subsequently transcribed into complementary DNA using QuantiTect Reverse Transcription Kit (Qiagen). 0.2µg of RNA for each condition were incubated in gDNA Wipeout Buffer for 2 min at
42°C to remove contaminating genomic DNA. The reverse transcription was carried out in thermowell tubes in 20\mu L final volume containing: template RNA, Quantiscript Reverse Transcriptase, Quantiscript RT Buffer, and RT Primer Mix. The retrotranscription was performed by incubating samples at 42°C for 30 min followed by an inactivation step at 95°C for 3 min.

Following reverse transcription, the real time PCR reaction was carried out in glass capillaries in 20\mu L final volume containing: QuantiFast SYBR Green RT-PCR Master Mix, forward and reverse primers (final concentration 1\mu M each) [for the amplification of the housekeeping gene GAPDH a mix of sense and antisense primers was used (GAPDH QuantiTect Primer Assay)], H2O and cDNA.

### Primers used in the study

<table>
<thead>
<tr>
<th>Gene</th>
<th>sense sequence</th>
<th>antisense sequence</th>
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<tbody>
<tr>
<td>IL-1(\beta)</td>
<td>5'-ACAGATGAAGTGCTCCTTCCA-3'</td>
<td>5'-GTCGGAGATTCGTAGCTGGAT-3'</td>
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<tr>
<td>IL-18</td>
<td>5'-CAAGGAATTGTCTCCCAGTGC-3'</td>
<td>5'-CAGCCGCTTTAGCAGCCA-3'</td>
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<td>Caspase-1</td>
<td>5'-GAAGGCCATTTGTGGGAAGAA-3'</td>
<td>5'-CATCTGGCTGCTCAAATGAA-3'</td>
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<tr>
<td>NLRP3</td>
<td>5'-AGCCACGCTAATGATCGACT-3'</td>
<td>5'-CAGGCTCAGAATGCTCATCA-3'</td>
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<tr>
<td>ASC</td>
<td>5'-GCCGAGGAGCTCAAGAAGTT-3'</td>
<td>5'-CAGGCTGGTGTAAGACTGAA-3'</td>
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After an initial denaturation step at 95°C for 5 min, real time PCR was performed for 40 cycles (95°C for 10 s and 60°C for 30 s for each cycle) by using LightCycler (Roche).

The mRNA levels were expressed in relative copy numbers normalised against GAPDH mRNA. Quantitative measurement of mRNA levels was expressed as fold increase compared to unstimulated cells.
7. SDS page and Western blot analyses

For immunoblotting MΦ1 were seeded in 12-well plates at a density of 2.5x10^6 cells/well. 24h after stimulation, cells were lysed in 100μL of RIPA buffer (Cell Signaling) (consisting of 20mM Tris-HCl pH 7.5, 150mM NaCl, 1mM Na₂EDTA, 1mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5mM sodium pyrophosphate, 1mM β-glycerophosphate, 1mM Na₃VO₄, 1μg/mL leupeptin). 1mM phenylmethanesulfonylfluoride (PMSF, protease inhibitor; Sigma Aldrich) was added just prior to use. The homogenates were then centrifuged at 4°C for 10 min at 14000xg and the supernatants were kept for Western blot analyses. The protein content was determined by Bradford assay.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the Laemmli method. Protein samples were completely denaturated by first adding the protein loading buffer (consisting of 400mM dithiothreitol (DTT), 6% SDS, 150mM Tris-HCl pH 6.8, 30% glycerol and 0.3% bromophenol blue) in 1:4 vol/vol, and subsequently boiling the mixture for 2 min.

Equal amount of protein were loaded on 12.5% polyacrylamide gels. As control, a mixture of purified proteins of known molecular weight (Protein Marker, Broad Range, New England Biolabs) was loaded next to the samples. Gels were inserted into the electrophoresis chamber vertically, filled with electrophoresis running buffer (consisting of 25mM Tris-HCl pH8.3, 250mM glycine and 0.1% SDS) (Invitrogen, Life technologies). The apparatus was connected to a constant current source (20mA per each gel) for 120 min. At the end of the electrophoretic run, proteins were transferred to a polyvinylidifluoride (PVDF) membranes (0.45μm) (Immuno-blot PVDF membrane, Millipore) by using a Mini Trans-blot cells (Bio-Rad). Transfer buffer consisted of 48mM Tris-HCl, 39mM glycine and 10% methanol. The apparatus was connected to a power supply and the electro-transfer was done at a constant current of 200mA for 90 min. Subsequently the membranes were blocked with 5% BSA dissolved in TTBS buffer (consisting of
20mM Tris pH 7.5, 0.5M NaCl, 0.01% Tween-20) (TTBS 5% BSA) at room temperature for 2h and incubated overnight with a human IL-1β specific antibody (Ab) (dil.:1/500, R&D systems MAB 201). A β-actin Ab (1/2000, Sigma Aldrich, clone AC-15) was used for normalisation of total protein amount in each sample.

The membranes were washed three times with TTBS 5% BSA and incubated with anti-mouse IgG (Fab specific)-peroxidase (secondary Ab A9917 Sigma-Aldrich) for 90 min. Finally the presence of specific proteins was evidenced by incubating the membranes with a solution consisting of 1.6mM 3,3’-diaminobenzidine tetrahydrochloride (peroxidase substrate) in 60mM Tris-HCl pH7.6, 0.03% CoCl2, 0.05% H2O2. The image of the membranes were acquired by a scanner (Canon) and the relative quantity of IL-1β was calculated as IL-1β density divided by β-actin density in each sample by using Fiji software (http://fiji.sc).

8. FLICA staining
To detect active caspase-1 inside the cells a FLuorochrome Inhibitor of CAspases assay (FLICA) (ImmunoChemistry Technologies) was used. After 24h of infection, cells were collected, washed and incubated for 1h with the fluorescein-labeled caspase-1 inhibitor FAM-YVAD-fmk (6-carboxyfluorescein-Tyr-Val-Ala-Asp-fluoromethyl ketone). After fixation with 1% paraformaldehyde in PBS for 24h the samples were acquired by a flow cytometer (FACSort) and analysed in computer (CellQuest Software).

9. Determination of apoptotic and dead cells
The percentage of death and apoptotic cells was determined by using Fixation and Dead cell discrimination kit (Miltenyi Biotec,) in combination with Annexin V staining of membrane alterations. After 24h of infection, Mφ1 were harvested in FACS tube, stained with Dead Cell Discriminator, a membrane-impermeant red fluorescence dye
which infiltrates selectively into dead cells because of their damaged membranes, and incubated in a horizontal position on ice under a 60W light bulb (distance 3-5 cm) for 10 min to fix the dye. Cells were then washed in 1mL 1x annexin binding buffer and incubated with saturating amounts of Annexin V-FITC (Miltenyi Biotec,) for 15 min in the dark. Following the incubation cells were washed with PBS and fixed by adding the Fixing solution. Finally, the Discriminator Stop reagent is added to the samples and the cells were kept at 4°C for 24h before being acquired by a flow cytometer (FACSort) and analysed using CellQuest Software. The cell population stained both by Dead cell discriminator and Annexin V represented the dead cells while the cells single stained by Annexin V were considered in apoptosis.

10. Statistical analysis
The statistical significance of the data was determined by Student’s t test for paired samples or by the nonparametric Wilcoxon matched pairs signed-rank test. A $P$ value of $<0.05$ was considered significant.