Supplement for:

Extracellular Vesicles of Minimalistic *Mollicutes* as Mediators of Immune Modulation and Horizontal Gene Transfer

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Supplementary Figures

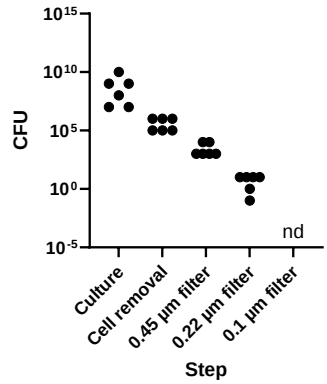

Figure S1: Removal of Mycoplasma bacterial cells via serial filtration. *Mmc* GM12 culture was centrifuged and subjected to serial filtration using 0.45, 0.22 and 0.1 µm filters, samples were collected at each step, plated on SP5 agar and CFUs were counted (nd = non detected).

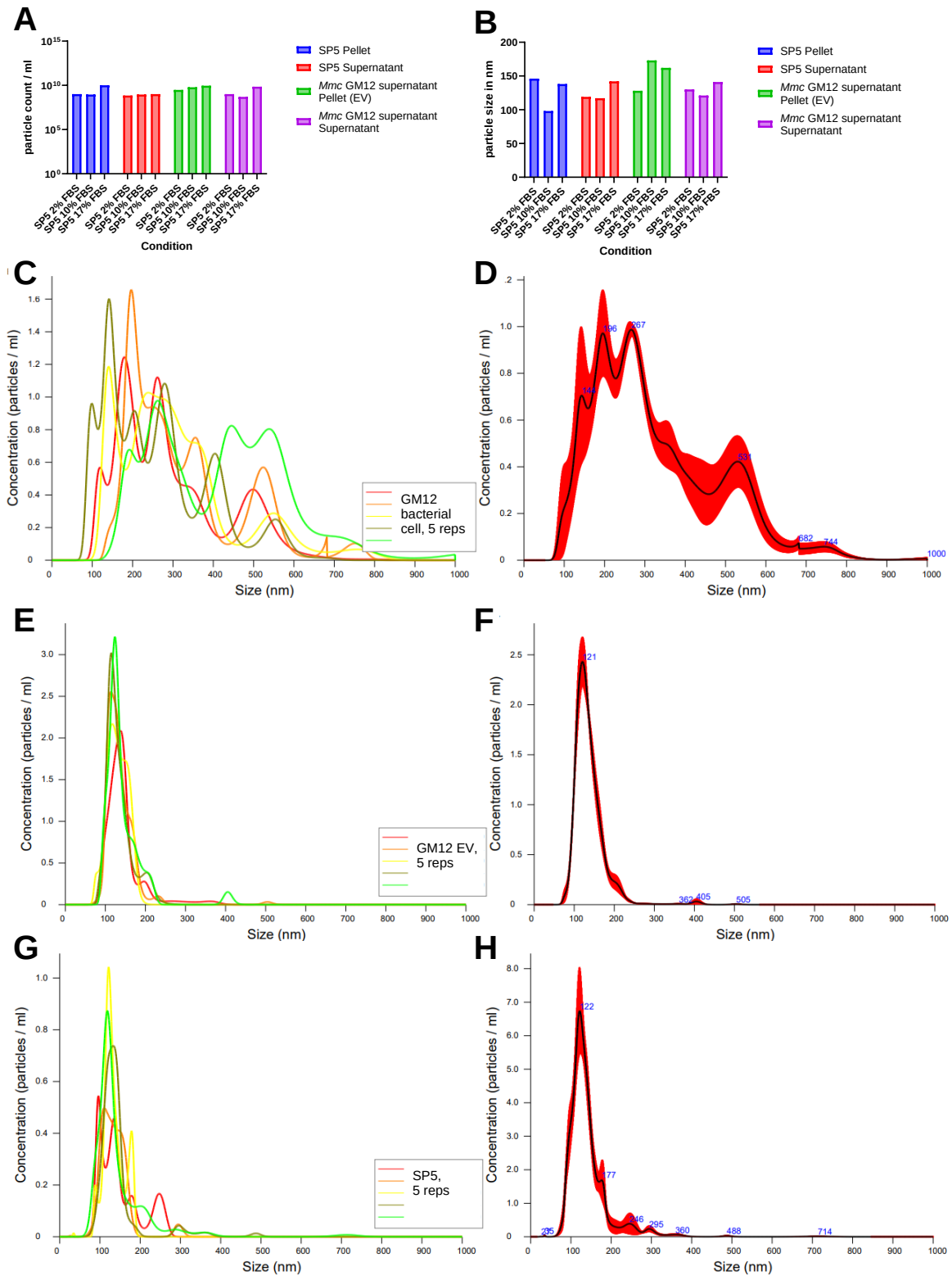

Figure S2: Nano particle tracking analysis of *Mmc* GM12 culture and its EVs using NanoSight. (A) Particle count per ml in the different samples. (B) Size estimation of particles in nm in the different samples. Particle concentration over size in nm for bacterial cells in standard SP5 (C and D), EVs (E and F) and SP5 medium (G and H). Left panels show the individual repeats while right panels show the averaged mean particle size derived from finite track length adjustment (FTLA) with NTA 3.4, error bars indicate ±1 standard error of the mean.

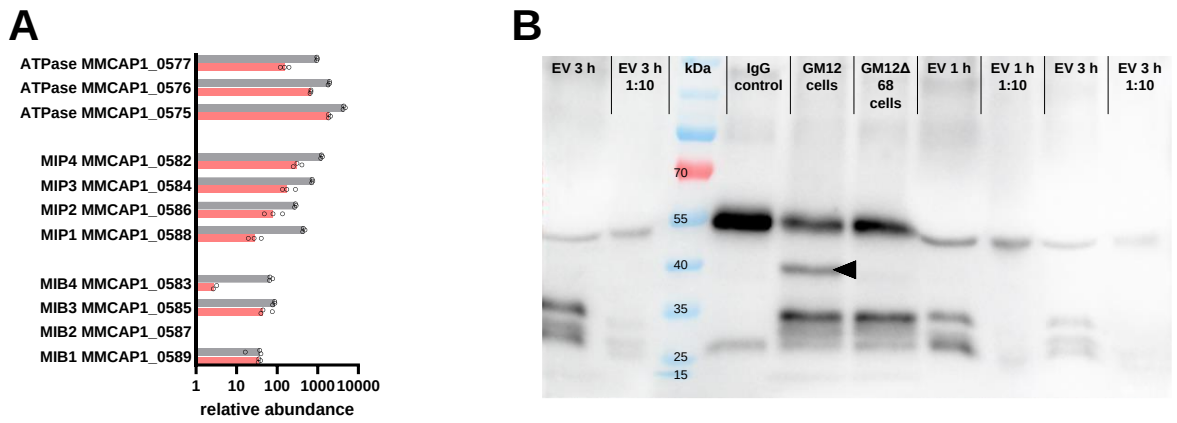

Figure S3: MIB-MIP protein presence and IgG cleavage assay. (A) relative abundance of IgG cleavage cluster related proteins in the proteomic profile of *Mmc* bacterial cells (Bac) and its EVs. (B) IgG cleavage activity determined by Western blot in *Mmc* GM12 and its deletion mutant Δ68 as negative control. Cleaved IgG heavy chain is indicated by an arrow.

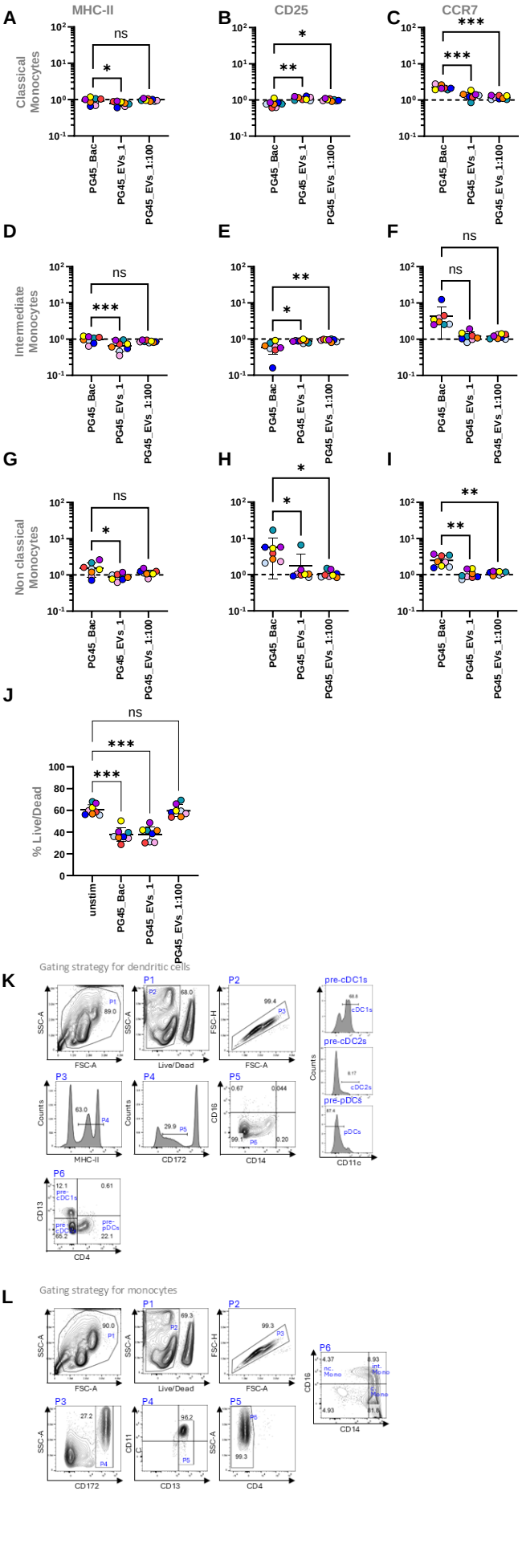
A
MHC-II
B
CD25
C
CCR7
Classical Monocytes
D
E
F
Intermediate Monocytes
G
H
I
Non classical Monocytes
J
% Live/Dead
unstim
PG45_Bac
PG45_EVs_1
PG45_EVs_1:100
ns
K
Gating strategy for dendritic cells
pre-cDC1s
pre-cDC2s
pre-pDCs
L
Gating strategy for monocytes

Figure S4: Ex vivo response of monocytes to bacteria or EVs of *M. bovis*. (A-C) Classical monocytes, (D-F) intermediate monocytes, (G-I) non-classical monocytes with the markers MHC-II (A, D, G), CD25 (B, E, H), and CCR7 (C, F, I). Values are expressed mean fluorescence intensity (MFI) normalized to MFI of unstimulated cells of the same animal, and each coloured circle represents PBMCs of one animal (n=8) stimulated for 16 h at 38.5°C (J) Percent of live bovine PBMCs upon stimulation with bacteria and EVs of *M. bovis* PG45 compared to unstimulated (unstim) (Significance based on *p* values as calculated by Repeated measures paired ANOVA with Dunnett's multiple comparisons test). (K, L) FCM gating strategy for phenotyping the different immune cell subsets in cattle. (K) Gating strategy for multiparameter FCM analysis of DCs, using Abs against MHC-II, CD172a, CD16, CD14, CD13, CD4, CD11c, CD25 and CCR7. A primary gate (P1) was set on FSC-A versus SSC-A. Then, the dead cell population (positive for Live/Dead) was excluded, followed by a FSC-H/FSC-A contour plot (to exclude doublets from the analysis). Among these cells, we defined the population positive for MHC-II and CD172a (low and intermediate only). Then, we defined among this population the cells negative for both CD14 and CD16 markers. Then, we defined among this population either the cells positive for CD13 and negative for CD4 markers (pre-cDC1s), either cell negative for CD13 and positive for CD4 markers (pre-pDCs), either cells negative for both (pre-cDC2s). Next, we considered CD11c marker to gate cDC1s and cDC2s (respectively pre-cDC1s and pre-cDC2s positive CD11c), as well as pDCs (pre-pDCs negative for CD11c). Maturation was evaluated based on the MFI of surface expression for MHC-II, CD25 and CCR7. (L) Gating strategy for multiparameter FCM analysis of monocytes, using Abs against CD172a, CD16, CD14, CD13, CD4, CD11c. A primary gate (P1) was set on FSC-A versus SSC-A. Then, the dead cell population (positive for Live/Dead) was excluded, followed by a FSC-H/FSC-A contour plot (to exclude doublets from the analysis). Next, we defined among this population the cells highly positive for CD172a marker ($CD172a^{high}$ cells), positive for CD13, and then negative for CD4. From this, classical monocytes (c.Mono) were gated as $CD14^{+}CD16^{-}$, intermediate monocytes (int.Mono) as $CD14^{+}CD16^{+}$, and nonclassical monocytes (nc.Mono) as $CD14^{-}CD16^{+}$. Maturation was then evaluated based on MFI of surface expression for MHC-II, CD25 and CCR7.

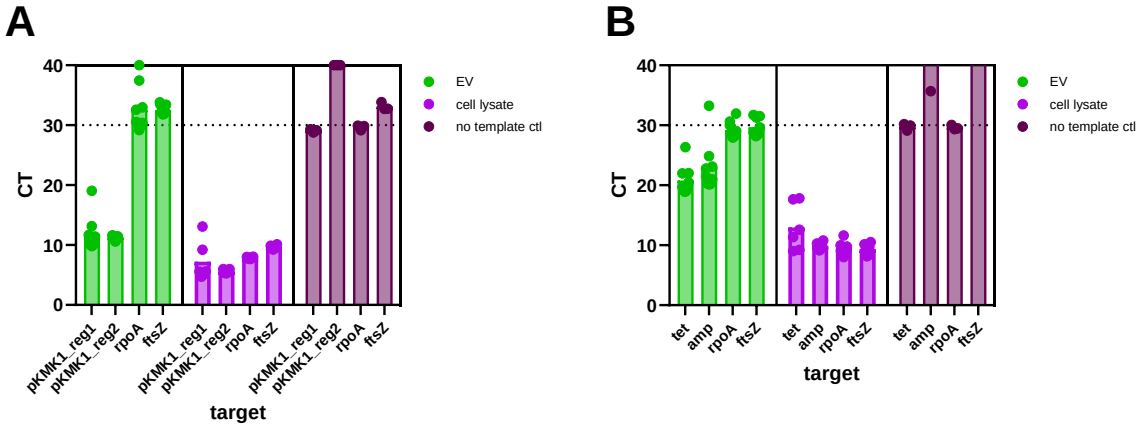

Figure S5: Presence of individual plasmid and chromosomal targets in *Mmc* derived EVs and *Mmc* bacterial lysatesas measured by qPCR. CT values for plasmid and chromosomal targets are shown in EVs, cell lysates and the non-template control, where symbols show the individual repeats and bars are median over n=6 of three biological and two technical replicates. (A) *Mmc* 152/93 with its plasmid pKMK1. (B) *Mmc* GM12 pIVB08 with its plasmid pIVB08.

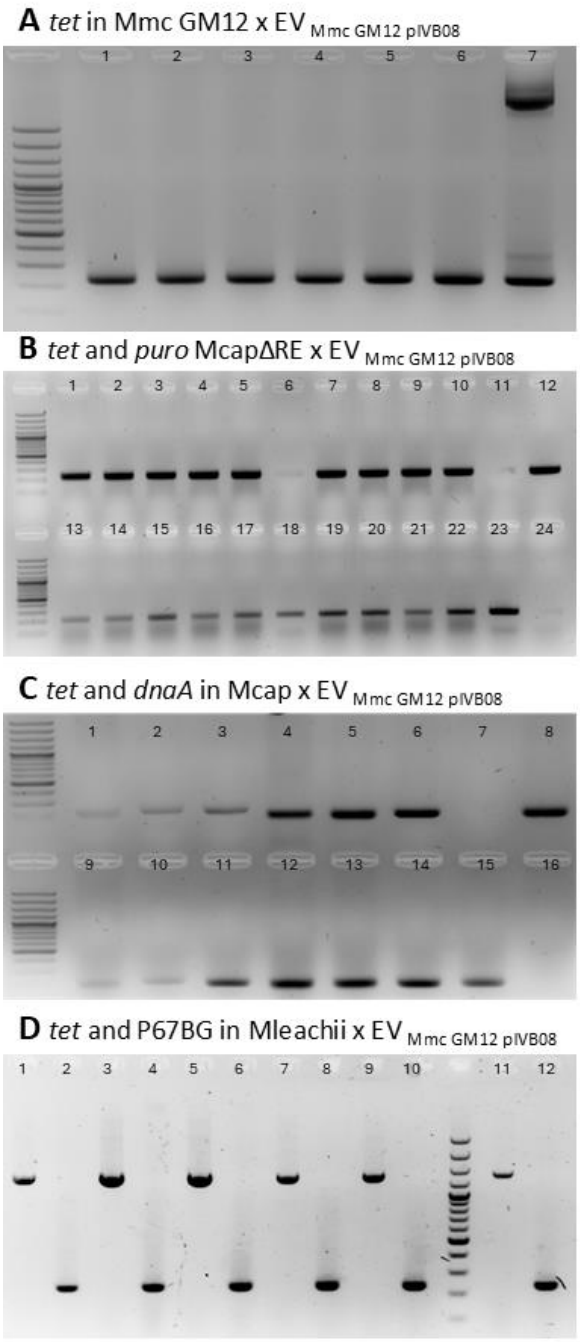

Figure S6: PCR screening of colonies grown in SP5 $_{tet5}$ after vesiduction experiments. A) *tet* amplicon in 6 colonies of *Mmc* GM12 x EV $_{Mmc\ GM12\ pIVB08}$ (lane 1 to 6) and plasmid pIVB08 as positive control (lane 7). B) *tet* (lane 1 to 10) and *puro* (lane 13 to 22) amplicons of 10 colonies of *Mcap* ΔRE x EV $_{Mmc\ GM12\ pIVB08}$ and *tet* in *Mcap* ΔRE (lane 11) and *Mmc* GM12 pIVB08 (lane 12) and *puro* in (lane 23) and *Mmc* GM12 pIVB08 (lane 24). C) *tet* (lane 1 to 6) and *dnaA* (lane 9 to 14) amplicons of 6 colonies of *Mcap* x EV $_{Mmc\ GM12\ pIVB08}$ and *tet* in *Mcap* (lane 7) and *Mmc* GM12 pIVB08 (lane 8) and *dnaA* in *Mcap* (lane 15) and *Mmc* GM12 pIVB08 (lane 16). D) *tet* and P67BG in *M. leachii* x EV $_{Mmc\ GM12\ pIVB08}$ (lane 1 to 10) and P67BG in *M. leachii* (lane 11) and *tet* in *Mmc* GM12 pIVB08 (lane 12).

Supplementary Tables

Table S1 Primers used in this study

Primer name	Primer sequence (5'-3')	Amplicon size (bp)	Target	Ref.
pKMK1_reg1_fw	GAAACCCGAATAAGAGTTTCTAAGT	139	Plasmid pKMK1	This study
pKMK1_reg1_rv	TGGTTCATACTTTCAGGTCAAGTG			
pKMK1_reg2_fw	ATTGAGTTTAAAAGTAACTGGGGTT	123		
pKMK1_reg2_rv	GCTCAACAAAAAGAAGTTAGGTCG			
rpoA_Fw	AAGTCTAAAACTCCACCTTCACT	102	Chromosomal target *Mmc*	This study
rpoA_Rv	AGGACCTGTTTATGCTGGTGA			
ftsZ_fw	TACCTCCTCCCATTCCAGCA	118		
ftsZ_rv	GGAGCTGGAGGAAATCCTGA			
amp_fw	GCACTTTTAAAGTTCTGCTATGTG	192	Plasmid pIVB08	Torres-Puig 2024
amp_rv	CAGTGTTATCACTCATGGTTATGG			
tet_fw	CTAAGTTATTTTATTGAACATATATCGTAC	232		
tet_rv	GAACATCGTAGACACTCAATTG			
puro_fw	GTTTGGGTTGCTGATGATGG	208	Chromosomal target *Mcap*ΔRE	This study
puro_rv	CAGGACTAACACCAACAGTAG			
dnaA_fw	GACATCTTCTTCAAAACCGT	272	Chromosomal target *Mcap*	Labroussaa 2016
dnaA_rv	GTTGATGGTGTAGAAGAACCT			
P67BG-F	GGTAATTCGAATAATGATCCT	1406	Chromosomal target *M. leachii*	
P67BG-R	TAAGTTTATTGAATTAAAGCG			

Ref.: Torres-Puig, S. *et al.* Functional surface expression of immunoglobulin cleavage systems in a candidate Mycoplasma vaccine chassis. *Commun. Biol.* **7**, 779 (2024).; Labroussaa, F. *et al.* Impact of donor–recipient phylogenetic distance on bacterial genome transplantation. *Nucleic Acids Res.* **44**, 8501–8511 (2016).

Supplementary dataset 1: Proteomic Profile of *Mmc* GM12 and *M. bovis* PG45 bacterial cells and EVs

➔ See Dataset xlsx