Non-canonical caspase-8 activation by cathepsin B drives anti-inflammatory human macrophage polarization

Running title: Cathepsin B-Caspase-8 axis in macrophage polarization

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Supplementary Materials and Methods

L-lactate secretion assay

100µL of 24H macrophages supernatants and control medium of culture is loaded in triplicates into 96-well plate. L-lactate secretion is evaluated on a YSI 2950D Biochemistry Analyzer by the difference between 24 vs 0H samples further rationalized to the number of secreting cells evaluated by flow cytometry.

Phagocytosis

Control macrophages are pre-treated with cytochalasin D (1µg/mL) for 30 minutes at 37°C and macrophages are then incubated with FITC-beads coated with E. Coli fragments (15µg/mL) for 30 minutes at 37°C (Vybrant™ Phagocytosis Assay kit, Invitrogen V6694). Macrophage phagocytic activity is then determined by flow cytometry by subtracting the mean fluorescence of macrophages and their control, diluted in trypan blue to eliminate background signals.

Supplementary Figure legends

Supplementary Fig. 1 Caspase activation during monocyte-to-macrophage differentiation and anti-inflammatory macrophages polarization. a Amino acid sequences of caspase substrates and their putative cleavage sites during human monocyte-to-macrophage differentiation. Human monocytes (d0) are differentiated with CSF-1 for 5 days and then treated with CSF-1 (M0) or polarized with IFN-γ + LPS for pro-inflammatory macrophages, IL-4 (M2), IL-4 ± IL-13, IL-6 or IL-10 for anti-inflammatory macrophages for 2 days. **b-d** Enzymatic measurement of non-apoptotic caspase activity in differentiating and polarized macrophages lysates using a specific fluorescent peptide (**b:** Ac-KWFD-AMC; **c:** Ac-IETD-AMC; **d:** Ac-DEVD-AMC). Results are expressed as A.U/min/mg of proteins and represent the mean ± SD of 3 independent experiments performed in triplicates. **e-f** Enzymatic measurement of recombinant caspase-8 (**e**) or caspase-3 (**f**) activity using specific fluorescent peptides (Ac-IETD-AMC, Ac-DEVD-AMC, Ac-NKFD-AMC and Ac-KWFD-AMC). Results are expressed as A.U/min/mg of proteins and represent the mean ± SD of 3 independent experiments performed in triplicates. **g** Caspase-8, caspase-7 and caspase-3 expression analysis by immunoblotting. HSP60 is used as a loading control. **h** Enzymatic measurement of non-apoptotic caspase activity using a specific fluorescent peptide (Ac-NKFD-AMC). Results are expressed as A.U/min/mg of proteins and represent the mean ± SD of 3 independent experiments performed in triplicates. ns : $p > 0.05$; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ and **** $p < 0.0001$ according to an ordinary one-way ANOVA.

Supplementary Fig. 2 CTS enzymatic activity and expression in polarizing macrophages. a Human monocytes (d0) are differentiated with CSF-1 for 5 days and then treated with CSF-1 (M0) or polarized with IFN-γ + LPS (M1) or IL-4 (M2) for 2 days. Enzymatic measurement of CTS using a specific fluorescent peptide Ac-FR-AMC. Results are expressed as A.U/min/mg of proteins and represent the mean ± SD of 3 independent experiments performed in triplicates. * $p < 0.05$ and ** $p < 0.01$ according to an ordinary one-way ANOVA.

b Anti-inflammatory polarizing macrophages are transfected with siRNA directed against Luciferase, CTSB, D, L, or S for 2 days. RT-qPCR analysis of CTSB, D, L and S gene expression. Results are expressed as relative expression and represent the mean ± SD of 3 independent experiments. ** $p < 0.01$ and **** $p < 0.0001$ according to a two-tailed unpaired Student's t test.

Supplementary Fig. 3 CTS and non-apoptotic caspase activities in anti-inflammatory macrophages upon pharmacological CTSB or caspase inhibition. Enzymatic measurement of CTSB+L and non-apoptotic caspase activities using a specific fluorescent peptide (Ac-RR-AMC and Ac-NKFD-AMC respectively). Results are expressed as A.U/min/mg of proteins and represent the mean ± SD of 3 independent experiments performed in triplicates. ns : $p > 0.05$; * $p < 0.05$ and ** $p < 0.01$ according to a two-tailed unpaired Student's t test.

Supplementary Fig. 4 Phenotype and functional profile of anti-inflammatory macrophages upon CTSB or caspase pharmacological inhibition. Human monocytes are differentiated in macrophages with CSF-1 for 5 days and polarized into anti-inflammatory macrophages during 2 days with IL-4 and further treated with Emricasan or CA-074 for 2 days. **a-b** Enzymatic measurement of CTSB and non-apoptotic caspase activities using specific fluorescent peptides (Ac-RR-AMC and Ac-NKFD-AMC respectively). Results are expressed as A.U/min/mg of proteins and represent the mean ± SD of 3 independent experiments performed in triplicates. **c-d** Lactate production analysis. Results are expressed as pg/mL/10^6 cells and represent the mean ± SD of 3 independent experiments. **e-f** Phagocytic capacity analysis of macrophages by flow cytometry. Results are expressed as MFI (Mean Fluorescence Intensity) and represent the mean ± SD of 3 independent experiments. ns : $p > 0.05$; * $p < 0.05$ and ** $p < 0.01$ according to a two-tailed unpaired Student's t test.

Supplementary Fig. 5 Phenotype and functional profile of anti-inflammatory macrophages upon genetic inhibition of CTSB, caspase-8, caspase-7, caspase-3 or CTSL. Anti-inflammatory macrophages are transfected with siRNA directed against Luciferase (used as a control of transfection), caspase-8, caspase-7, caspase-3, CTSB or CTSL during 2 (**a**) or 3 days (**b, c**). **a** RT-qPCR analysis of CTSB, caspase-8, caspase-7, caspase-3 and CTSL gene expression. Results are expressed as relative expression and represent the mean ± SD of 4 independent experiments. ** $p < 0.01$; *** $p < 0.001$ and **** $p < 0.0001$ according to a two-tailed unpaired Student's t test. **b** Enzymatic measurement of CTSB and non-apoptotic caspase activities using specific fluorescent peptides (Ac-FR-AMC and Ac-NKFD-AMC respectively). Results are expressed as A.U/min/mg of proteins and represent the mean ± SD of 5 independent experiments performed in triplicates. **c** Phagocytic capacity analysis by flow cytometry. Results are expressed as MFI (Mean Fluorescence Intensity) and represent the mean ± SD of 3 independent experiments. ns : $p > 0.05$; * $p < 0.05$; ** $p < 0.01$ and **** $p < 0.0001$ according to an ordinary one-way ANOVA.

Supplementary Fig. 6 Transcriptomic reprogramming of anti-inflammatory macrophages under genetic inhibition of CTSB. Anti-inflammatory macrophages are transfected with siRNA directed against Luciferase (M2 siLuc), used as a control of transfection, caspase-8 (M2 siC8) or CTSB (M2 siCTSB) for 2 days. **a** RT-qPCR analysis of caspase-8 or CTSB gene expression. Results are expressed as relative expression and represent the mean ± SD of 4 or 3 independent experiments respectively. ns : $p > 0.05$; * $p < 0.05$; ** $p < 0.01$ and *** $p < 0.001$ according to according to a two-tailed unpaired Student's t test. **b** Unsupervised 2D projection of the six transcriptome samples principal component analysis. **c** Volcano plot representation of significant dysregulated genes between M2 siCTSB compared to M2 siLuc. **d** Dot plot representation of the top 20 biological processes from gene ontology analysis.