Expanded View Figures

Figure EV1. Structural modeling of *Mtb* IniA and IniC.

A Sequence alignment of the GTP binding domains of the *Mtb* DLPs and other members of the DLP superfamily. *Bacilllus subtilis* (Bs) DynA (P54159), *Nostoc punctiforme* (*Np*) BDLP1 (B21ZD3) and BDLP2 (B21ZD2), *Campylobacter jejuni* (*Cj*) *BDLP1* (CJ0411) and BDL2 (CJ0412), *Escherichia coli* (*Ec*) LeoA (E3PN25), human mitochondrial OPA1 (0060313) and *M. smegmatis* (*Ms*) IniA and IniC. *Indicates the conserved lysine (K) residue mutated in IniA in this study.

B Crystal structure of the *Msm* IniA (left) and structures predicted by AlphaFold2 of *Mtb* IniA (center) and *Mtb* IniC (right). Structures are color coded to show the GTPase domain (red), bundle signal element/HB1/neck domain (green), HB2/stalk/trunk domain (blue), and C-terminal helical tail domain in IniC (orange).

C Shown is the top ranked structural model of *Mtb* IniAC complex predicted by AlphaFold-Multimer prediction. The conserved lysine (K) residue mutated in IniA, in this study is indicated (light blue).

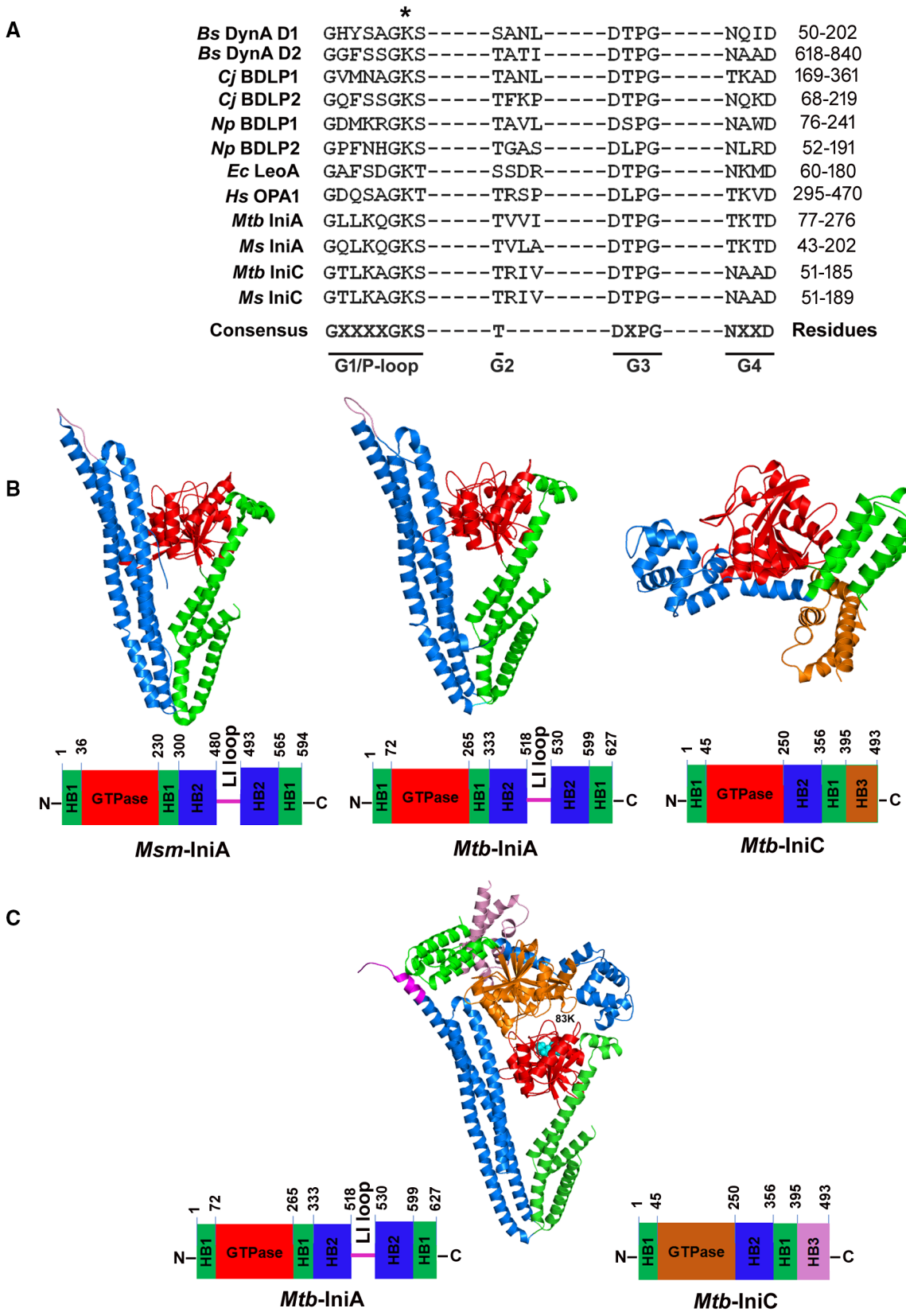
A
*
Bs DynA D1 GHYSAGKS-----SANL-----DTPG-----NQID 50-202
Bs DynA D2 GGFSSGKS-----TATI-----DTPG-----NAAD 618-840
Cj BDLP1 GVMNAGKS-----TANL-----DTPG-----TKAD 169-361
Cj BDLP2 GQFSSGKS-----TFKP-----DTPG-----NQKD 68-219
Np BDLP1 GDMKRGKS-----TAVL-----DSPG-----NAWD 76-241
Np BDLP2 GPFNHGKS-----TGAS-----DLPG-----NLRD 52-191
Ec LeoA GAFSDGKT-----SSDR-----DTPG-----NKMD 60-180
Hs OPA1 GDQSAGKT-----TRSP-----DLPG-----TKVD 295-470
Mtb IniA GLLKQGKS-----TVVI-----DTPG-----TKTD 77-276
Ms IniA GQLKQGKS-----TVLA-----DTPG-----TKTD 43-202
Mtb IniC GTLKAGKS-----TRIV-----DTPG-----NAAD 51-185
Ms IniC GTLKAGKS-----TRIV-----DTPG-----NAAD 51-189
Consensus GXXXXGKS-----T--------DXPG-----NXXD Residues
G1/P-loop
G2
G3
G4
B
N
HB1
GTPase
HB1
HB2
LI loop
HB2
HB1
C
1 36 230 300 480 493 565 594
Msm-IniA
1 72 265 333 518 530 599 627
Mtb-IniA
1 45 250 356 395 493
HB3
Mtb-IniC
C
83K
1 72 265 333 518 530 599 627
Mtb-IniA
1 45 250 356 395 493
Mtb-IniC

Figure EV1.

Figure EV2. IniA and IniC are both required for EV release in mycobacteria.

A Quantitation of MEV released by *Mtb* strains based on quantification of protein and lipid in purified MEV isolated from biological triplicates and normalized to CFUs. The graph shows that individually *iniA* or *iniC* do not complement the mutant; both genes are needed. Data are presented as mean ± SEM. $^{**}P \leq 0.001$; $^{****}P \leq 0.0001$ (Student's *t*-test).

B Representative Immunodot blot of one of the MEV preparations analized in panel (A) using anti-MEV polyclonal antiserum as primary antibody.

C Schematic representation of the *Msm* genomic region containing *iniA* and *iniC*, indicating the location of the primers (P1048, P1106) used to validate allelic exchange and gene deletion of *iniC*.

D Validation of *Msm iniC deleted mutant* (*MsmΔiniC*) showing the PCR product amplified with (P1048 and P1106).

E Quantitation of EVs released by three independent cultures of *Msm* strains based on EV protein and lipid normalized to CFU number. Data are presented as mean ± SEM. $^{***}P \leq 0.001$ (Student's *t*-test).

Source data are available online for this figure.

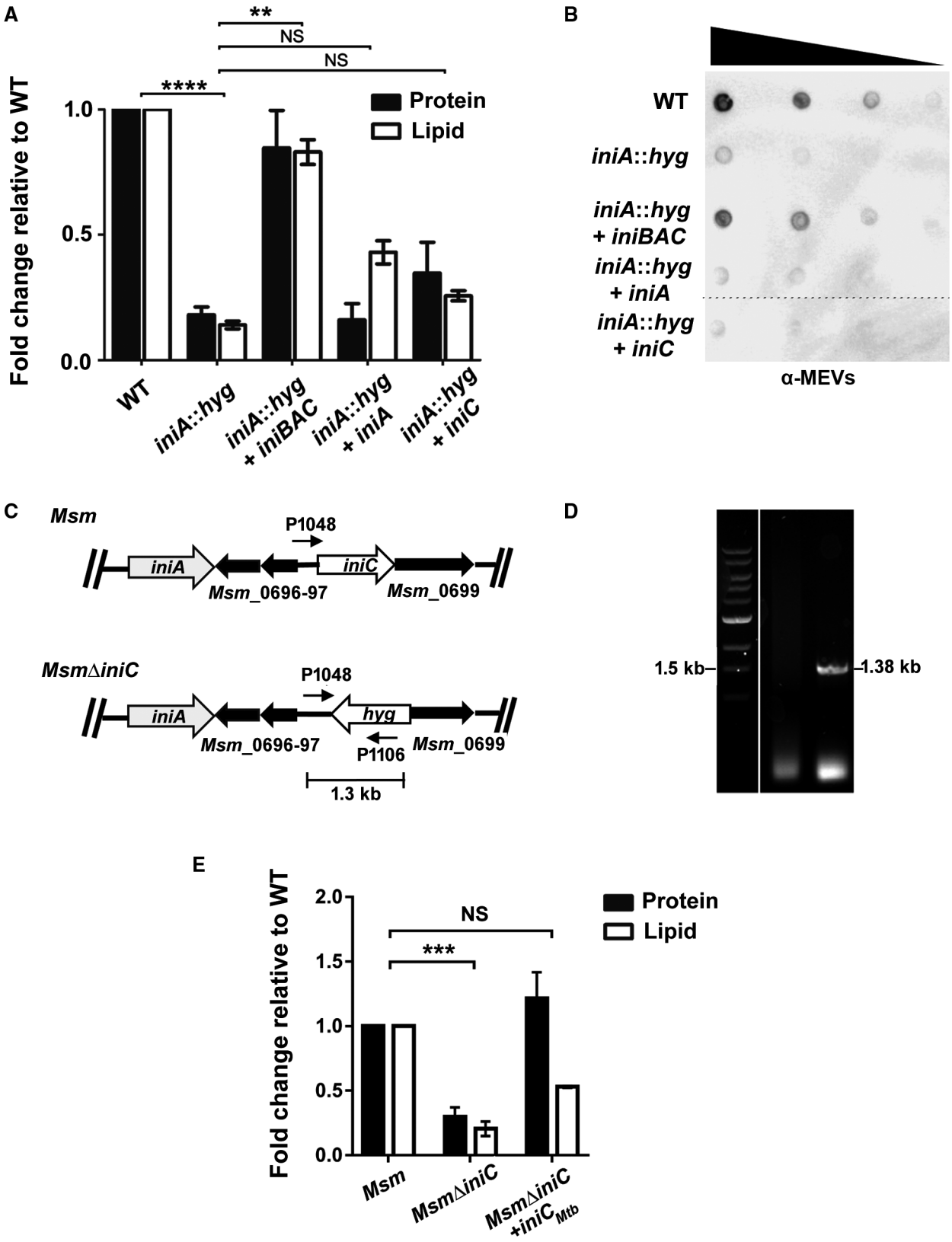
A
**
NS
NS
****
Protein
Lipid
Fold change relative to WT
1.0
0.5
0.0
WT
iniA::hyg
iniA::hyg + iniBAC
iniA::hyg + iniA
iniA::hyg + iniC
B
WT
iniA::hyg
iniA::hyg + iniBAC
iniA::hyg + iniA
iniA::hyg + iniC
α-MEVs
C
Msm
P1048
iniA
iniC
Msm_0696-97
Msm_0699
MsmΔiniC
P1048
iniA
hyg
Msm_0696-97
P1106
Msm_0699
1.3 kb
D
1.5 kb
1.38 kb
E
Protein
Lipid
NS
***
Fold change relative to WT
2.0
1.5
1.0
0.5
0.0
Msm
MsmΔiniC
MsmΔiniC +iniC Mtb

Figure EV2.

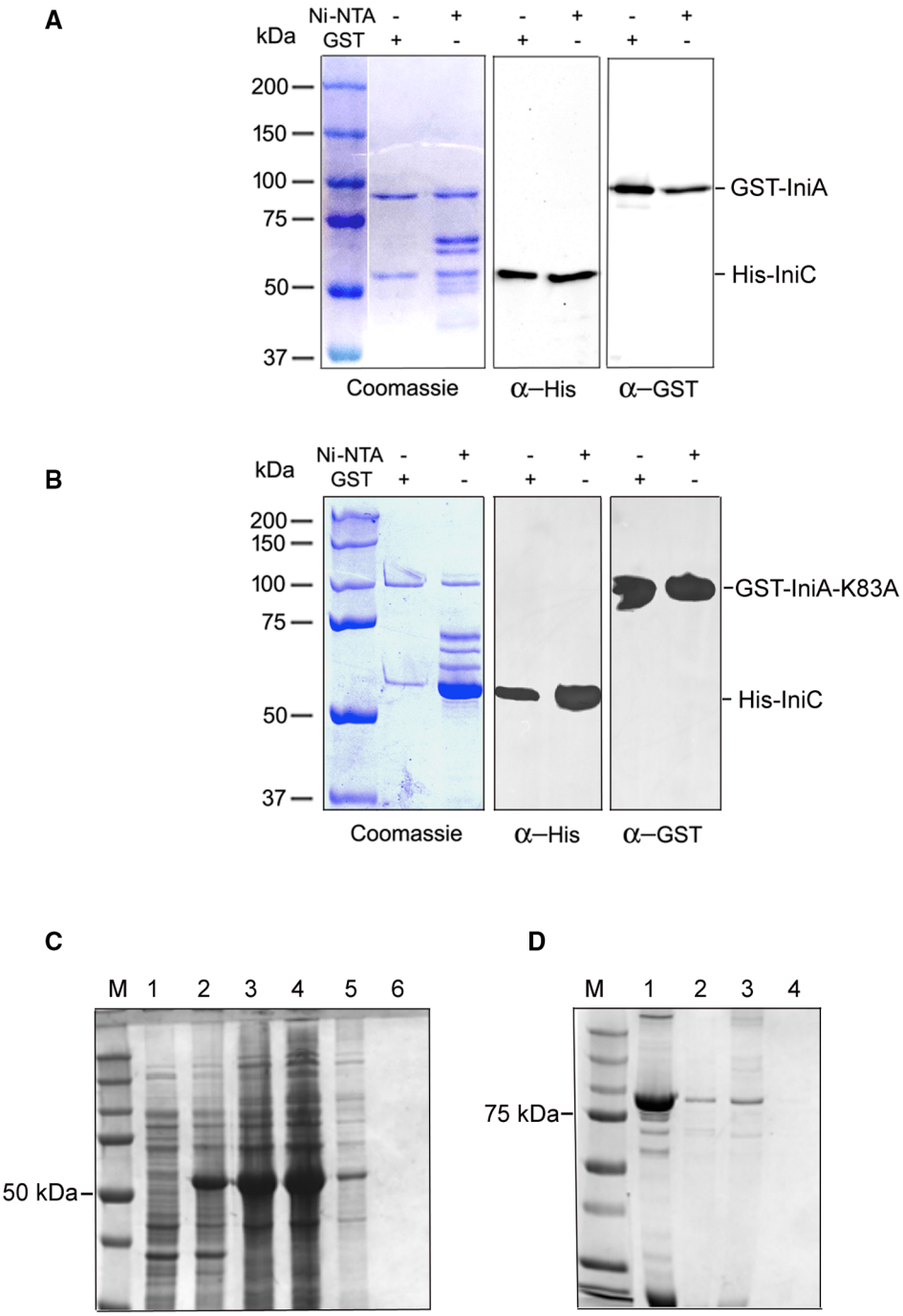

Figure EV3. IniA and IniC interaction.

A Coomassie-stained SDS–PAGE and corresponding Western blots showing co-purification of *Mtb* GST-IniA and His-IniC by affinity chromatography. Anti-tag (GST and His) specific antibodies were used in the Western blot. The experiment was repeated twice with similar results.

B Coomassie-stained SDS–PAGE and corresponding Western blots showing co-purification of *Mtb* GST-IniAK83A and His-IniC by affinity chromatography. Anti-tag (GST and His)-specific antibodies were used in the Western blot. The experiment was repeated twice with similar results.

C Control for nonspecific binding of His-IniC to the GST column. A cell lysate of *E. coli* BL21 transformed with a plasmid expressing His-IniC (~51KDa) uninduced (lane 1) or IPTG induced (lane 2) loaded on a glutathione affinity column; flow through and washes with loading buffer (lanes 3–5); material bound and eluted with 10 mM reduced glutathione (lane 6).

D Control for nonspecific binding of GST-IniA to Ni^{+2}-NTA. Partially purified GST-IniA (lane 1) was loaded into a Ni^{+2}-NTA column. Flow through (lane 2), wash (lane 3) and bound material eluted with 250 mM imidazole (lane 4).

Figure EV4. Cell surface changes in *Msm* constitutively overexpressing *Mtb iniAC*.

A, B Transmission electron micrograph of *Msm* WT. Scale bars are 500 nm in panel (A) and 100 nm in panel (B). Asterisks mark cell blebs.

C–E Transmission electron micrograph of *Msm* overexpressing *iniAC* (*iniAC*$_{Mtb}$::pMV261). Scale bars are 500 nm panel (C) and 100 nm in panels (D) and (E). Asterisks mark cell blebs.

F, G Detail of a cryoelectron micrograph of the cell envelope of *Msm* WT (F) and *Msm* overexpressing *iniAC* (*Msm iniAC*$_{Mtb}$::pMV261) (G). Asterisks mark membrane blebs. Scale bars are 50 nm.

H Quantitation of blebs on cells examined in panels (A–E). Thirty individual cells per strain were counted from two different TEM grids. The error bars indicate mean ± SD. $^{**}P < 0.05$. (Mann–Whitney Test).

I Quantitation of cell membrane blebs along a defined longitudinal section of the cell membrane (700–750 nm) on cells examined in pannels F and G. Fifteen cells per strain were counted from a single vitrified sample. The error bars indicate mean ± SD. $^{****}P > 0.0001$ (Mann–Whitney Test).

Source data are available online for this figure.

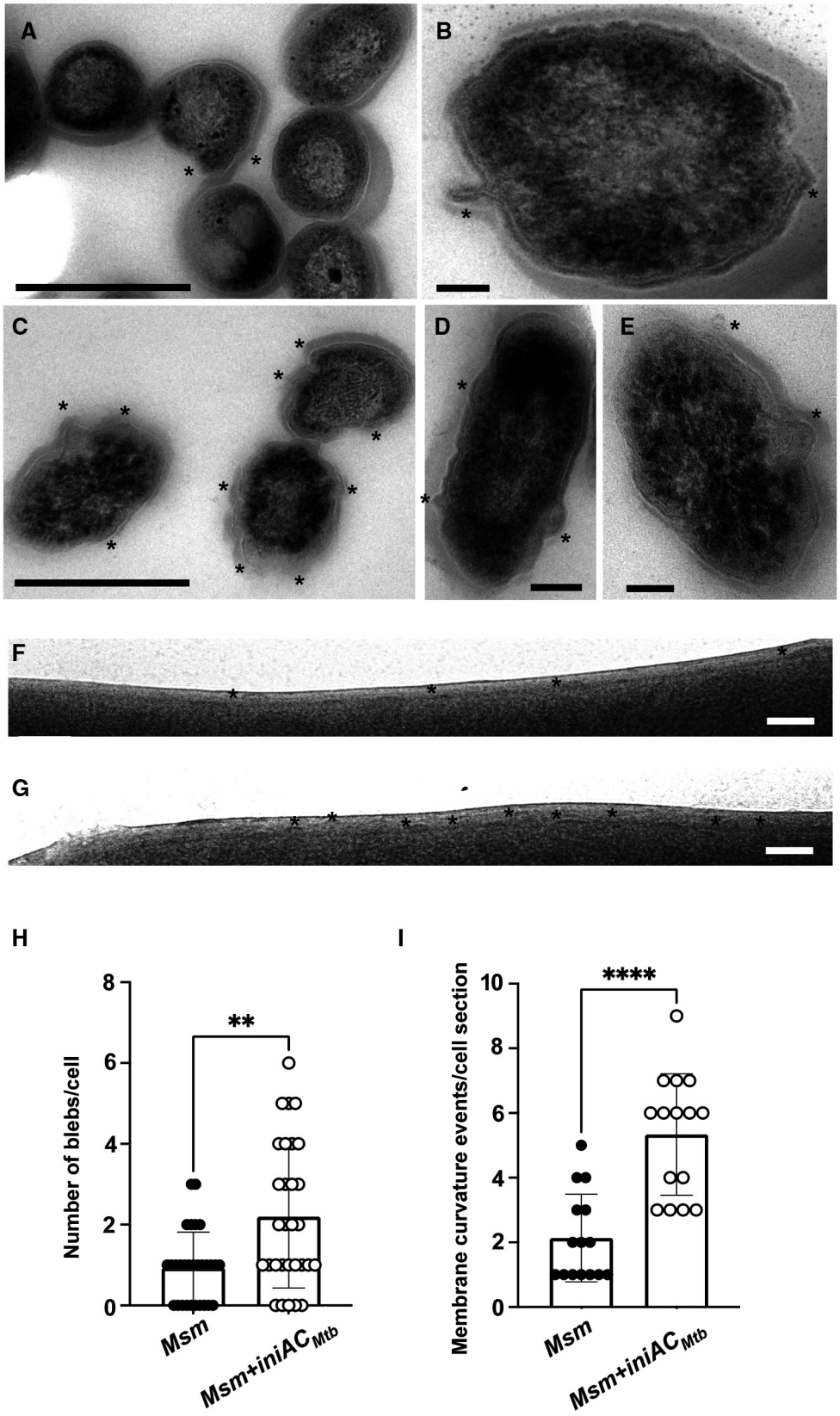

Figure EV4.

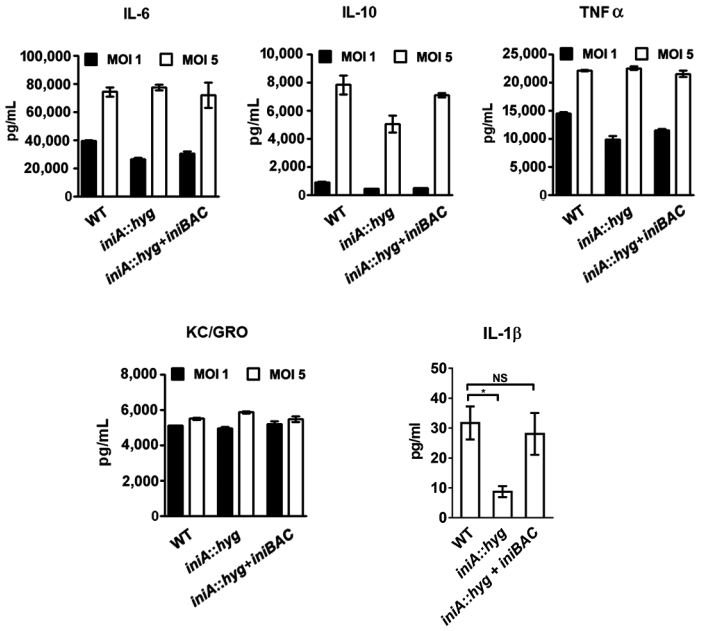

Figure EV5. Cytokine response of macrophages infected with *Mtb*.

Shown are the levels of IL-6, IL-10, TNF-α, KC/GRO, and IL-1 β detected in the culture supernatant of B-MDMs infected at MOI of 1 or 5 (5 in the case of IL-1β) with WT, *iniA::hyg* and complemented strain. Background cytokine levels from uninfected macrophage culture filtrates were subtracted before plotting. Data are presented as means ± SD of triplicate infections. Data are presented as mean ± SD. $*P \leq 0.05$ (Student's *t*-test). Nonsignificant (NS).