Microsporidia Ser/Thr Protein Phosphatase PP1 Targets DCs MAPK Pathway and Impair Immune Functions

Jialing Bao[1,2,*], Yunlin Tang[1,2], Yebo Chen[1,2], Jiangyan Jin[1,2], Xue Wang[1,2], Guozhen An[1,2], Lu Cao[1,2], Biying Mo[1,2], Huarui Zhang[1,2],Gong Cheng[3], Guoqing Pan[1,2], Zeyang Zhou[1,2],

1- The State Key Laboratory of Resource Insects, Southwest University, Chongqing, 400715, China

2- Chongqing Key Laboratory of Microsporidia Infection and Control, Southwest University, Chongqing 400715, China

3- Tsinghua University-Peking University Joint Center for Life Sciences, School of Medicine, Tsinghua University, Beijing 100084, China

Correspondence: Jialing Bao, baojl@swu.edu.cn

Running Title: Microsporidia impair DCs function

Highlights

- Persistence of Microsporidia within host would impair dendritic cell functions such as phagocytosis, maturation, antigen presentation and T cell priming, thereby disrupting both innate and adaptive immunities and making host more vulnerable to secondary infections
- Microsporidia impairs DCs function via Serine/Threonine Protein Phosphatase PP1 directly targeting at DCs p38α/MAPK pathway
- Latent Microsporidia infection and persistence would be a great threat to public health when fighting acute and emerging pathogens

Abstract

Microsporidia are difficult to be completely eliminated once infected,and the persistence may disrupt host cell functions. Here in this study, we aimed to elucidate the impairing effects and consequences of microsporidia on host dendritic cells. *Enterocytozoon hellem*, one of the most commonly diagnosed human-infecting microsporidia species was applied. In vivo models demonstrated that E. hellem infected mice were more susceptible to further pathogenic challenges, and DCs were identified as the most affect groups of cells. In vitro assays revealed that E. hellem infection impaired DCs immune functions as reflected by down-regulated cytokines expressions, lower extent of maturation and antigen presentations. E. hellem infection detained DCs' potencies to prime and stimulate T cells, therefore host immunities were disrupted. We further demonstrated that *E. hellem* Ser/Thr protein phosphatase PP1 directly interact with host p38α(MAPK14) to manipulate p38 α (MAPK14)/NFAT-5 axis of the MAPK pathway. Our study is the first to elucidate the molecular mechanisms of the impairing effects of microsporidia on host DCs immune functions. The emerging of microsporidiosis may be great threat to public health.

Key words: Microsporidia, *Enterocytozoon hellem*, Dendritic cells, Serine/Threonine Protein Phosphatase, MAPK

Introduction

Microsporidia is a huge group of intracellular pathogens that can infect all animals, from invertebrates to vertebrates including human beings (1, 2). At least 15 species can infect humans and the four most common ones are *Enterocytozoon bieneusi* (*E. bieneusi*), *Encephalitozoon hellem* (*E. hellem*), *Encephalitozoon cuniculi* (*E. cuniculi*) and *Encephalitozoon intestinalis* (*E. intestinalis*) (3, 4). Immune-compromised individuals were believed to be more vulnerable to microsporidia infections (5). However, accumulating evidence showed that immune-competent individuals would also be infected, and the outcomes are often asymptomatic and cause latent infections (6-8). For instances, one study conducted in Cameroon revealed that 87% teenagers and 68% healthy asymptomatic individuals were actually had subclinical microsporidial infections and were shedding spores (9). In addition, co-infections of microsporidia with other pathogens such as HIV, cryptosporidia and *M. tuberculosis* are under-estimated, but actually quite common and usually has exacerbated outcomes compared to single pathogen infection alone (10, 11). These findings revealed the latent infection and persistence of microsporidia in immune-competent individuals are much higher than we used to think. Although the issues about the latent infection of microsporidia in immune-competent individuals had long been under-estimated, it should now be paid more attentions since the wide existence of microsporidia in nature and the wide host ranges of them may lead to emerging infections and serious public health problems (8, 12).

As the obligate intracellular pathogen, the interactions between microsporidia and host cells remain to be the spotlight, and researchers are very interested to elucidate how microsporidia modulate host cell functions especially the immune cell functions. By far, most studies were carried out using genetically knock-out or immune-deficient mice to increase microsporidia infection and colonization rates, and usually focused on host adaptive immunity (13, 14). Yet, using immune-competent animal models and elucidating the roles of hosts' sentinel innate immunity during microsporidia-host interactions are much in demand (15, 16).

Dendritic cells (DCs), the professional antigen presenting cells and the bridge of innate and adaptive immunities, are found participating in defending microsporidia infections (17-19). Moretto et al showed that *in vitro* infection of *E. cuniculi* to DCs can stimulate CD8+ T cells to release IFNγ which is known to be cytotoxic to microsporidia; Bernal et al found that *E. intestinalis* interferes DCs cell differentiation and production of pro-inflammatory cytokine IL-6 (14, 15). Moreover, studies revealed that DCs differentiation influences host's overall anti-microbial capability (20, 21). Therefore, DCs might be the master regulator of host immune responses against microsporidia and other pathogen co-infections.

The MAPK signaling pathway is essential in regulating many cellular processes including inflammation and stress responses. Along the signal transduction route, there are several key points (proteinases) that can be modulated thus affect the signaling outcomes. For example, the p38a (MAPK14), MAP Kinase Kinases (MKKs) have been reported to be regulated by several pathogens and medicines (22, 23). In addition, downstream of MAPK pathway, there is an essential transcriptional factor NFAT5 (24, 25). NFAT (Nuclear Factors of activated T cells) was originally identified as a key transcription factor involved in maintaining cellular homeostasis against hypertonic.

Emerging evidence pointed out the immune-regulatory function of NFAT5, and is achieved by inducing different target genes and different signaling pathways. Expressions of pro-inflammatory genes such as *IL-6, IL-2*, *H2Ab* are all directly controlled NFAT-5 (26, 27).

Ubiquitous serine/threonine protein phosphatase (PP1) is a single domain catalytic protein that is exceptionally well conserved in all eukaryotes, from fungi to human, in both sequence and function (28). In human body, PP1 is responsible for about 30% of all de-phosphorylation reactions (29, 30). Pathogenic microbes usually express the serine/threonine proteinase as modulator of host. For example, *Mycobacterium tuberculosis* express PknG within host macrophage to regulate host protein phosphorylation and interferes with autophagy flux, thus greatly affect host cell functions (31, 32). Intracellular pathogen microsporidia possess reduced genome to bare only minimum of essential genes. However, we are very excited to found the existence of *PP1* gene in microsporidia genome, indicating its key functions in pathogen growth and in pathogen-host interactions. It is therefore of great interest to exploit the regulation effects of microsporidia PP1 on dendritic cell functions.

Here in current study, we plan to utilize murine model as well as cells cultured *in vitro* to thoroughly investigate the influences of microsporidia infection on DCs. Our study will elucidate the regulation mechanisms of microsporidia on host immune responses, and will shed light on prevention of pathogens co-infections and emerging diseases.

Materials and Methods

Pathogens

Encephalitozoon hellem (*E. hellem*) strain (ATCC 50504) is gift of Prof. Louis Weiss (Albert Einstein College of Medicine, USA). Spores were inoculated and propagated in rabbit kidney cells (RK13, ATCC CCL-37), cultured in Minimum Essential Medium Eagle (MEM) with 10% fetal bovine serum (FBS) (ThermoFisher, USA). The spores were collected from culture media, purified by passing them through 5 μm size filter (Millipore, Billerica, MA), and stored in sterile distilled water at 4°C(33). Spores were counted with a hemocytometer before usages.

Staphylococcus aureus (S. aureus) was gifted by Dr. Xiancai Rao (Department of Microbiology, College of Basic Medical Sciences, Army Medical University, Chongqing, China). The microbe was modified on the basis of strain of N315 to express EGFP for visibility during observations, and cultured on TSB medium (34).

Animals

Wild-type C57BL/6 mice (six-week, female) were reared in animal care facility according to Southwest University-approved animal protocol (SYXK-2017-0019). At the end of experiment, all mice were euthanized using carbon dioxide narcosis and secondary cervical dislocation.

Cells and cell lines

The primary dendritic cells were isolated from mice mesenteric lymph nodes, spleen and bone marrow. Mesenteric lymph node aligned with the intestine were moved by forceps, washed with cold PBS and teased into single cell mix in harvesting medium such as RPMI Medium 1640. Bone marrow were flushed by RPMI Medium

1640 from the femur of dissected mice. Mice spleens were digested in spleen dissociation medium (STEMCELL Technologies, Canada) at room temperature, followed by gently passing several times through a 16 Gauge blunt-end needle attached to a 3 cc syringe and then through a primed 70μm nylon mesh filter. The single cell suspension, either from bone marrow or spleen, was then subjected to the EasySep Mouse Pan-DC Enrichment Kit (STEMCELL Technologies, Canada) to isolate dendritic cells only. Briefly, add Enrichment Cocktail and subsequently Biotin Selection Cocktail to sample. Next incubate sample with Magnetic Particles and use magnet to negatively select the dendritic cells portion. The isolated DCs were counted and cultured in RPMI Medium 1640 (supplemented with 10% FBS and penicillin/streptomycin) (Gibco, USA) in a 37°C, 5% CO2 incubator.

DCs cell line, DC2.4 (Sigma-Aldrich SCC142) was purchased from BeNa Culture Collection, China. The cells were cultured in RPMI Medium 1640 supplemented with 10% FBS and penicillin/streptomycin (Gibco, USA) in a 37°C, 5% CO_2 incubator.

The suspension T cell line, Jurkat cells (Clone E6-1, ATCC) was purchased from FuDan IBS Cell Center, China.The cells were cultured in RPMI1640 medium, supplemented with 10% (v/v) FBS and 1% (v/v) penicillin/streptomycin (Gibco, USA) in a 37℃, 5% CO2 incubator.

Microsporidia infection

In vivo infection of microsporidia to wild-type mice was achieved as follows. $1X10^7$ *E. hellem* spores/mice/day were inoculated into wild-type mice for two days, the mice were transiently pre-treated with dexamethasone (Aladin, Cas 2392-39-4, China) to increase infection rates but no significant effects on immune cells as assessed in our established murine model and other reports (35-38). The control groups were either treated with PBS, LPS (5mg/kg), or *S. aureus* ($1X10^7$ CFU/mice). The body weights were monitored. At the endpoint of the experiment, mice were sacrificed by CO_2 inhalation. Samples of blood, urine, faeces and organs were collected for further investigations.

In vitro infection of microsporidia were achieved by adding *E. hellem* spores (30:1/spores: cells) to primary DCs or DC2.4 cells cultures.

Successful invasion and colonization of *E. hellem* within cells or in host organs could be verified by immuno-fluorescence assays, or qPCR using *E. hellem* primers targeting at the conserved SSU-rDNA (5'-TGAGAAGTAAGATGTTTAGCA-3'; 5'-GTAAAAAGACTCTCACACTCA-3') (5).

***S. aureus* infection**

For *in vivo* infection, the bacteria were inoculated into mice via intraperitoneal injection at the dose of 10^7 CFU/mice. For *in vitro* infection, the bacteria were added to cell cultures at the ratio of 5:1 (DCs: *S. aureus*) or at the ratio of 1:1 (DCs: *S. aureus*), for various experimental purposes.

Co-culture of DCs with T cells

DCs were infected with *E. hellem* (30:1/spores: cells) for 24 hours. The controls were either un-infected controls or infected by *S. aureus* (5:1/DCs: *S. aureus*). These DCs were washed with PBS to get rid of excess pathogens, and then Jurkat cells were added to the culture groups (1:1 DCs/T cells), respectively. The innate immune cells

and the lymphocytes were co-incubated for 12 hours. After that, gently stir the flasks to collect the suspension cells (T cells), the bottom-attached cells were collected as DCs, and were used for further analysis respectively.

Flow cytometry analysis

The immune cell profiles and cell characterization, the expressions of cell surface markers were assessed by flow cytometry analysis. Single cell suspensions, from various treatments, were washed with 1x PBS/0.3 % BSA, and then stained with fluorochromes-conjugated antibodies (all purchased from BioLegend, USA) for 30 min at 4°C. Samples were subjected to analysis via FACSCanto II flow cytometer (BD Biosciences, USA), and the data were analyzed with FACSDiva software (v6.1.2).

Cytokines expressions

Cytokines such as interleukin-6 and interleukin-12expression levels were detected by ELISA Kits (ThermoFisher Scientific, USA). Samples were either mice plasma or cell culture supernatants. Mice peripheral blood was drawn with anti-coagulant sodium citrate added, the blood samples were then centrifuged at 400x g for 10 minutes to get the supernatant plasma.

qPCR

To assess transcription levels of target genes, total RNAs of different cells such DCs would be extracted by TRIzol (Ambion, USA). The RNA samples were then reversely transcribed to cDNAs using High-Capacity RNA to cDNA Kit (Yeasen Biotechnology, China). qPCR assay was carried out according to Hieff® qPCR SYBR Green Master Mix instructions (Yeasen Biotechnology, China). The genes/primers information was shown in supplementary data **(S-Table 1)**.

Label free quantitative mass spectrometry

The total proteins of DC2.4 cells, either un-infected or infected by *E. hellem*, were extracted respectively. The protein samples were then subjected to label free quantitative mass spectrometry analysis performed on a Q Exactive mass spectrometer (Thermo Scientific). The MS data were analyzed using MaxQuant software version 1.5.3.17 (Max Planck Institute of Biochemistry in Martinsried, Germany). The biological functions of proteins were annotated by Gene Ontology (GO) Annotation (Blast2GO, http://www.blast2go.com) and the online Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://geneontology.org/). Differentially expressed proteins between the two groups were screened using fold change greater than 1.5 fold (up to 1.5 fold or less than 0.5). Those selected proteins were explored following bioinformatic analysis, including hierarchical cluster by ComplexHeatmap R (version 3.4), KEGG Functional Enrichment analysis, and Protein-Protein Interact Network using IntAct molecular interaction database (http://www.ebi.ac.uk/intact/). Statistical significance was analyzed using Student's t test based on P-value < 0.05.

Statistics

Statistical analysis of results was conducted by using Student's T-test or Two-way ANOVA and to identify the differences between two groups, with P < 0.05 being considered a significant difference.

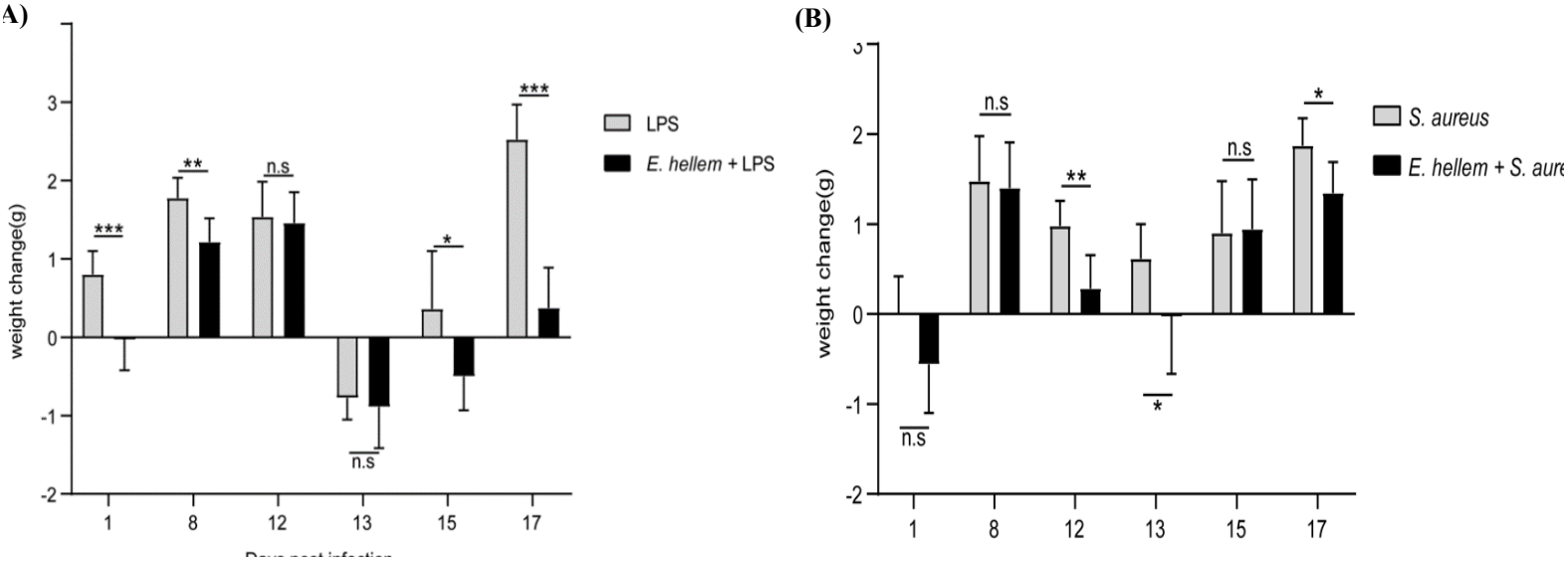
Results

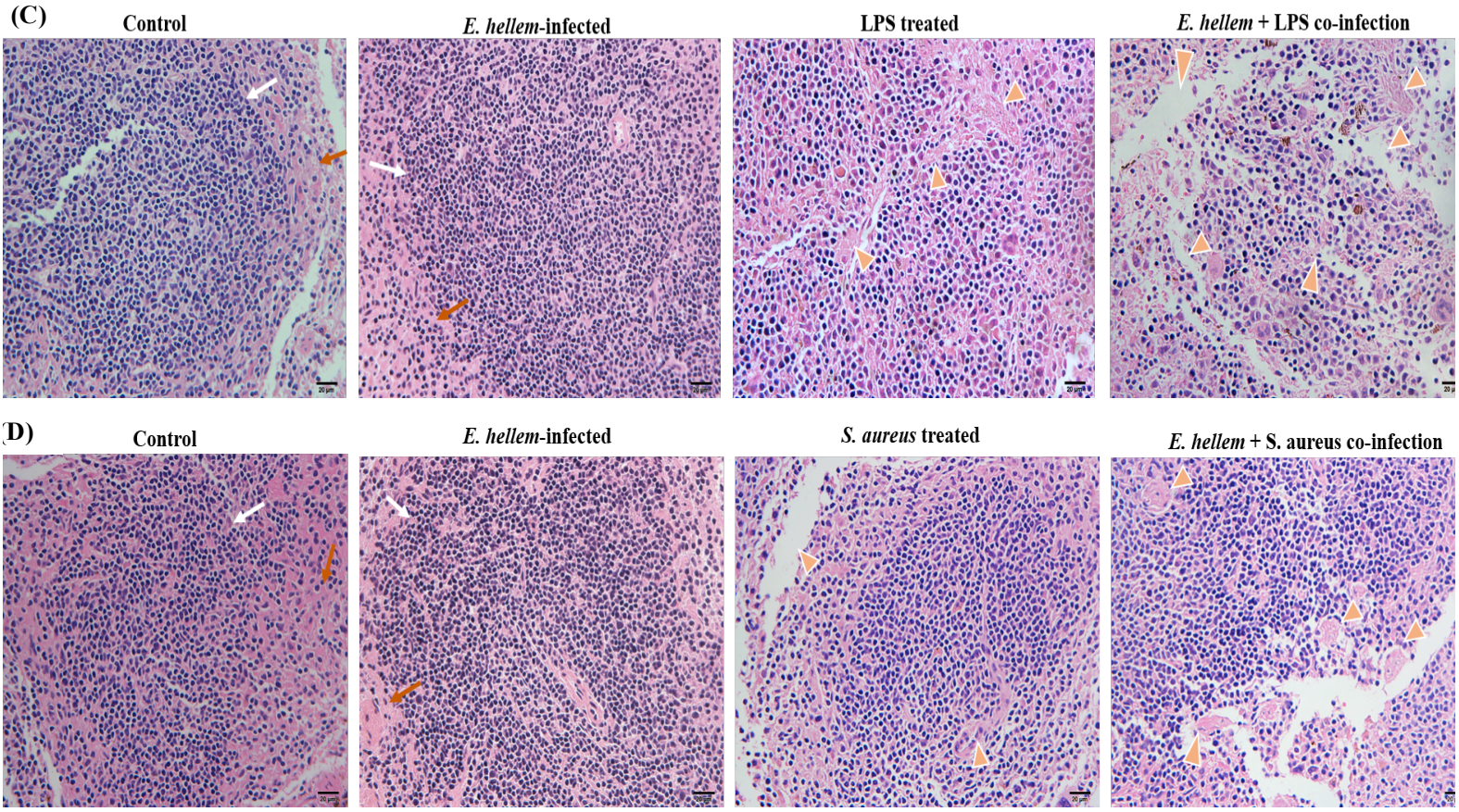
Persistent infection of *E. hellem* increases host disease susceptibility and disturbs dendritic cell populations

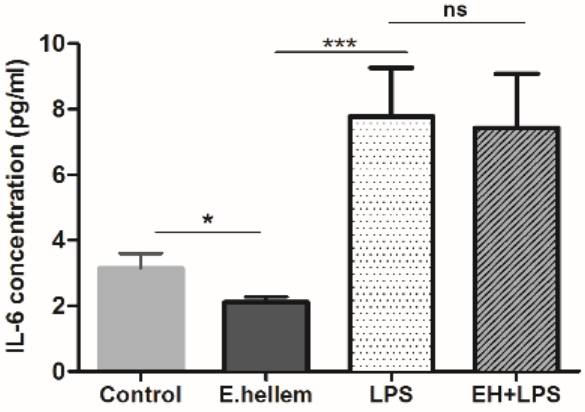
Microsporidia infections are usually asymptotic, however the covert infection and persistent existence within host may disturb immune responses therefore making the host more vulnerable to further challenges. Here in this study, we firstly utilized our previously established microsporidia infection model to infect wild-type C57BL/6 mice with *E. hellem*. As the result, *E. hellem* infection were covert as proved by no obvious symptoms such as spleen edema or significant weight loss (supplementary **S-Fig. 1A, 1B)**, but *E. hellem* could persist as proved by detection of the spores in host blood, stool and urine samples even after more than half a month past infection (supplementary **S-Fig. 1C**).

Interestingly, when *S. aureus* or endotoxin lipopolysaccharide (LPS) were inoculated into the *E. hellem* pre-infected mice, the hosts showed increased susceptibility to the secondary challenges. As shown, *E. hellem* pre-infection plus secondary infections (co-infection) caused significantly more weights loss and slower weigh re-gain in mice, compared to secondary challenges alone (**Fig. 1A-1B**). *E. hellem* infection alone had no significant irritations to host organs such as spleen, but *E. hellem* pre-infection plus secondary infections (co-infection) lead to more irritations in tissues compared to secondary challenges alone (**Fig. 1C-1D**). In addition, ELISA analysis of plasma cytokine levels revealed that *E. hellem* pre-infection not only down-regulated the expressions of pro-inflammatory cytokines IL-6 and IL-12, but also detained the increase or even decreased the cytokine levels when facing secondary infections, compared to secondary challenges alone (**Fig. 1E-1H**).

Since the major infection route of microsporidia is through ingestion, the assaulting pathogens were majorly sampled by phagocytes in the intestinal mucosa such as dendritic cells (DCs) and drained to mesenteric lymph nodes (MLN). Therefore, we are very interested to investigate whether *E. hellem* infection dysregulated the immune cell populations and which group is affected most in the MLN. Flow cytometry analysis showed that dendritic cells (DCs) populations were significantly altered after infection, but not the lymphatic T cells or B cells, nor the inflammatory monocytes (**Fig. 1I**). Taken together, these data demonstrated that covert infection of *E. hellem* would persist within host, making host more vulnerable to further challenges, and the most affected sentinel cell populations are dendritic cells (DCs), therefore may play key roles in microsporidia-host interactions.




(C)
Control
E. hellem-infected
LPS treated
E. hellem + LPS co-infection
(D)
Control
E. hellem-infected
S. aureus treated
E. hellem + S. aureus co-infection


(E)
IL-6 concentration (pg/ml)
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6
8
10
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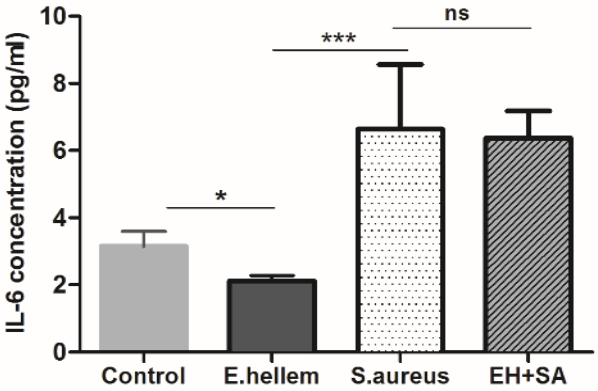
ns
Control
E.hellem
LPS
EH+LPS


(F)
IL-6 concentration (pg/ml)
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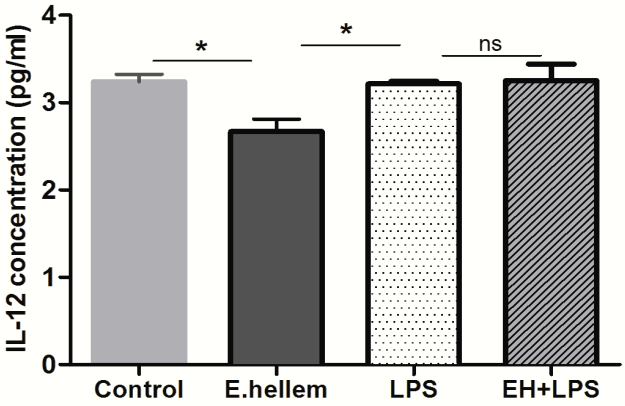
Control
E.hellem
S.aureus
EH+SA


(G)
IL-12 concentration (pg/ml)
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1
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3
4
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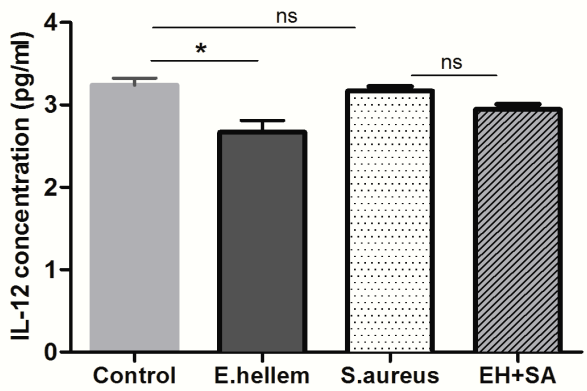
Control
E.hellem
LPS
EH+LPS


(H)
IL-12 concentration (pg/ml)
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3
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*
ns
Control
E.hellem
S.aureus
EH+SA

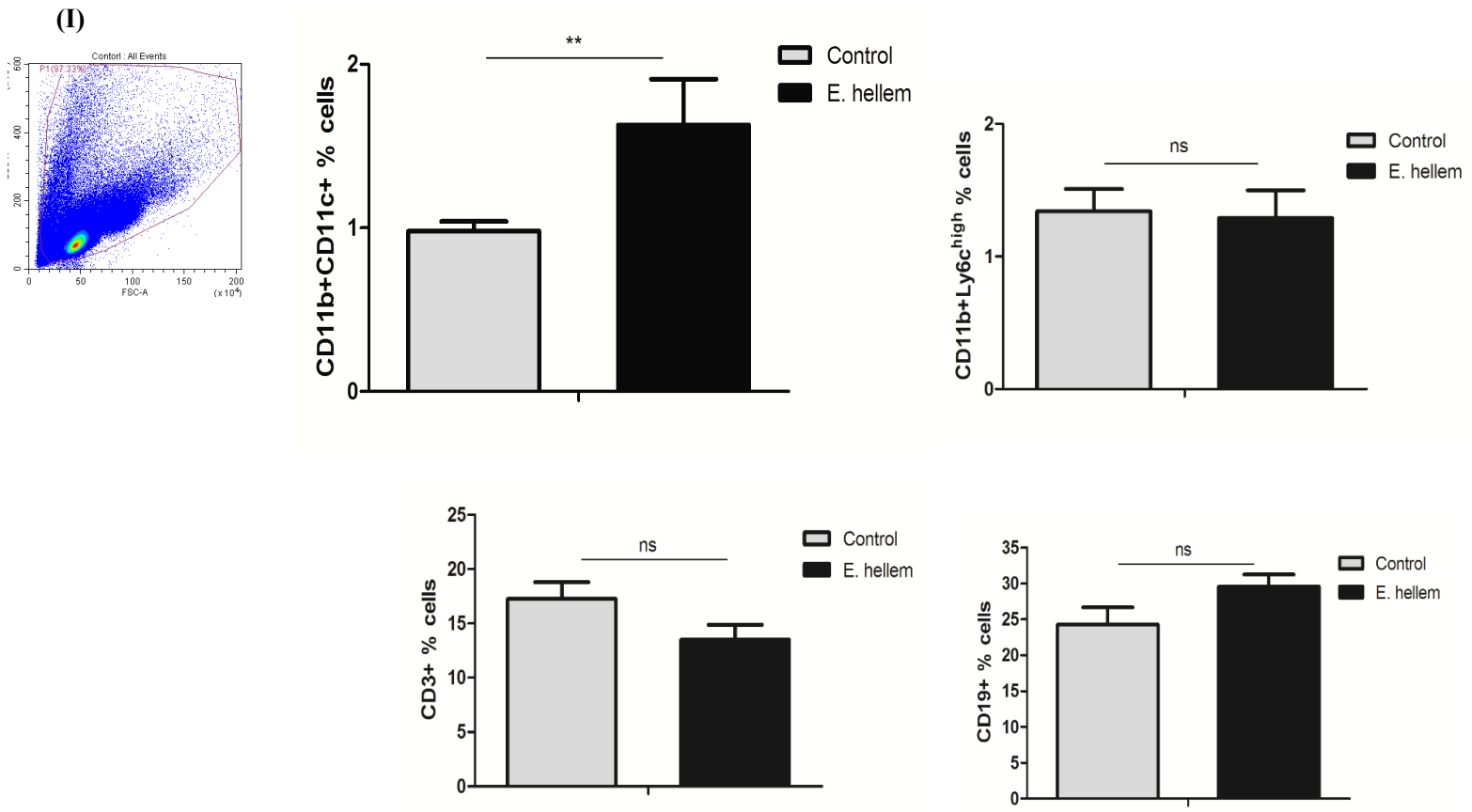


Figure 1. *E. hellem* infection and persistence increases host disease susceptibility and affect dendritic cells. **(A, B)** LPS treatment or *S. aureus* infection in *E. hellem* pre-infected mice (*E. hellem*+*S. aureus*, *E. hellem*+LPS) caused more body weight loss and slower/less body weight re-gain. (n=8 per group). (*= P<0.05, **=P<0.01, ***=P<0.001). **(C, D)** Hematoxylin-eosin staining of spleen samples. *E. hellem* infection alone had no obvious effects on tissue pathology compared to control, as white pulps (white arrows) and red pulps (orange arrows) arranged normally. *E. hellem* pre-infection plus LPS treatment, or *E.hellem* pre-infection plus S. aureus infection, caused more damages to tissues compared to single challenges alone, as shown by more enlarged/distorted cells/cytosols and vacuolations (golden arrow heads) (Scale bar=20 μm). **(E-H)** ELISA assay showed that the *E. hellem* infection (E. hellem) significantly down-regulate the expression of IL-6 and IL-12 compared to un-infected (Control), and were significantly lower than s. aureus infection (S. aureus). *E. hellem* pre-infection (*EH*+*SA*) did not arose higher but caused a slight lower levels of cytokines compared to S. aureus infection alone. (n=8 per group). (*= P<0.05, **=P<0.01). **(F, G)** ELISA assay showed that the *E. hellem* infection (E. hellem) significantly down-regulate the expression of IL-6 and IL-12 compared to un-infected (Control). Single challenges such as LPS treatment (LPS) or *S. aureus* infection (SA) would arose IL-6 and IL-12 expressions, however *E. hellem* pre-infection (*EH*+LPS, or EH+SA) did not arose higher expressions but caused a slight lower levels of cytokines compared to single challenges. (n=8 per group). (*= P<0.05, **=P<0.01). **(I, J)** Flow cytometry analysis of the immune cells profiles from mouse mesenteric lymph nodes. Mesenteric lymph nodes were isolated from control or *E. hellem* infected mice, and were teased into single cell mixture (n=3-5 lymph nodes from each mouse, 8 mice per group). Results showed that dendritic cells (CD11b+CD11c+) were significantly disturbed after *E. hellem* infection, but not inflammatory monocytes (CD11b+Ly6c^{high}), T cells (CD3+), nor B cells (CD19+) were affected (**=P<0.01, ns=no significance; n=8/group).

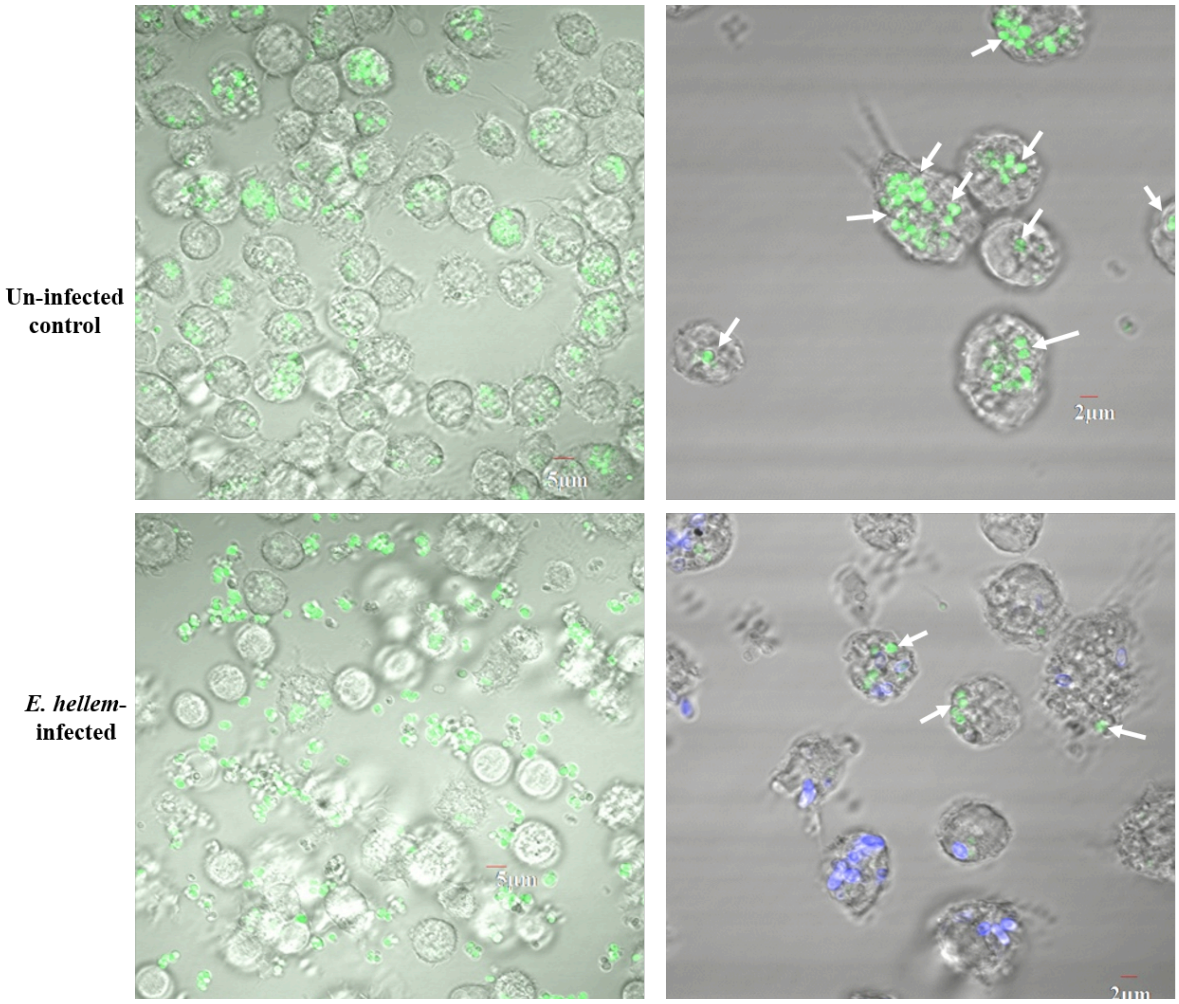
***E. hellem* interferes DCs immune functions and maturation**

The major immune functions of DCs include pathogen phagocytosis, cytokine expressions, antigen presentation and so on. The phagocytosis abilities of DCs, either from *E. hellem* infected mice or controls, towards the fluorescently-labelled *S. aureus* were compared and analysed. As shown, control DCs sufficiently engulfed fluorescent-labelled *S. aureus*, while the DCs from *E. hellem*-infected mice were reluctant to engulf *S. aureus* **(Fig. 2A,** and supplementary videos **S-Video1-4)**.

Full maturation and efficient homing of DCs from MLN to T cell-region constitute crucial aspects of DCs functions. Therefore, we collected immune cells from the spleens of either *E. hellem*-infected, LPS-treated or un-infected mice respectively. Flow cytometry analysis demonstrated that, the matured and specialized DCs populations were retained at comparable levels to un-infected controls even after *E. hellem* infection, but the LPS-treatment significantly up-regulated matured DCs populations in spleen (**Fig. 2B**). Flow cytometry analysis further confirmed that *E. hellem* detained the expressions of maturation and co-stimulatory surface markers, CD40 and CD86, on splenic DCs (**Fig. 2C-2D**). Next, cytokines expressions of splenic DCs were assessed by qPCR analysis and confirmed that DCs from *E. hellem* infected group expressed significantly lower levels of *IL-12p40*, *IFNγ*, *IL-6* and *IFNα,* compared to DCs from un-infected controls or LPS treated groups (**Fig. 2E-2H**).

Taken together, these findings demonstrated that *E. hellem* infection interferes the full immune functions and full maturation processes of DCs, which would explain the above showed detained cytokine levels in serum and increased host susceptibility to secondary pathogens.

(A)



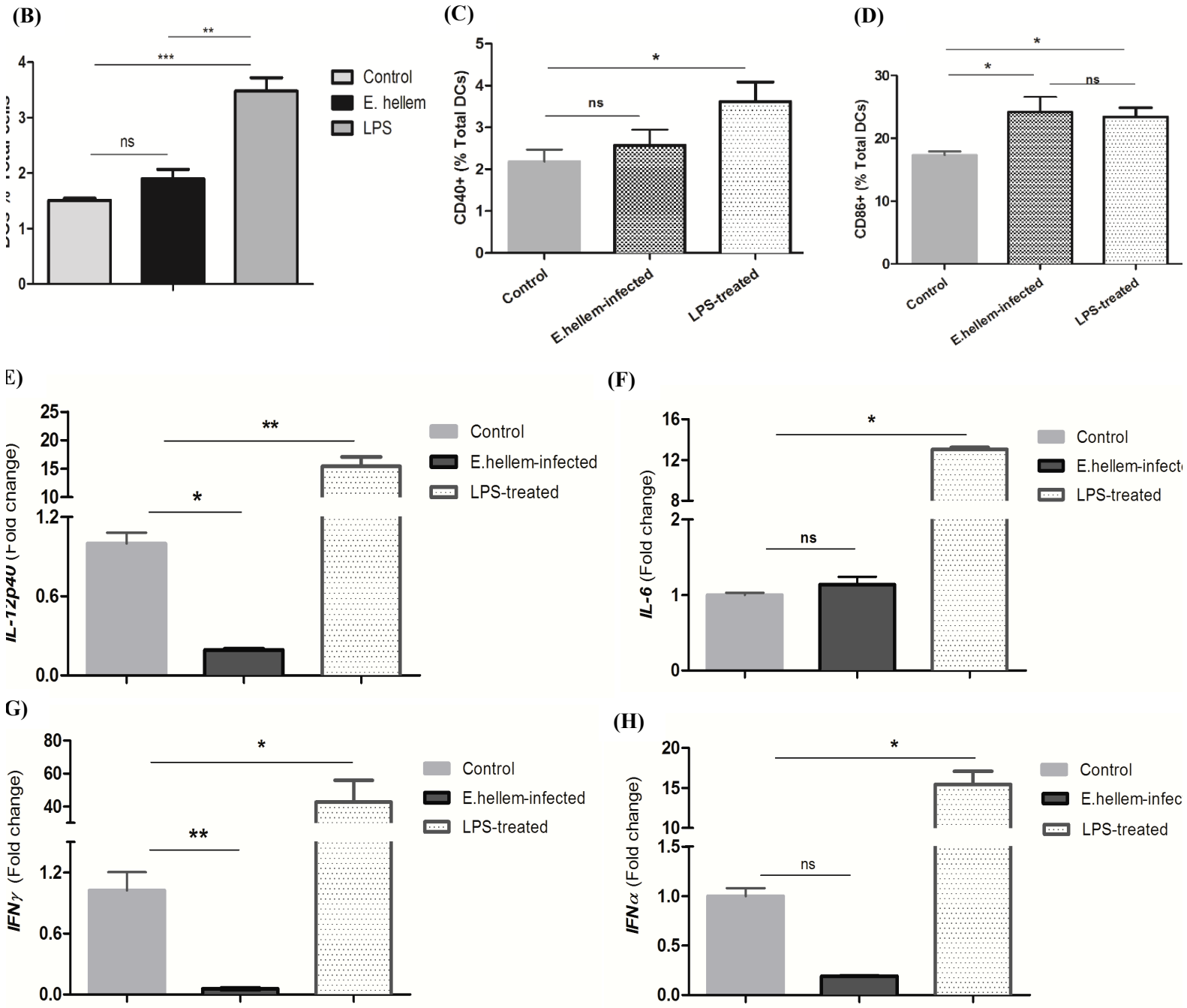


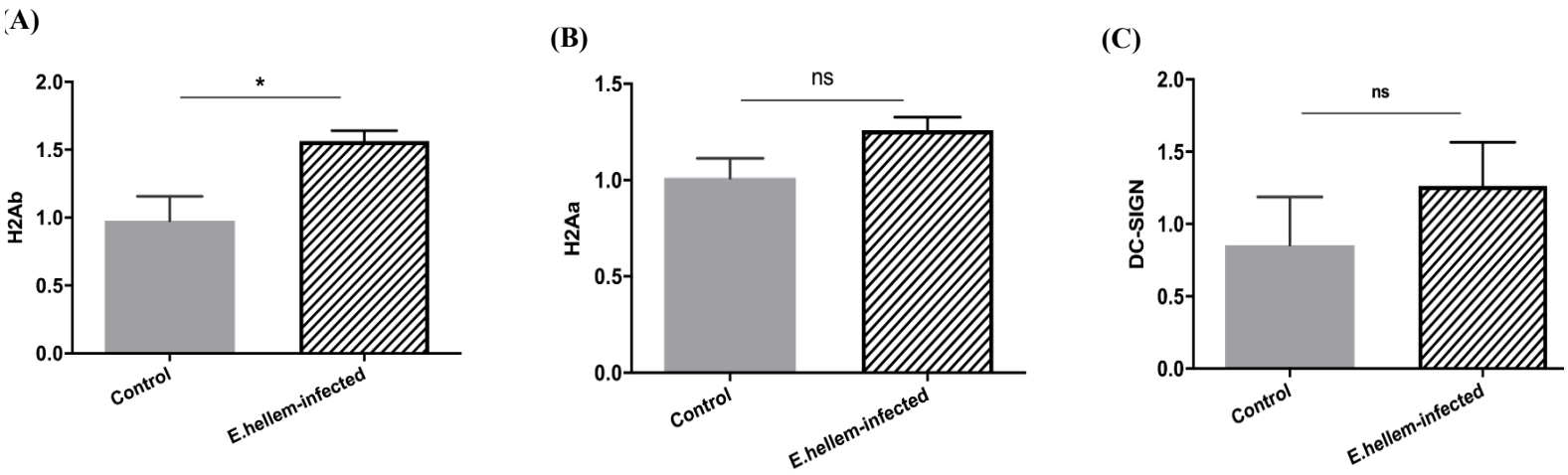
Figure 2. *E. hellem* interferes DCs immune functions and maturation. **(A)** DCs were isolated from normal mice or *E. hellem* infected mice, fluorescent (GFP) labelled *S. aureus* were then added to DCs culture (MOI=20:1). The phagocytic ability was assessed by fluorescent microscopy. Most of the added-in *S. aureus* (green) were engulfed by control DCs; while most added-in *S. aureus* (green) were floating outside of DCs isolated from *E. hellem*-mice (scale bar=5 μm). Zoomed-in images showed that DCs from *E. hellem*-infected group engulfed significant less *S. aureus* (green; arrows) compared to DCs from un-infected controls. Persistence of *E. hellem* spores was manifested by calcofluor white stain (blue) (scale bar=2 μm). **(B)** Flow cytometry analysis showed that *E. hellem* infection retained the splenic dendritic cells (33D1+) to the comparabel level as the un-infected controls. But LPS treatment significantly up-regulated the dendritic cells (n=8/group) (ns=no significance, **=P<0.01, ***=P<0.001) **(C)** *E. hellem* infection, but not LPS treatment, inhibited the expressions of CD40 on dendritic cells. The expression of CD86 were at comparable level between *E. hellem* infection and LPS treatment, both significantly higher than un-infected control. (n=8/group) (ns=no significance, *=P<0.05)**. (E-H)** Cytokine expressions from splenic dendritic cells. **(E)** *IL-12p40* expression level of DCs from *E. hellem* infected mice was significantly down-regulated compared to un-infected controls, and was significantly lower than the LPS-treated ones. **(F)** *IL-6* expression level of DCs from *E. hellem* infected mice was retained from un-infected controls, and was significantly lower than the LPS-treated ones. **(G)** *INFγ* expression level of DCs from *E. hellem* infected mice was significantly down-regulated compared to un-infected controls, and was significantly lower than the LPS-treated ones. **(H)** *IFNα* expression level of DCs from *E. hellem* infected mice was retained from un-infected controls, and was significantly lower than the LPS-treated ones. (n=8/group) (ns=no significance, *=P<0.05, **=P<0.01, ***=P<0.001)

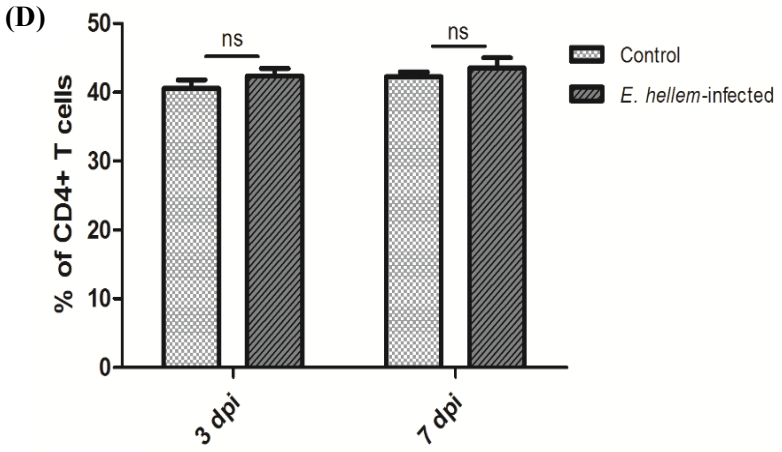
***E. hellem* detained DCs antigen presentation and T cell priming potencies**

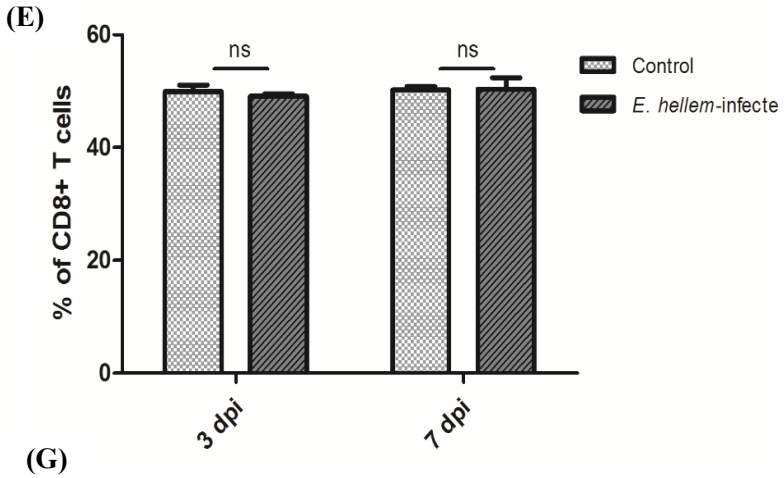
Fully functioning DCs would present the processed antigens to T cells and prime T cell activation. Therefore, we analyzed the expressions of *H2Aa* and *H2Ab* in MHC-II complex, and *DC-SIGN*, all are essential for antigen presentation and T cell priming (39). qPCR assay showed that, although *E. hellem* infection caused the up-regulation of *H2Ab*, it detained the up-regulations of *H2Aa* in the MHC-II complex and the essential antigen presentation marker *DC-SIGN* (**Fig. 3A-3C**), indicating that the antigen presentation abilities of dendritic cells were severely impaired. Next, we analysed the alterations of T cells populations and stimulations from spleens by flow cytometry. Results showed that the neither CD4+ T cells nor CD8+ T cells showed significant stimulation after *E. hellem* infection (**Fig. 3D-3E**). qPCR analysis of the expressions of *Ctla4* and *Tigit*, the known markers for T cells action, confirmed that there were no significant changes after *E. hellem* infection (**Fig. 3F-3G**). These data demonstrated that *E. hellem* detained the DCs antigen presentation abilities and T cell priming potencies, as reflected by no up-regulation of MHC-II complex or DC-SIGN, no changes of T cell populations and stimulation markers expressions.

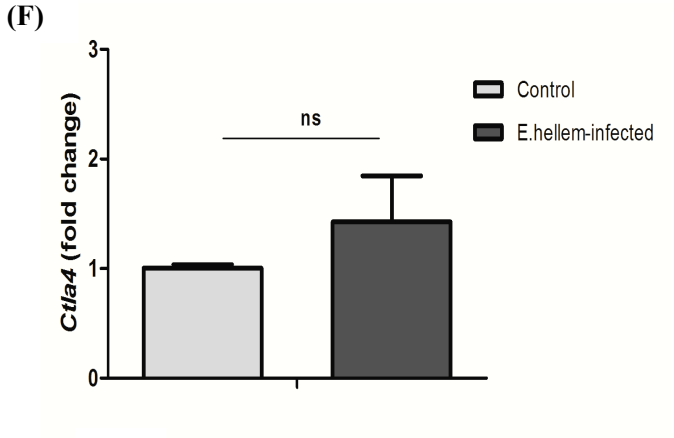
Next, we took one more step forward by co-culturing DC2.4 cells and Jurkat-T cells together *in vitro,* to further demonstrate the interference of *E. hellem* on DCs functions and reluctance of T cell priming. Here, DC2.4 cells were infected by *E. hellem* or *S. aureus* and then co-cultured with Jurkat-T cells *in vitro* for 12 hrs. DCs and T cells were then separated and total RNAs were extracted respectively for qPCR analysis. Results again confirmed that, the *E. hellem*-infected DCs were reluctant to up-regulate expressions of *H2Aa*, *H2Ab* and *DC-SIGN* compared to *S. aureus* infected controls (**Fig. 3H**); While the T cells co-cultured with *E. hellem*-infected DCs detained or even significantly down-regulated the expressions of T cell activation markers such as *CD4*, *Ctla4*, and *PD-1* (**Fig. 3I**).

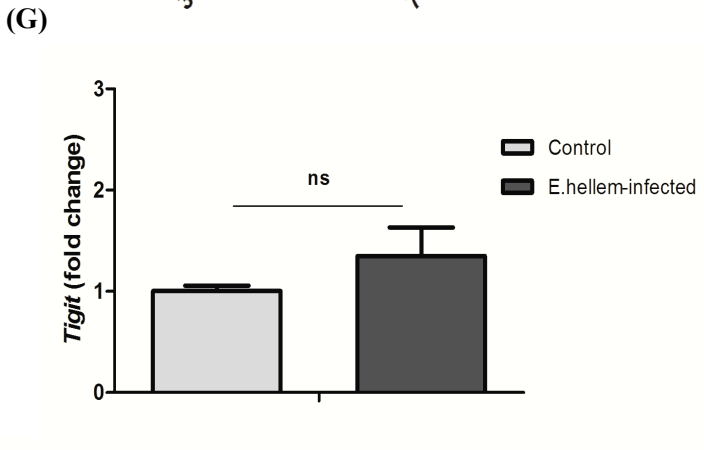
Taken together, our findings demonstrated that *E. hellem* infection defers DCs immune functions and T cell priming potencies, the impaired innate immune responses by DCs together with the reluctance of T cell stimulations would severely weaken host immunity against any further co-infections or challenges.

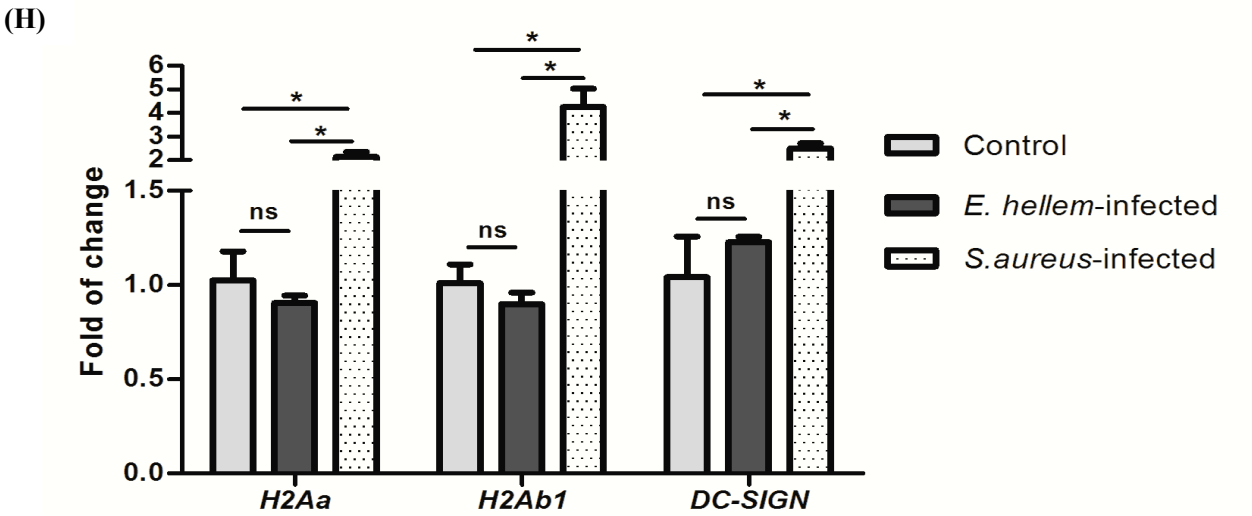



(D)
% of CD4+ T cells
50
40
30
20
10
0
ns
ns
3 dpi
7 dpi
Control
E. hellem-infected


(E)
% of CD8+ T cells
60
40
20
0
ns
ns
3 dpi
7 dpi
Control
E. hellem-infecte


(F)
Ctla4 (fold change)
3
2
1
0
ns
Control
E.hellem-infected


(G)
Tigit (fold change)
3
2
1
0
ns
Control
E.hellem-infected


(H)
Fold of change
6
5
4
3
2
1.5
1.0
0.5
0.0
ns
ns
ns
H2Aa
H2Ab1
DC-SIGN
Control
E. hellem-infected
S.aureus-infected

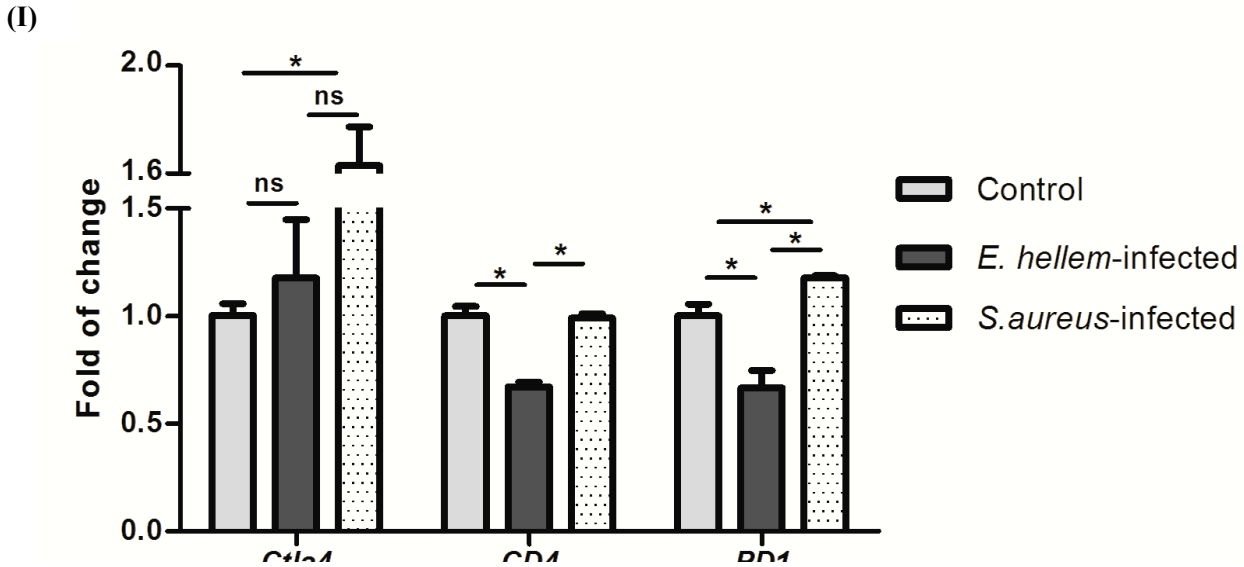
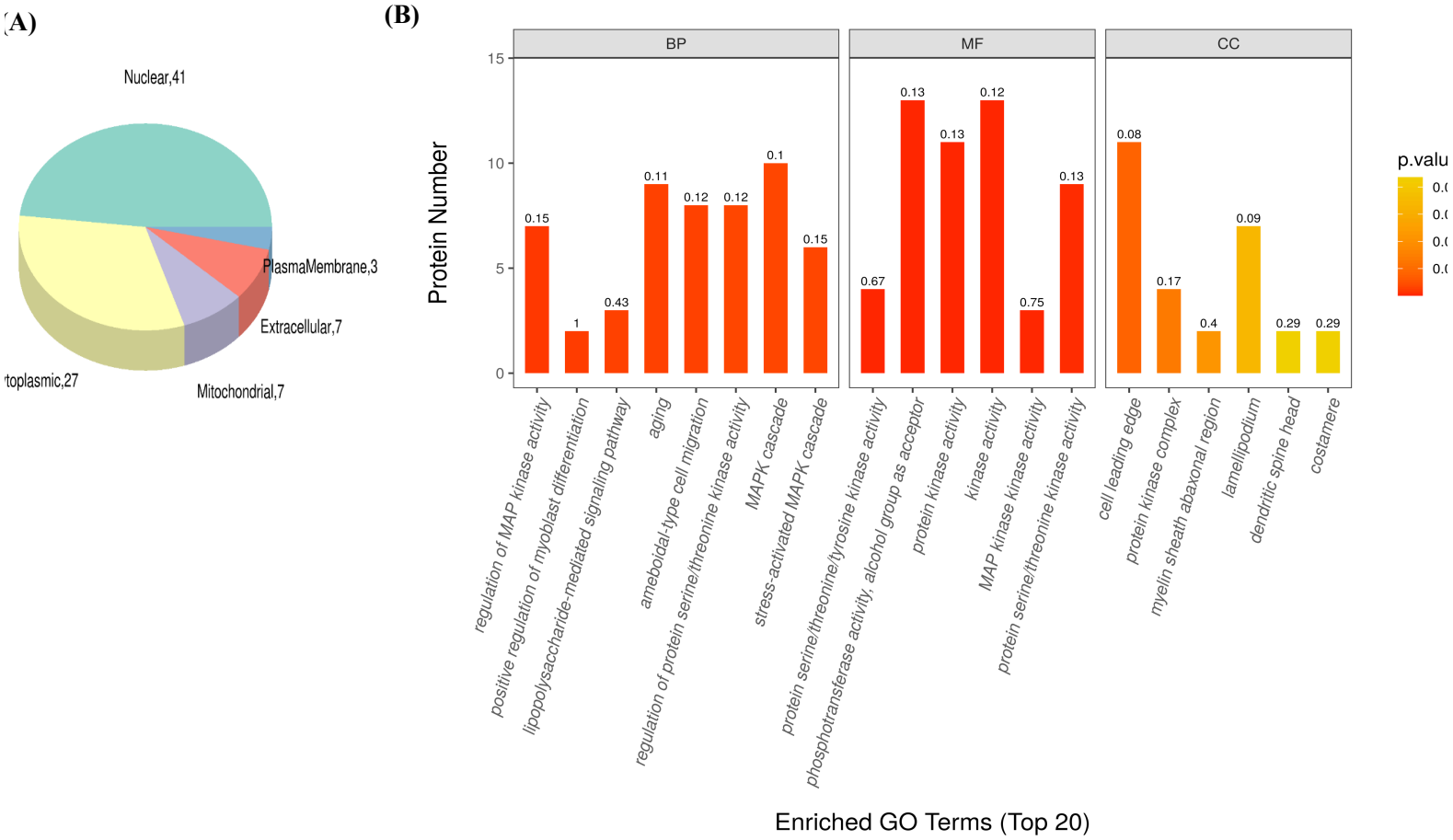

(I)
Fold of change
2.0
1.6
1.5
1.0
0.5
0.0
ns
ns
Control
E. hellem-infected
S.aureus-infected

Figure 3. *E. hellem* detained the antigen presentation abilities and T cell priming potencies of DCs. **(A-C)** The expressions of DCs antigen-presentation related surface markers were assessed by qPCR assay. Results showed that *E. hellem* infection caused the up-regulation of *H2Ab*, but detained the up-regulations of both *H2Aa* and *DC-SIGN* (ns=no significance; *=P<0.05). **(D-E)** CD4+ T cells and CD8+ T cells were analysed for population changes after *E. hellem* infection from 3 days post infection (3 dpi) and 7 dpi. Results showed there were no significant stimulations or changes of populations after *E. hellem* infection (n=8/group) (ns=no significance). **(F-G)** The expressions of *Ctla4* and *Tigit* were analysed by qPCR analysis. Results demonstrated that there was no statistical significance between *E. hellem* infection and un-infected control (ns=no significance). (**H**) DC2.4 cells were either infected by *E. hellem*, *S. aureus* or un-infected controls, were co-cultured with Jurkat-T cells; The DC2.4 cells were isolated later for surface makers analysis. qPCR results showed the representative antigen-presentation markers were detained by *E. hellem* infection, compared to *S. aureus* infections. **(I)** The Jurkat T cells isolated from co-culture with DC2.4, were also analysed by qPCR. Results confirmed that T cells co-cultured with *E. hellem*-infected DCs reluctant to up-regulate the *Ctla4*, and significantly down-regulated the expressions of T cell activation surface markers *CD4* and *PD-1* (n=10/group) (ns=no significance; *=P<0.05).

The MAPK-NFAT5 signaling pathway is key for *E. hellem* -DCs interaction and modulations

We have demonstrated that *E. hellem* down-regulated the expressions of several immune related genes such as *IL-6*, *H2Ab* and *H2Aa* in DCs. It is known that in immune cells, the expressions of these genes were often regulated by transcriptional factor NFAT5, often activated by p38α-MAPK signaling cascade. Therefore, we firstly investigated the proteome changes of DCs after *E. hellem* infection by mass spectrometry (Top differentiated DCs proteins were listed in supplementary **S-Table 2).** Sub-cellular localization analysis revealed that most of the differentially expressed DCs proteins were localized in the cytoplasm or nucleus (**Fig 4A**), indicating that many interactions and modulations occurred in cytoplasm or nucleus such as the signaling pathways and the consequent transcriptional modifications. Gene Ontology (GO) enrichment as well as KEGG analysis showed that many cellular events in DCs include the MAPK signaling pathways/cascade were indeed affected by *E. hellem* infection (**Fig. 4B**). Among the top differentially expressed DCs proteins, representative ones which are associated with cellular responses, signal transductions, and transportations. The representatives were listed in **Table 1**. The protein-protein interactions (PPI) network analysis of top identified proteins revealed that MAPK signaling pathway would be one of the central links of these representative proteins (**Fig. 4C**).

Next, we assessed the expressions and localizations of NFAT5 after *E. hellem* infection. Western blot assays showed that the NFAT5 expressions in DCs, *in vitro* cell culture or isolated from *in vivo* murine model, were suppressed by *E. hellem* infection (**Fig. 5A**). Immuno-fluorescent microscopy proved again with visual evidence that *E. hellem* was able to persistence and proliferate within DCs. More importantly, the trans-localization of transcriptional factor NFAT5 from cytoplasm into nucleus was severely inhibited by *E. hellem* infection (**Fig. 5B**). To verify the essential roles of NFAT5 in DCs responding *E. hellem*, we knocked-down the NFAT5 in DC2.4 cells by RNAi assay and demonstrated that the proliferations of *E. hellem* within DCs were increased (**Fig. 5C-5D**). These findings suggest that NFAT5 and related MAPK signaling pathway are the key axis in responding *E. hellem* infection, therefore may be the major regulation targets of pathogen-host interactions.



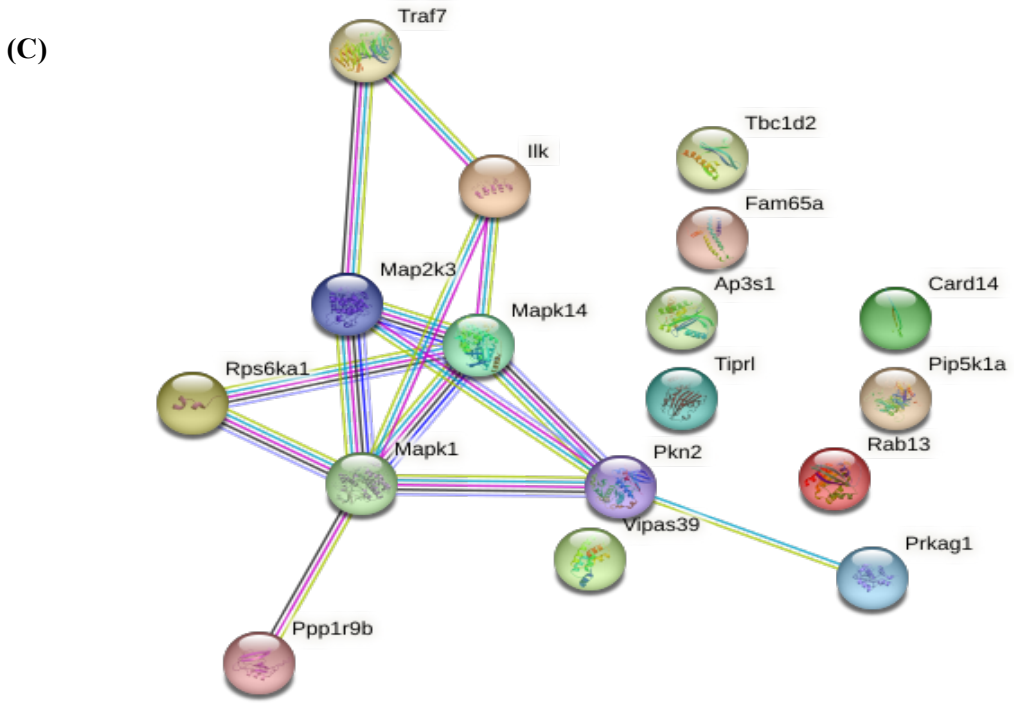
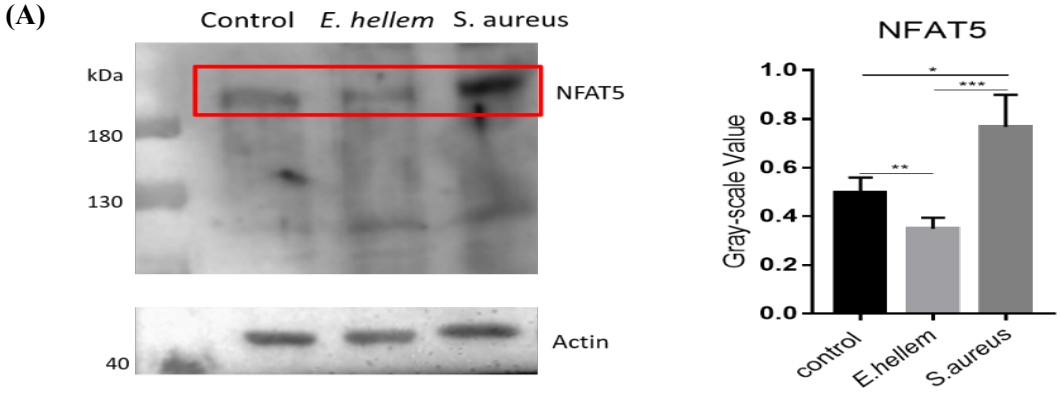
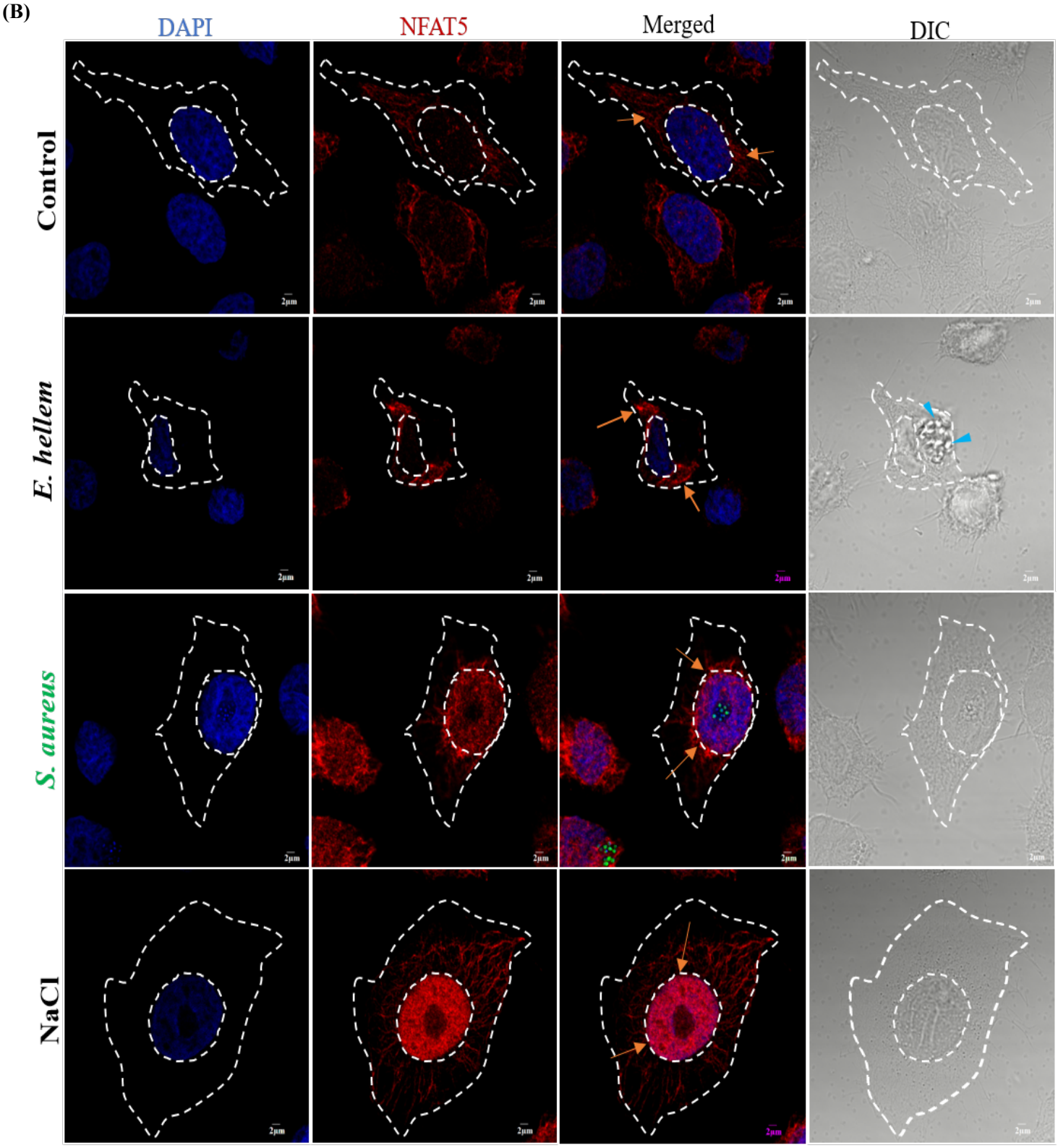


Figure 4. **p38α /MAPK signaling pathway is key for *E. hellem* -DCs interaction and modulations.** **(A)** Pie chat of sub-cellular localizations of top differentially expressed DCs proteins after *E. hellem*. The top localizations are cytoplasm and nucleus. **(B)** GO enrichment analysis of top differentially expressed DCs proteins after *E. hellem* infection. Many signaling pathway include MAPK pathway were among the most affected cellular events. **(C)** Protein-protein interaction network showed that top differentially expressed DCs proteins were associated with MAPK signaling pathway.

Table 1. Representative DCs proteins differentially expressed after *E. hellem* infection

Protein ID	Protein Name	Gene Name	Function	Alterations
P47811.3	Mitogen-activated protein kinase 14	Mapk14	MAP kinase p38. Essential component of the MAPK signal transduction pathway	Down
P63085	Mitogen-activated protein kinase 1	Mapk1	Serine/threonine kinase which acts as an essential component of the MAP kinase signal transduction pathway	Down
O09110	Dual specificity mitogen-activated protein kinase kinase 3	Map2k3	Essential components of MAP kinase signal pathway, catalyzes the concomitant phosphorylation of a threonine and a tyrosine residue in the MAP kinase p38	Down
P18653	Ribosomal protein S6 kinase alpha-1	Rps6ka1	Serine/threonine-protein kinase that acts downstream of ERK (MAPK1/ERK2 and MAPK3/ERK1) signaling and mediates activation of activation of the transcription factors CREB1, ETV1/ER81, NR4A1 and so on.	Down
Q922B6	E3 ubiquitin-protein ligase TRAF7	Traf7	Auto-ubiquitination regulated by MAP3K3. Potentiates MEKK3-mediated activation of the NF-kappa-B in signaling	Down
O55222	Integrin-linked protein kinase	Ilk	Act as a mediator of inside-out integrin signaling	Down
B1AVH7	TBC1 domain family member 2A	Tbc1d2	GTPase-activating protein for RAB7A, and signaling effector	Down
Q9DCR2	AP-3 complex subunit sigma-1	Ap3s1	Facilitates the budding of vesicles from the Golgi membrane and may be directly involved in trafficking to lysosomes	Down
Q8BH58	TIP41-like protein	Tiprl	Allosteric regulator of serine/threonine-protein phosphatase 2A (PP2A)	Down
Q8BGQ1	Spermatogenesis-defective protein 39 homolog	Vipas39	Involved in endosomal maturation, and lysosomal trafficking	Down
Q6R891	Neurabin-2	Ppp1r9b	Scaffold protein in multiple signaling pathways.	Up
Q99KF0	Caspase recruitment domain-containing protein 14	Card14	Scaffolding protein that can activate the inflammatory transcription factor NF-kappa-B and p38/JNK MAP kinase signaling pathways.	Up
Q68FE6	Rho family-interacting cell polarization regulator 1	Ripor1	Effector protein for Rho-type small GTPases that plays a role in cell polarity, signaling and directional migration	Up
O54950	5-AMP-activated protein kinase subunit gamma-1	Prkag1	AMP/ATP-binding subunit of AMP-activated protein kinase (AMPK), a kinase that plays key roles	Up
Q8BWW9	Serine/threonine-protein kinase N2	Pkn2	PKC-related serine/threonine-protein kinase and Rho/Rac effector protein that participates in specific signal transduction during cellular singling	Up
P70182	Phosphatidylinositol 4-phosphate 5-kinase type-1 alpha	Pip5k1a	Catalyzes the phosphorylation of PtdIns4P to form PtdIns(4,5)P2, involves in cellular phagocytosis, migration, signaling.	Up
Q9DD03.1	Ras-related protein Rab-13	Rab13	Small GTPases key regulators of intracellular membrane trafficking	Up


(A)
Control
E. hellem
S. aureus
kDa
180
130
40
NFAT5
Actin
NFAT5
Gray-scale Value
1.0
0.8
0.6
0.4
0.2
0.0
*
***
**
control
E.hellem
S.aureus


(B)
DAPI
NFAT5
Merged
DIC
Control
E. hellem
S. aureus
NaCl

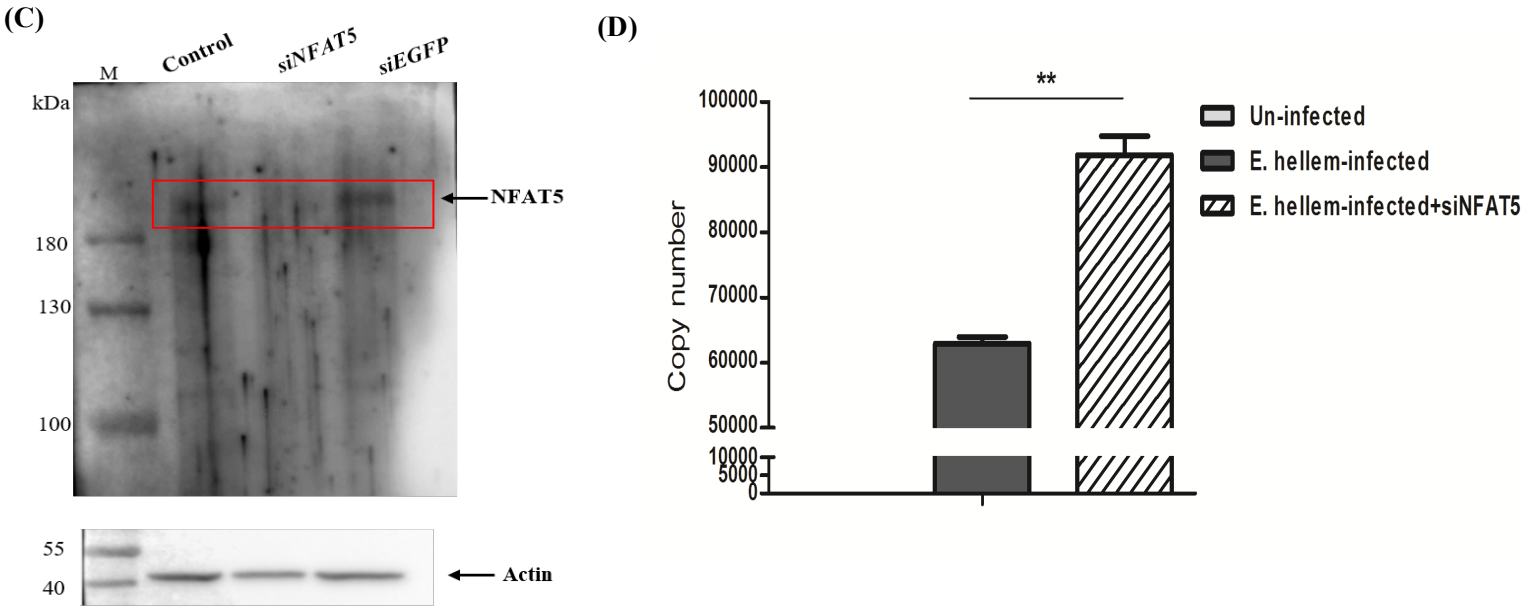


Figure 5. NFAT5/MAPK signal axis is essential during *E. hellem* infection and cellular modulation. (A) Western blot analysis of NFAT5 protein levels in DCs showed that NFAT5 expression was suppressed by *E. hellem* infection, compared to un-infected or S. aureus infection controls. **(B)** Immunofluorescent microscopy of NFAT5 localization in DCs. NFAT5 (Alex Flour 594) is constitutively expressed in the cytoplasm (orange arrows) of un-infected control DCs. The localization of NFAT5 was retained in the cytoplasm (orange arrows) after *E. hellem* infection. The *E. hellem* was able to proliferate in DCs and form parasitophorous vacuole (blue arrows). *S. aureus* infection and osmotic stimulation control (NaCl) were applied to DCs, and both can lead to up-regulation of NFAT5 expressions and re-localization into nucleus (orange arrows). (Scale bar=2 μm). **(C)** Western blot analysis showed that NFAT5 protein levels are significantly down-regulated after RNAi interference. **(D)** qPCR analysis of *E. hellem* proliferation within host DC2.4 cells, as reflected by the copy numbers of *E.hellem* specific *PTP4* (n=8/group; **=P<0.01).

E. hellem Serine/Threonine Protein Phosphatase (PP1) targets DCs p38α (MAPK14)/MAPK

To identify the regulating factors from *E. hellem*, we analyzed *E. hellem*-derived proteins within infected DCs by mass spectrometry (*E. hellem* proteins identified in DCs were listed in supplementary **S-Table 3**). Results revealed that most of these proteins are associated with protein binding, cellular responses and signal transduction. Moreover, we found that the serine/threonine protein phosphatase PP1 is one of the top expressed *E. hellem*-derived proteins in DCs (Representatively identified *E. hellem* derived proteins in **Table 2**).

PP1 is a major Ser/Thr phosphatase and highly conserved in all eukaryotes, and known to regulate p38α/MAPK signaling at several levels including direct interacting with component proteins in the pathway. To verify the direct interactions, we utilized yeast two dihybrid assay, and confirmed that *E. hellem* PP1 directly interact with DCs P38α (MAPK14) (**Fig. 6**). Moreover, we expressed *E. hellem*-derived PP1 in normal DC cells (**Fig.7A**), and proved that the heterologous expressed *E. hellem* PP1 co-localized with host p38α (MAPK14) (**Fig.7B**). In addition, the expressions of representative genes from MAPK pathways such as NFAT5, IL-6, H2Aa and H2Ab were all significantly down-regulated by heterologous expressed *E. hellem* PP1 (**Fig.7C**).

Taken together, this is the first clear evidence of microsporidia-derived proteins directly target and modulate host MAPK signaling pathway, thus affect host immune cell functions. The impaired immune functions of DCs affect both innate and adaptive immune responses and makes host more vulnerable for further pathogen infections and co-infections (**Fig. 8**).

Table 2. Representative *E. hellem* proteins identified in infected DCs

Protein ID	Gene ID	Protein Name/Function
XP_003888309.1	13466767	PP1 serine/threonine phosphatase
XP_003886772.1	13467540	Ras-like GTP binding protein
XP_003887182.1	13468215	GTP-binding nuclear protein
XP_003887892.1	13466917	Rab GTPase
XP_003886950.1	13467595	Beta-tubulin
XP_003886661.1	13466496	Eukaryotic translation initiation factor 2 subunit gamma
XP_003886837.1	13467379	RAD3-like DNA-binding helicase
XP_003887105.1	13467910	Nop56p-like protein
XP_003887061.1	13468065	DNA topoisomerase II
XP_003886739.1	13467235	Dihydrofolate reductase
XP_003887411.1	13467332	Histidyl-tRNA synthetase
XP_003887230.1	13468325	Hypothetical protein
XP_003887352.1	13467170	Hypothetical protein
XP_003887011.1	13467738	Hypothetical protein
XP_003887459.1	13467461	Hypothetical protein

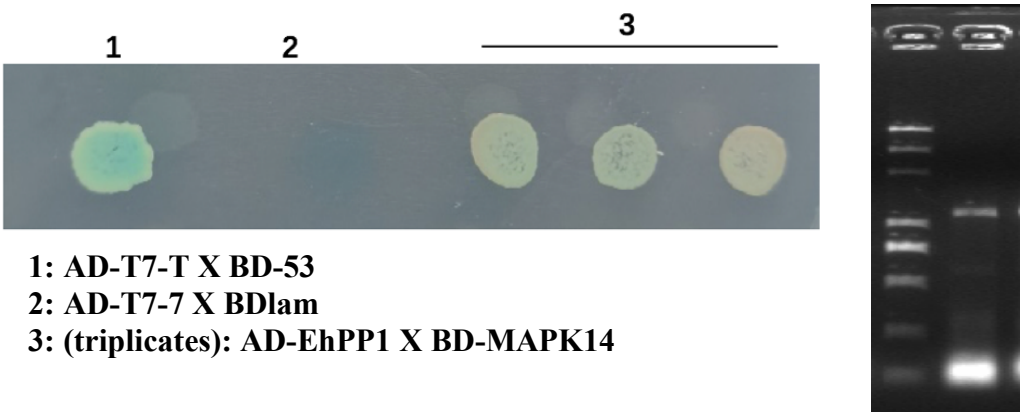
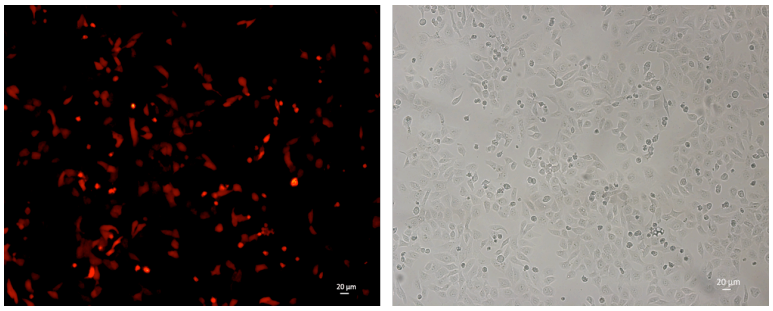
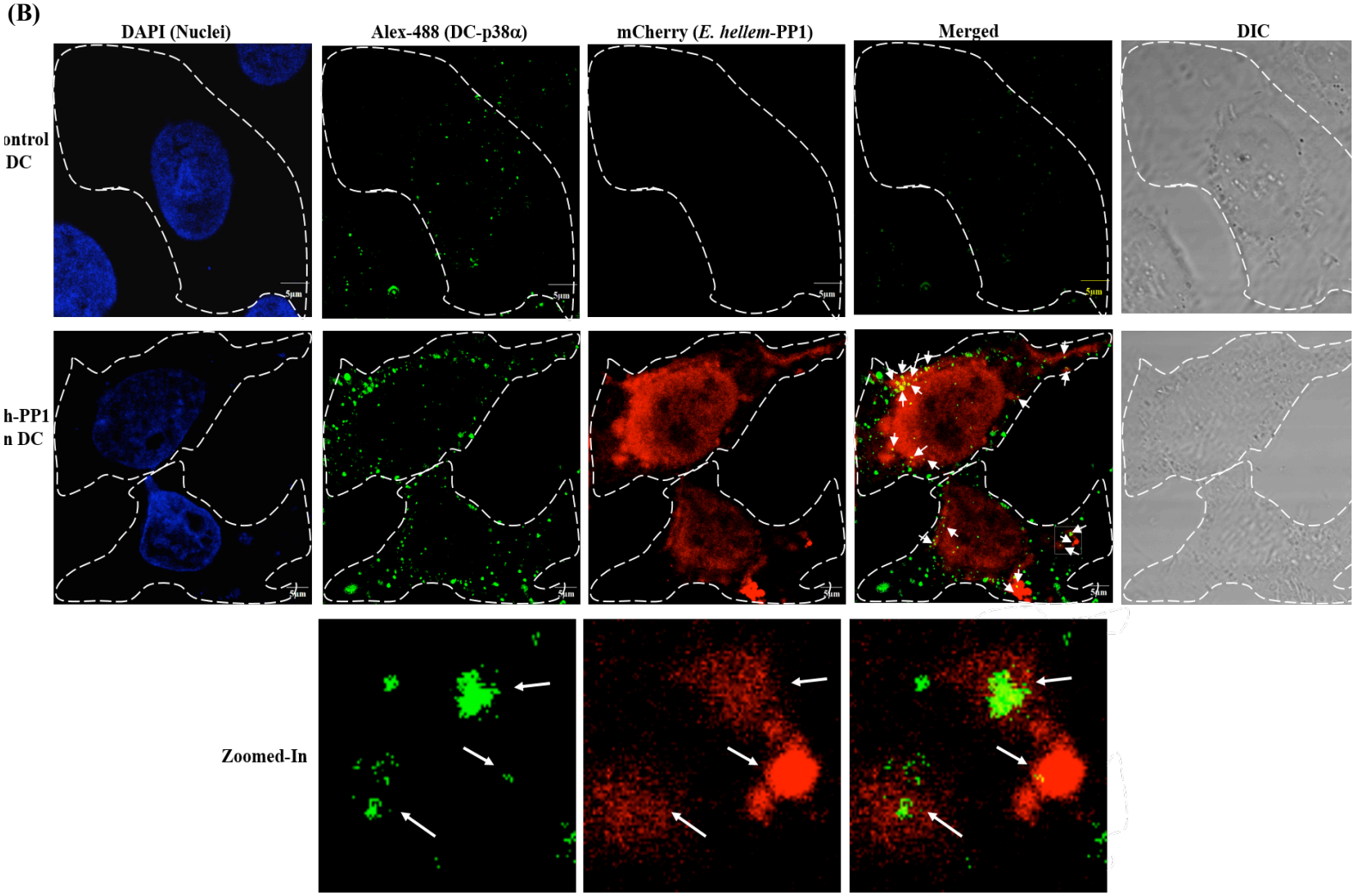


Figure 6. Direct binding of *E. hellem*-PP1 with DC- p38α(MAPK14), shown by yeast two-hybrid assay. DC-MAPK14 was cloned into pGBK7 plasmid (BD-MAPK14), and *E. hellem*- Serine/Threonine Protein Phosphatase PP1 was cloned into pGADT7 plasmid (AD-EhPP1). The plasmids were transformed into competent yeast cells and the binding was validated in synthetic dropout-Leu-Trp -Ade-His medium supplemented with Xα-gal. The fusion strain of pGBKT7-53 with pGADT7-T was used as the positive control, fusion strain of pGBKT7-lam with pGADT7-T were used as the negative controls. The EhPP1 and MAPK14 fused clones were subjected to PCR and gel electrophoresis to confirm the existence of target sequences.

(A)



(B)


DAPI (Nuclei)
Alex-488 (DC-p38α)
mCherry (E. hellem-PP1)
Merged
DIC
ontrol
DC
h-PP1
n DC
Zoomed-In

(C)

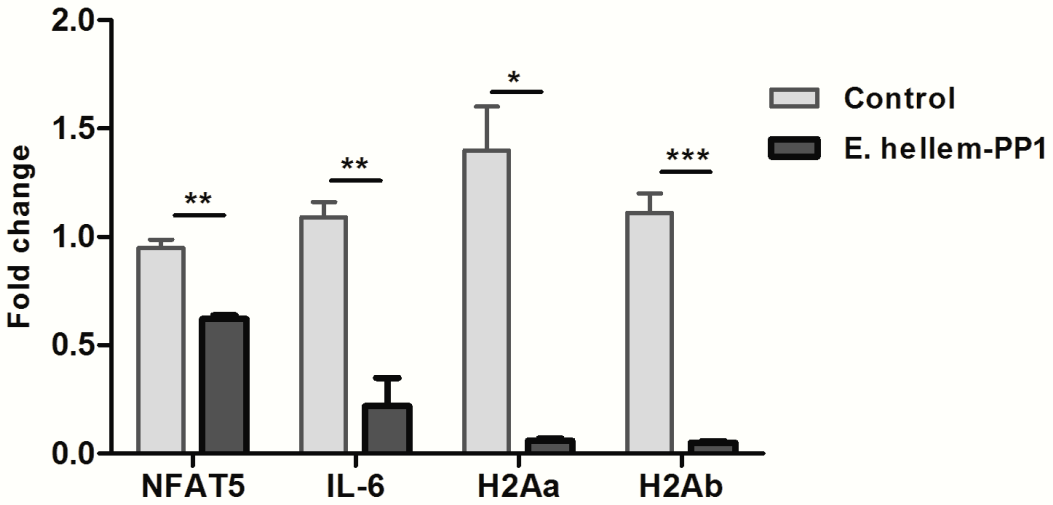

Fold change
2.0
1.5
1.0
0.5
0.0
**
**
*
***
Control
E. hellem-PP1
NFAT5
IL-6
H2Aa
H2Ab

Figure 7. Direct interaction of *E. hellem*-PP1 with DC- p38α (MAPK14) and the down-regulated MAPK pathway genes expressions. (A) Expressions of *E. hellem*-PP1 in DCs. Fluorescent microscopy confirmed the expression of *E. hellem* derived PP1 (pCMV-mCherry-PP1) in the cytoplasm of DCs (red color). (Scale bar=20 μm). **(B)** Immunofluorescent microscopy confirmed the co-localization of *E. hellem*-PP1 with DCs p38α (MAPK14). Heterologous expressed *E. hellem* PP1 (mCherry, red color) is expressed in the cytoplasm of DCs. The DCs p38a(MAPK14) is labeled as Alex Flour 488 (green color) and is constitutively expressed in the cytoplasm of DCs. *E. hellem*-PP1 co-localize with DCs p38α (MAPK14) (arrows). (Scale bar=5 μm). **(C)** qPCR analysis of DCs *NFAT5, IL-6, H2Aa* and *H2Ab* of MHC-II complex. All these genes were significantly down-regulated when DCs have heterologous expressed *E. hellem* PP1 (n=12/group; *=P<0.05, **=P<0.01, ***=P<0.001).

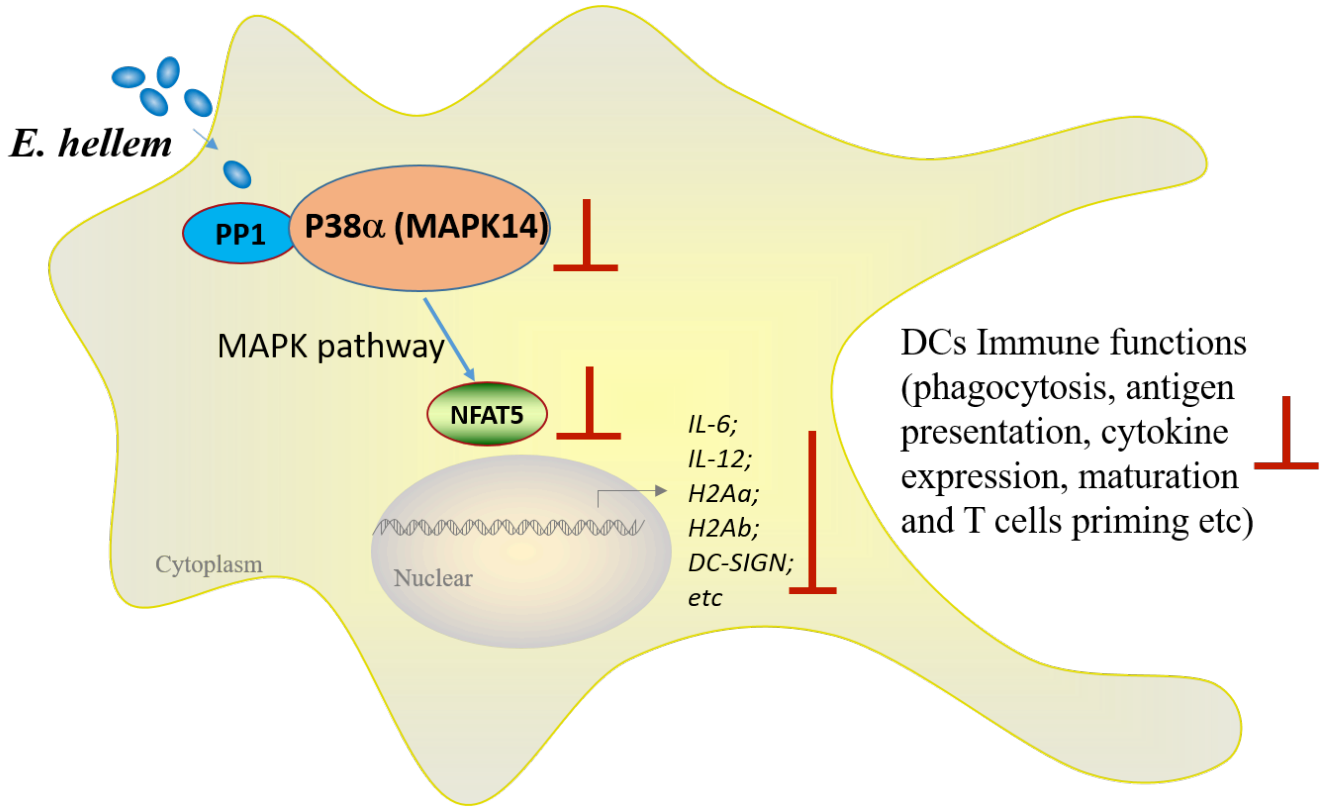


Figure 8. Image illustration and summary of *E. hellem* expressed the Serine/Threonine Protein Phosphatase PP1, directly interacted with DCs MAPK14. The direct interaction would interfere normal functions of MAPK14, and the subsequent transduction of the MAPK pathway therefore the downstream transcriptional factor NFAT5 expression and localization were disrupted. Eventually various immune related genes of DCs were down-regulated and the immune functions of DCs were severely impaired.

Discussions

Our study is the first to elucidate the molecular mechanism of microsporidia manipulation of host dendritic cells, and more importantly to prove the damaging effects of microsporidia persistence to the immune systems and the increased chances of being co-infected by other pathogens. In this study, we used the frequently diagnosed and zoonotic species of microsporidia, *E. hellem* as the pathogen. In the mesenteric lymph nodes of *E. hellem*-infected C57BL/6 mice, we found that dendritic cells (DCs), but not monocytes nor lymphocytes, were the most affected immune cells group. Analysis of immune cells in the spleen further confirmed that the DCs' immune functions were severely detained and impaired after *E. hellem* infection, as reflected by the phagocytosis ability, maturation process, cytokine expressions, and T cell priming potentials. *In vitro* and *in vivo* infection models both confirmed that, not only the priming process of DCs to T cells were inhibited, but also the *E. hellem* infection makes the mice more vulnerable to further infections as reflected by more weight change and slower recovery rate. To identify the regulation mechanisms, mass spectrometry and yeast two hybrid analysis applied and demonstrated that the DCs p38α(MAPK14)/NFAT-5 axis of the MAPK signaling pathway was the key mediator, and *E. hellem* serine/threonine protein phosphatase PP1 directly interact with p38α(MAPK14) to manipulate DCs immune functions. RNAi assays showed that knocking-down of transcriptional factor NAFT5 in the p38α(MAPK14)/NFAT-5 axis lead to increased proliferation of *E. hellem* within host, confirming the essential role of MAPK signaling pathways during pathogen-host interactions and providing possible targets of disease prevention and control.

The MAPK singling pathway is known for its important roles in innate immune responses by regulating the production of inflammatory and anti-inflammatory cytokines, innate immune cell viability, and the function of antigen-presenting cells (APCs) (40). We demonstrated by mass spectrometry that many proteinases in the pathway were disturbed after *E. hellem* infection, such as p38α(MAPK14), MAPK1, Map2k3 and many of them cross-interact with each other. Therefore, the pathogen could target any of them to manipulate the whole signal transduction pathway. In fact, we proved the direct interaction between pathogen-derived Serine/threonine-protein phosphatase 1 (PP1) with p38α(MAPK14). The direct interaction indeed impaired the whole transduction process and the downstream outcomes, as we demonstrated in this study that the expressions of transcriptional factors and pro-inflammatory genes/cytokines of DCs were severely detained or inhibited. Beside the proteins in the MAPK pathway, we also identified other differentially expressed proteins such as Neurabin-2, ribosomal protein S6 kinase alpha-1, E3 ubiquitin-protein ligase TRAF7 and so on. These proteins are functioning as scaffold proteins or in other cellular pathways such ERK and ubiquitination (41-43). It's thus reasonable to infer that these different signaling pathways were cross-linked together in responding to *E. hellem* infection and modulation. In addition to cross-talks, we are also very interested to identify the which pattern recognition receptors were responsible for reception of *E. hellem* and activation of MAPK signaling in the upstream. Actually,

the mass spectrometry analysis provided some candidates such as the Integrin-linked protein kinase, which is and adaptor of integrin-related signal reception and transduction(44).

The essential roles and the manipulations of DCs during *E. hellem*-host interactions have been elucidated in our study. Yet, we should not neglect the involvement of other immune cells and processes. For instance, the involvement and protective roles of CD8+ T cells against microsporidia infection have been demonstrated in a mouse model study (45). These findings were in accordance with our findings that, when the T cell priming capabilities were disrupted in DCs, less of effector T cells such CD8+T cells would be stimulated therefore the host had worse outcomes from *E. hellem* infection and increased susceptibility to co-infecting secondary pathogens. In addition, the infection of microsporidia in other immune cells may also manipulate normal cell functions. For instances, it's known that p38α(MAPK14) regulates oncogenic process, autophagy and apoptosis of many cells such as inflammatory monocytes and B lymphocytes (46, 47). The microsporidia persistence within those cells may not affect immune responses as they do in DCs, but arose other problems such as increased autophagy or apoptosis so that the whole immune systems were compromised. Therefore, it's of great importance to fully investigate the effects of microsporidia on host cells so that to prevent microsporidiosis and consequent co-infections.

Serine/threonine-protein phosphatase 1 (PP1) is a highly conserved protein phosphatase in all eukaryotes, which regulates critical cellular processes including cell cycle progression, apoptosis and metabolism. In mammalian cells, the involvement of PP1 in several oncogenic pathways has become evident, and has been recognized as a potential drug target in cancer (48). In eukaryotic pathogens, the important regulation roles of PP1 are getting more attentions in recent years. For instance, PP1 have been found to regulate pathogen cell maturation, proliferation and metabolism (49, 50). As the result, the existence of pathogen-derived PP1 within host cells may be of great importance for both pathogen cells' intracellular proliferation as well as host cell manipulation. Our study is the first and very excited to identified the *E. hellem*-derived Serine/threonine-protein phosphatase 1 (PP1) and verified the direct interaction with host p38α (MAPK14) in the MAPK signaling pathway. Interestingly, it's reported that PP1 would also interact and dephosphorylate RPS6KB1, homologous to our identified ribosomal protein S6 kinase alpha-1. Considering the intrinsic cross-talks and links of these host proteins, it will be not surprising to identify multiple effects of microsporidia derived PP1 on host immune responses and other cellular processes in future studies.

In the era of wide-spreading emerging pathogens, shortage of antibiotics and aging of populations, the co-existence and co-infections of multiple pathogens would become great public health threats. Microsporidia exist widely in nature, caused nearly no symptoms when persist in host. But our study firstly pointed out the persistence of microsporidia within host would bring damaging outcomes, as they impair host's innate as well as adaptive immunities and increase host susceptibility to co-infections. Therefore, microsporidia detection, prevention and control should get enough attentions in future.

Author Contributions

JB designed the study and conducted experiments, interpreted the data, and wrote the manuscript. JJ, YT, XW, YC, LC, GA, BM, HZ participated in conducting experiments and analysis of data. GC contributed to study design and manuscript editing. GP and ZY contributed in manuscript editing. All authors contributed to the article and approved the submitted version.

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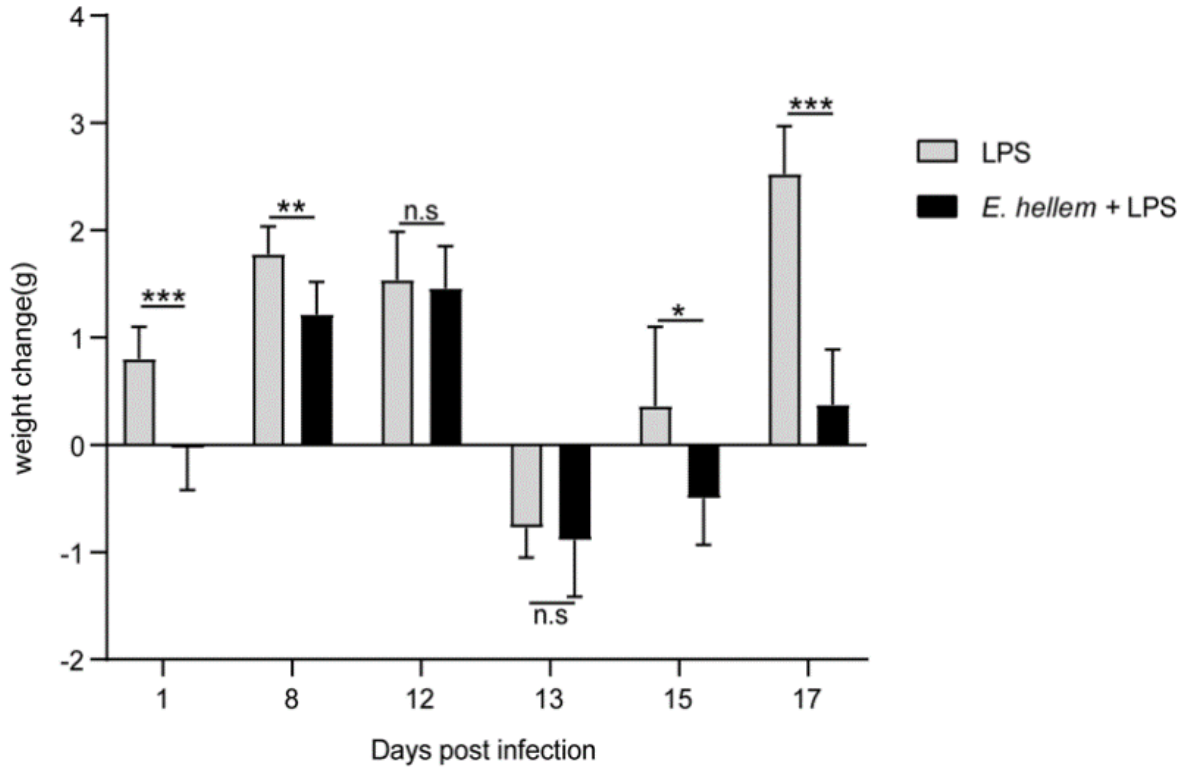
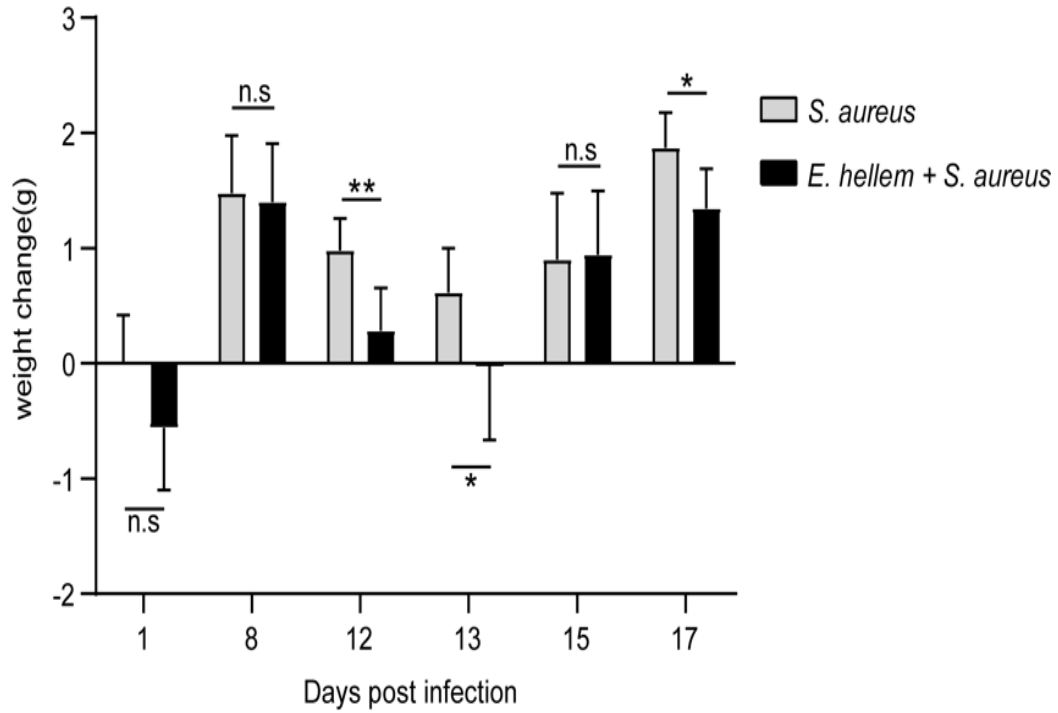
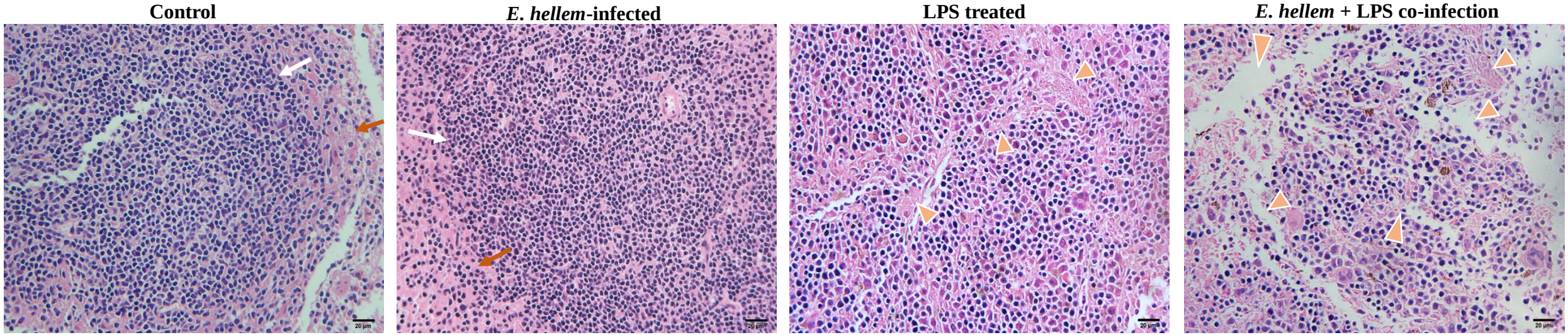
(A)


4
3
2
1
0
-1
-2
weight change(g)
***
**
n.s
n.s
*
***
1
8
12
13
15
17
Days post infection
LPS
E. hellem + LPS

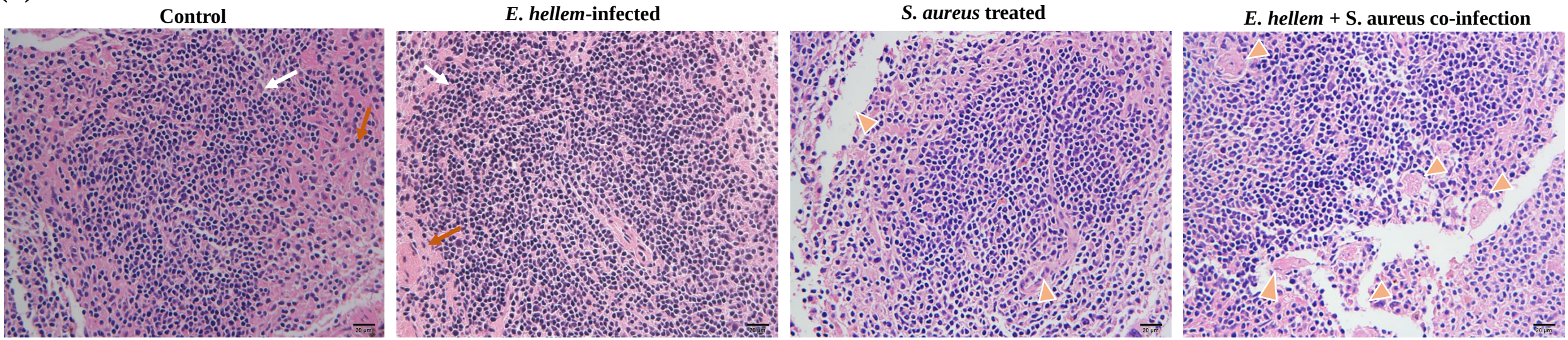
(B)


3
2
1
0
-1
-2
weight change(g)
n.s
n.s
**
*
n.s
*
1
8
12
13
15
17
Days post infection
S. aureus
E. hellem + S. aureus

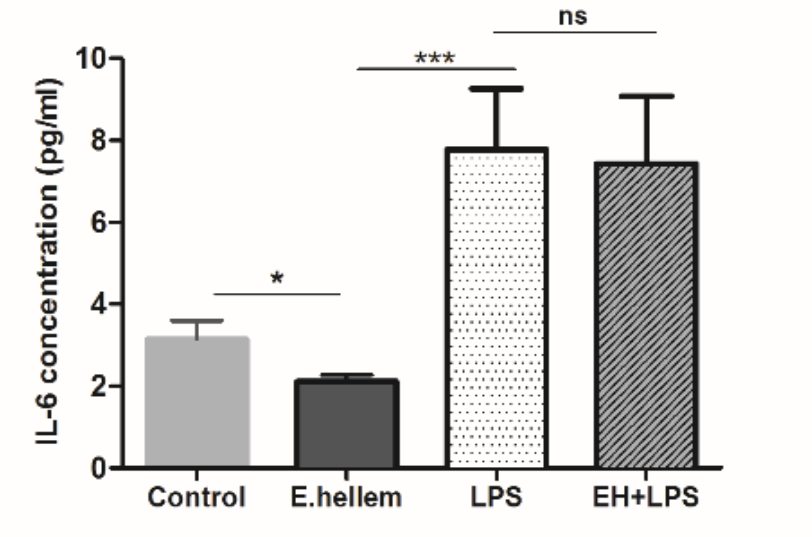
(C)


Control
E. hellem-infected
LPS treated
E. hellem + LPS co-infection

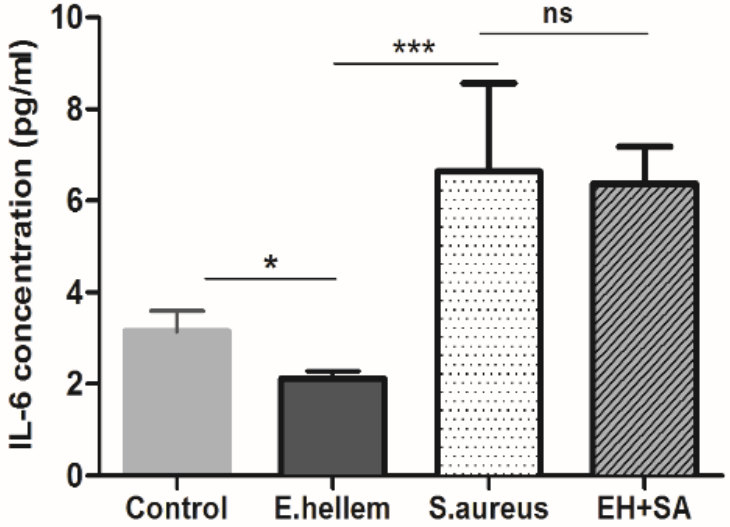
(D)


Control
E. hellem-infected
S. aureus treated
E. hellem + S. aureus co-infection

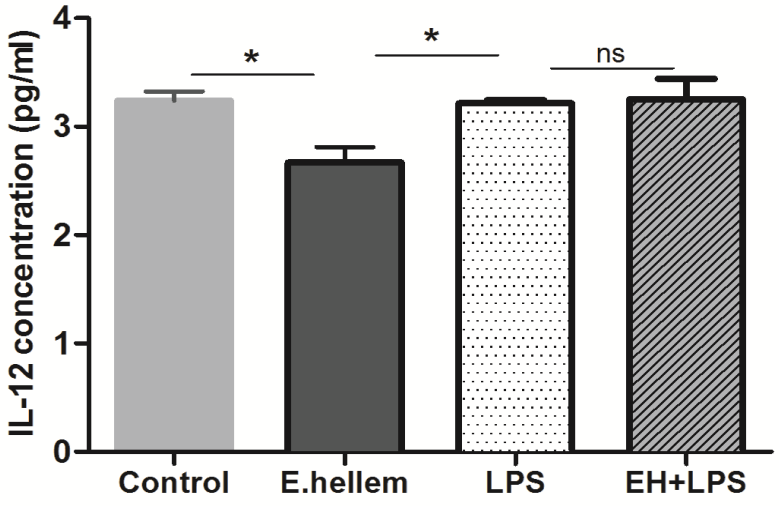
(E)


IL-6 concentration (pg/ml)
10
8
6
4
2
0
*
***
ns
Control
E.hellem
LPS
EH+LPS

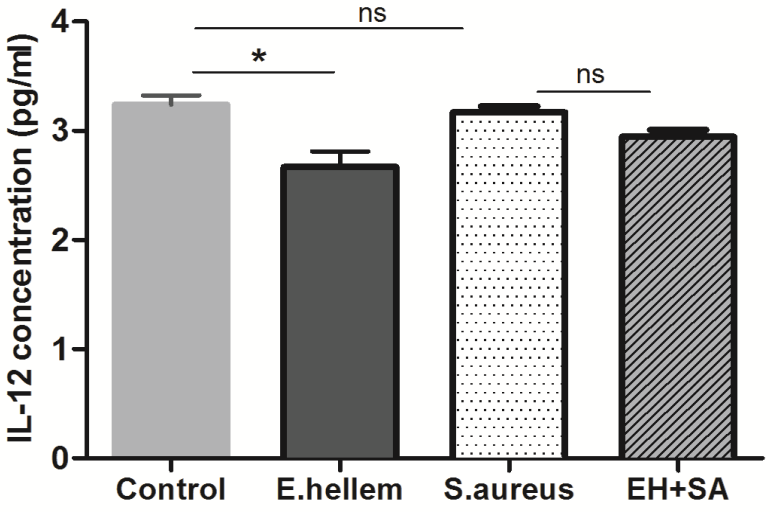
(F)


IL-6 concentration (pg/ml)
10
8
6
4
2
0
*
***
ns
Control
E.hellem
S.aureus
EH+SA

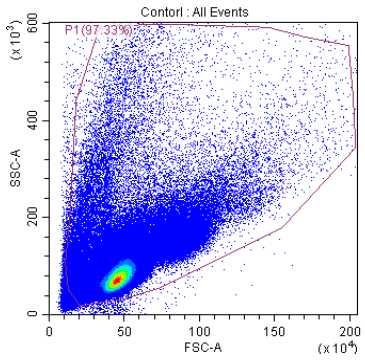
(G)

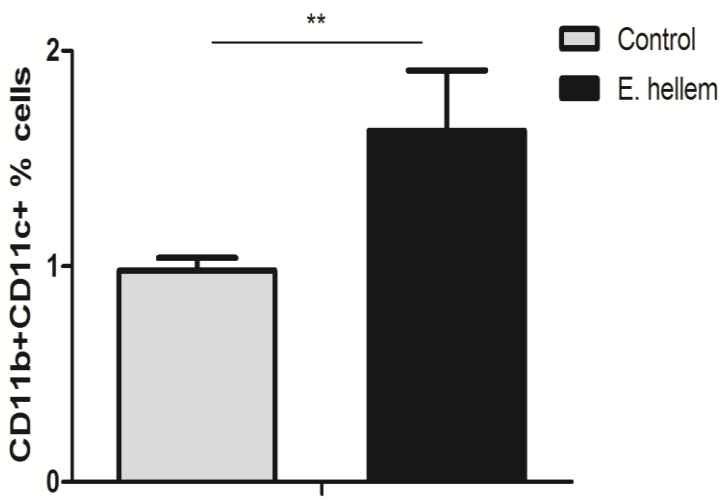

IL-12 concentration (pg/ml)
4
3
2
1
0
*
*
ns
Control
E.hellem
LPS
EH+LPS

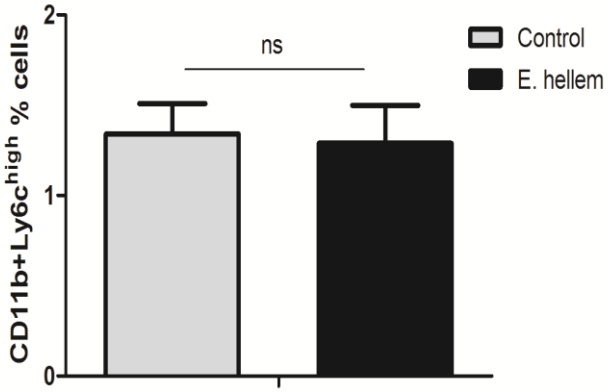
(H)

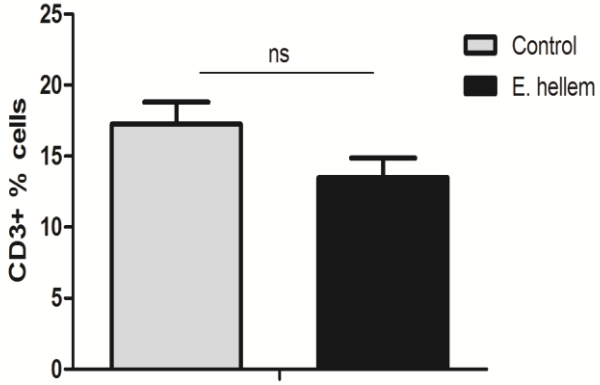

IL-12 concentration (pg/ml)
4
3
2
1
0
ns
*
ns
Control
E.hellem
S.aureus
EH+SA

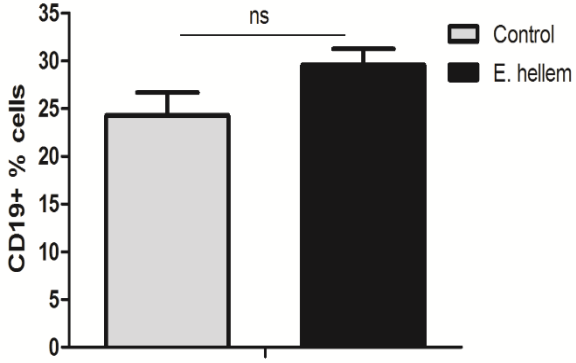
(I)


Contorl : All Events
P1(97.33%)
SSC-A
FSC-A
(x10³)
(x10⁴)


**
Control
E. hellem
CD11b+CD11c+ % cells


ns
Control
E. hellem
CD11b+Ly6c^high % cells

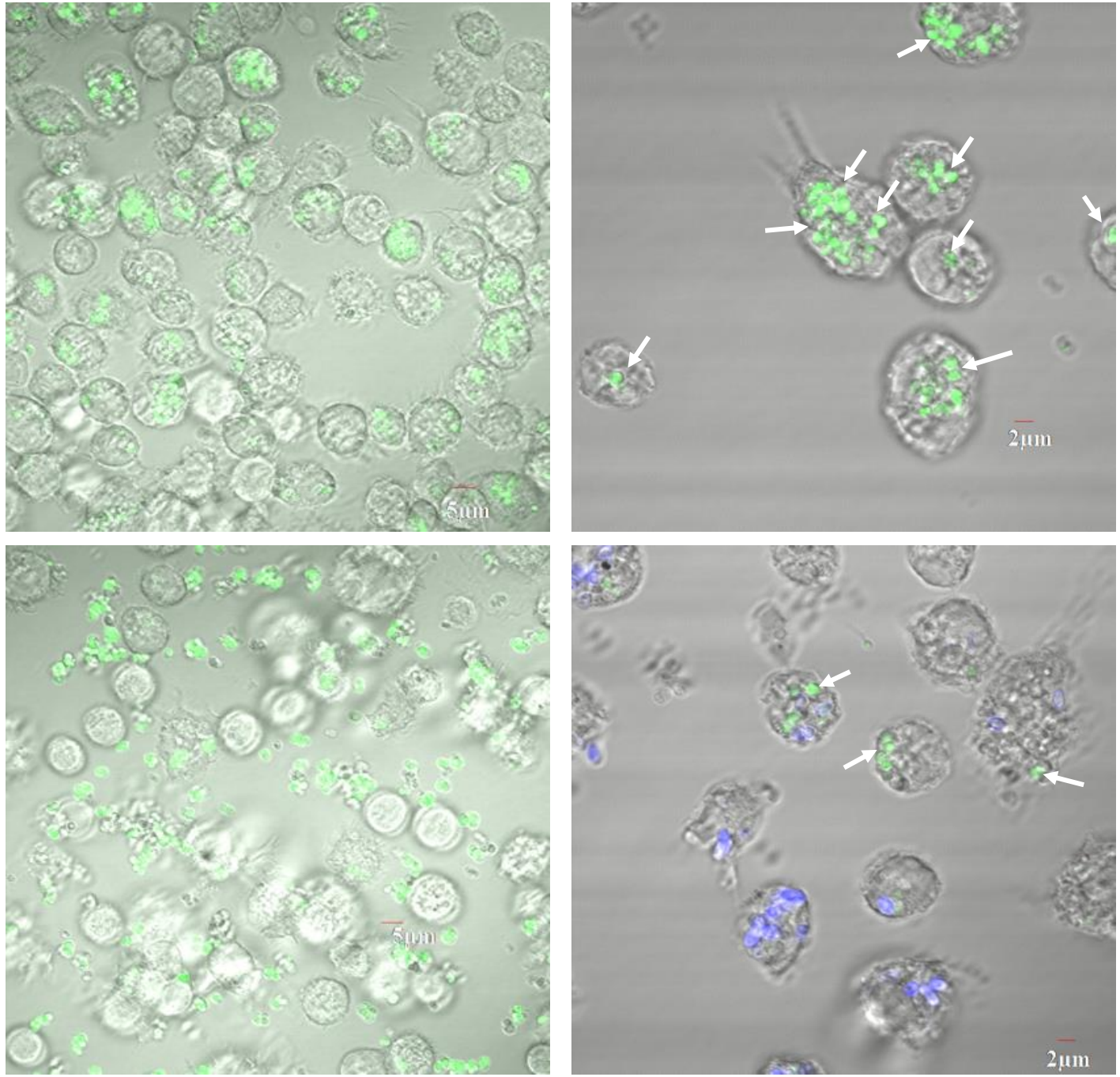

ns
Control
E. hellem
CD3+ % cells

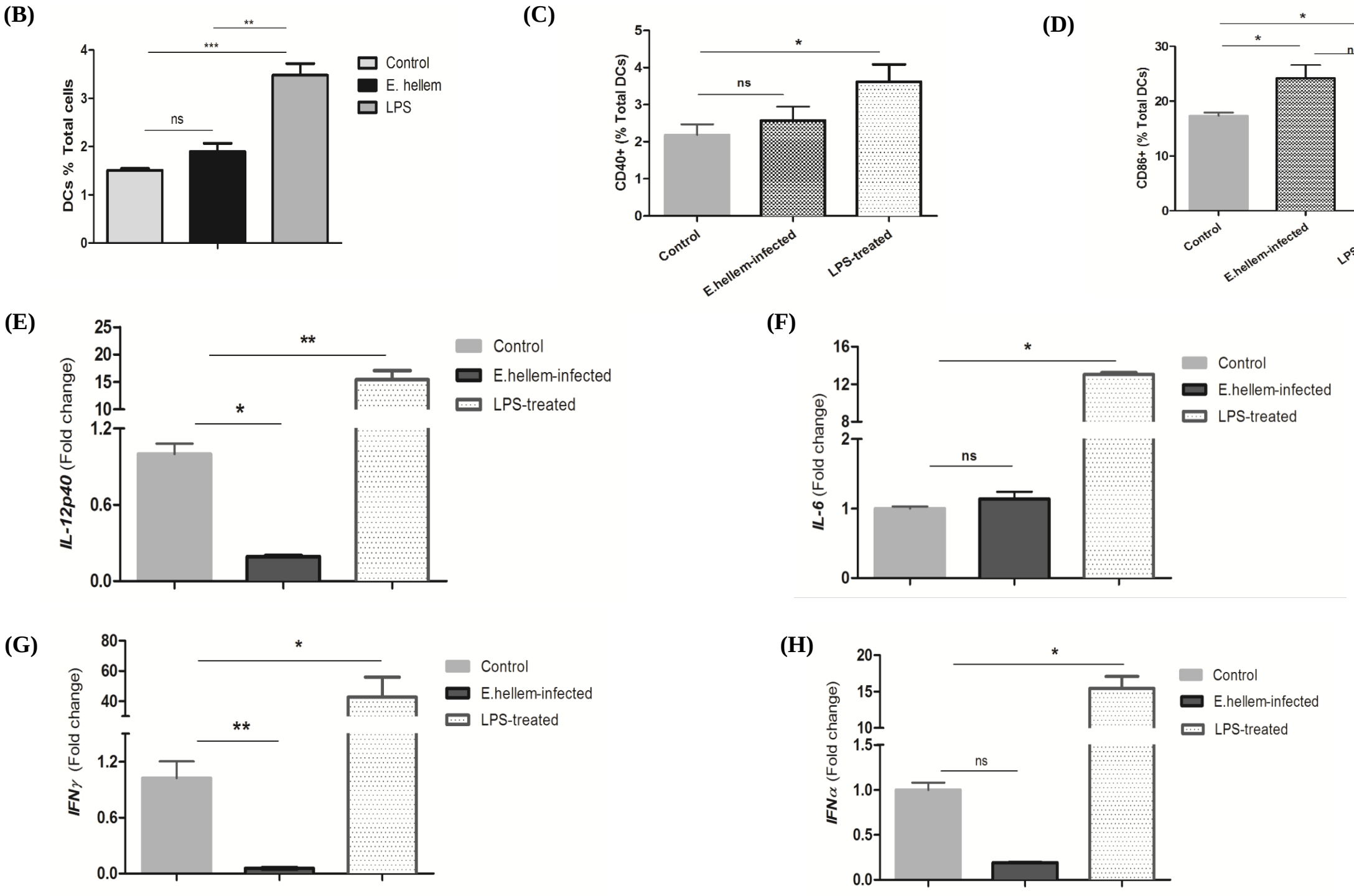

ns
Control
E. hellem
CD19+ % cells

(A)

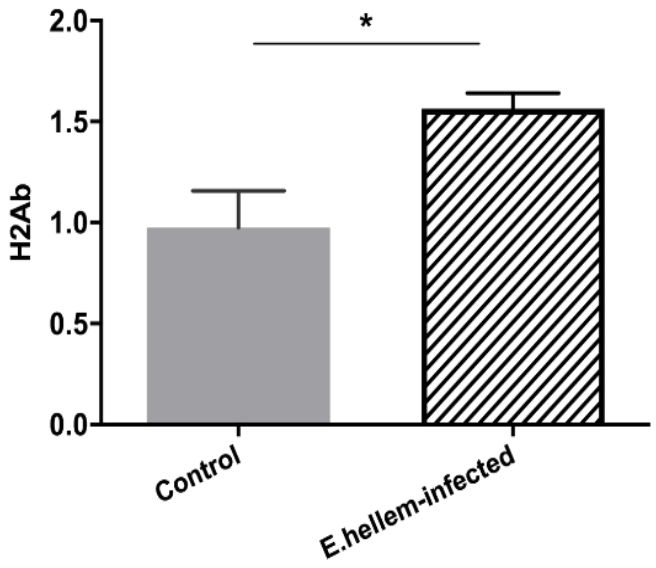
Un-infected control

***E. hellem*-infected**

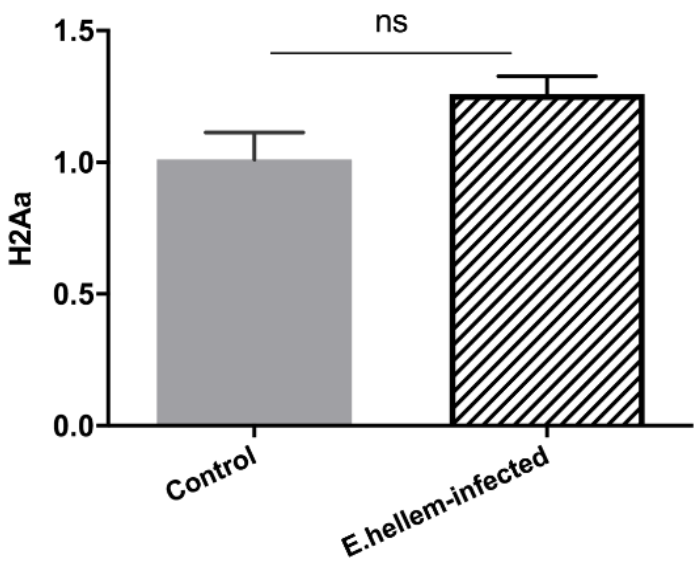



(B)
DCs % Total cells
Control
E. hellem
LPS
ns
***
**
(C)
CD40+ (% Total DCs)
Control
E.hellem-infected
LPS-treated
ns
*
(D)
CD86+ (% Total DCs)
Control
E.hellem-infected
LPS-treated
*
ns
(E)
IL-12p40 (Fold change)
Control
E.hellem-infected
LPS-treated
*
**
(F)
IL-6 (Fold change)
Control
E.hellem-infected
LPS-treated
ns
*
(G)
IFNγ (Fold change)
Control
E.hellem-infected
LPS-treated
**
*
(H)
IFNα (Fold change)
Control
E.hellem-infected
LPS-treated
ns
*

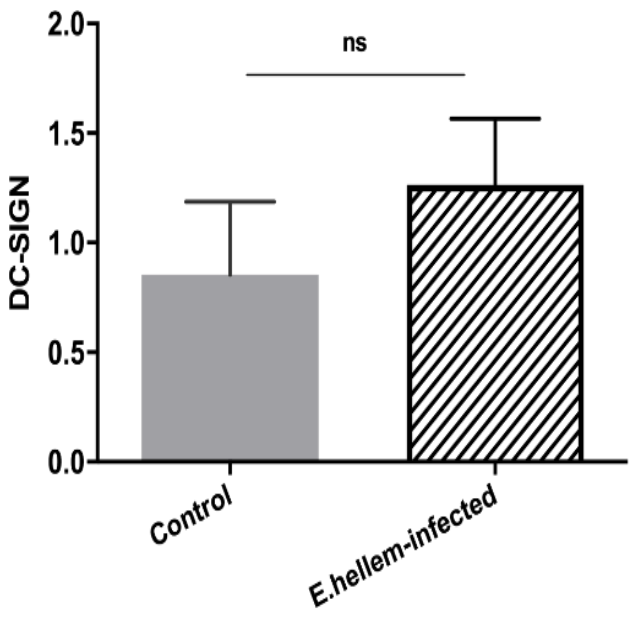
(A)


*
2.0
1.5
1.0
0.5
0.0
H2Ab
Control
E.hellem-infected

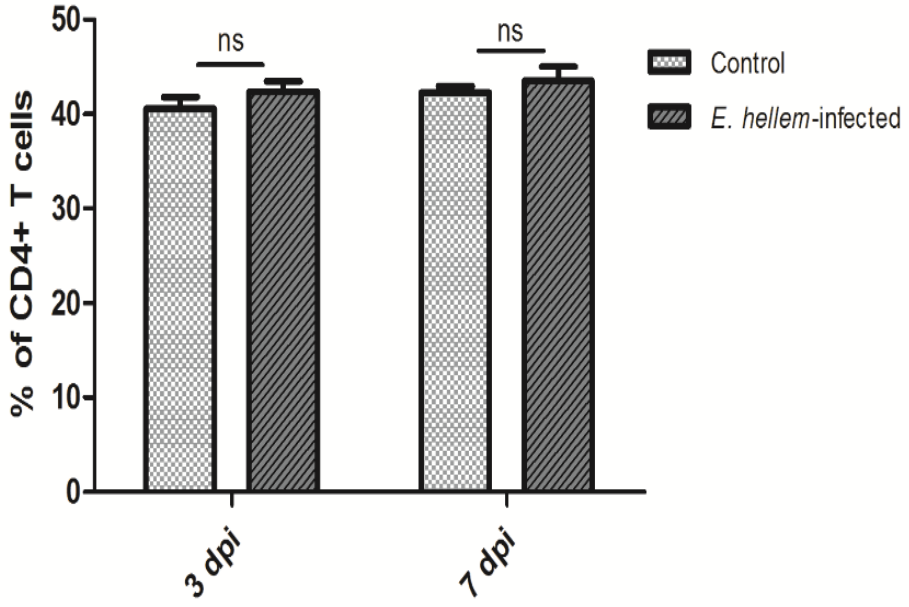
(B)


ns
1.5
1.0
0.5
0.0
H2Aa
Control
E.hellem-infected

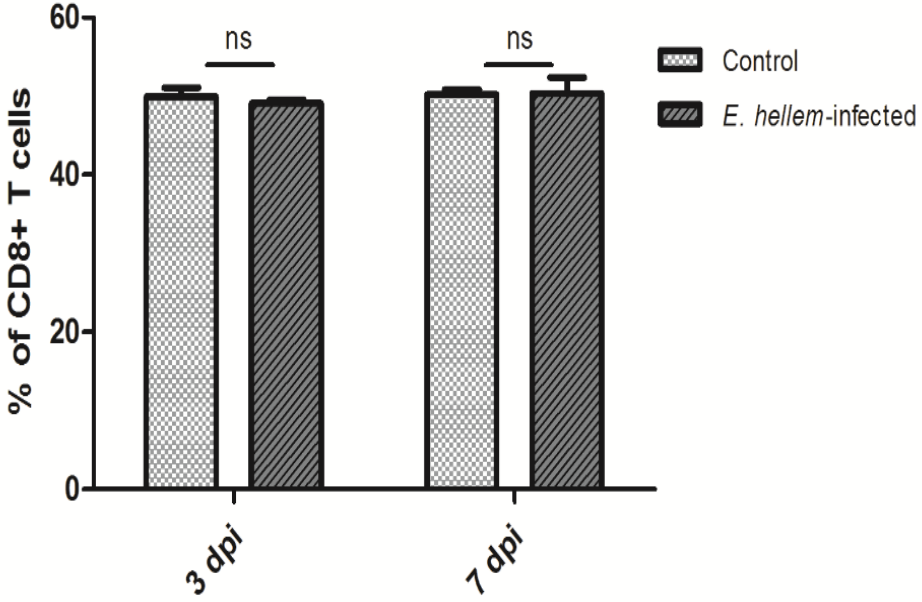
(C)


ns
2.0
1.5
1.0
0.5
0.0
DC-SIGN
Control
E.hellem-infected

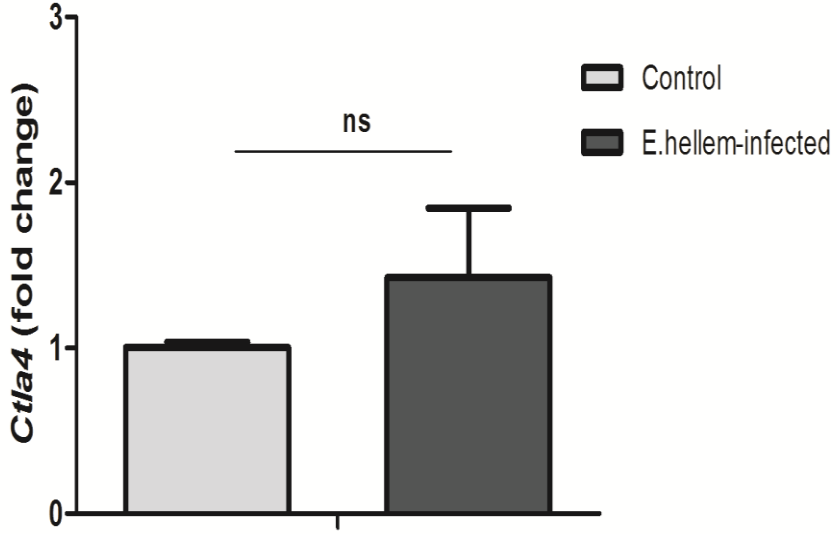
(D)


% of CD4+ T cells
50
40
30
20
10
0
ns
ns
3 dpi
7 dpi
Control
E. hellem-infected

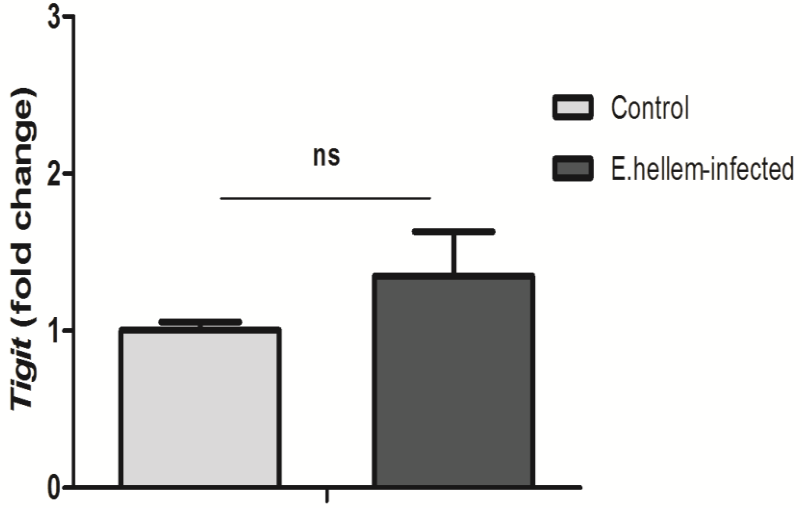
(E)


% of CD8+ T cells
60
40
20
0
ns
ns
3 dpi
7 dpi
Control
E. hellem-infected

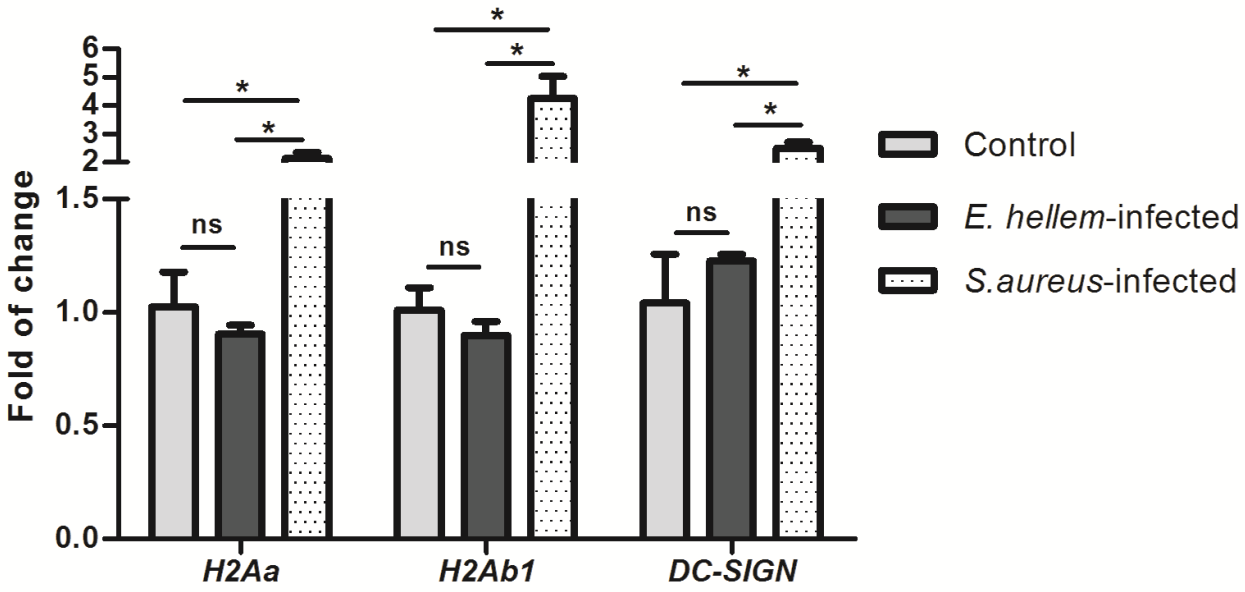
(F)


Ctla4 (fold change)
3
2
1
0
ns
Control
E.hellem-infected

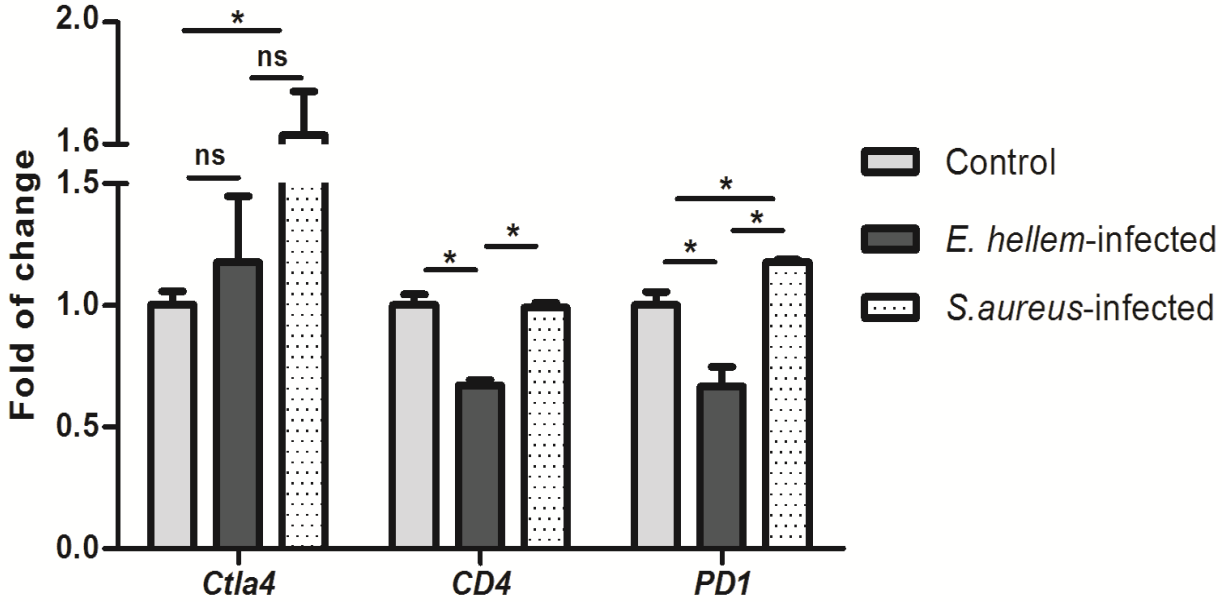
(G)


Tigit (fold change)
3
2
1
0
ns
Control
E.hellem-infected

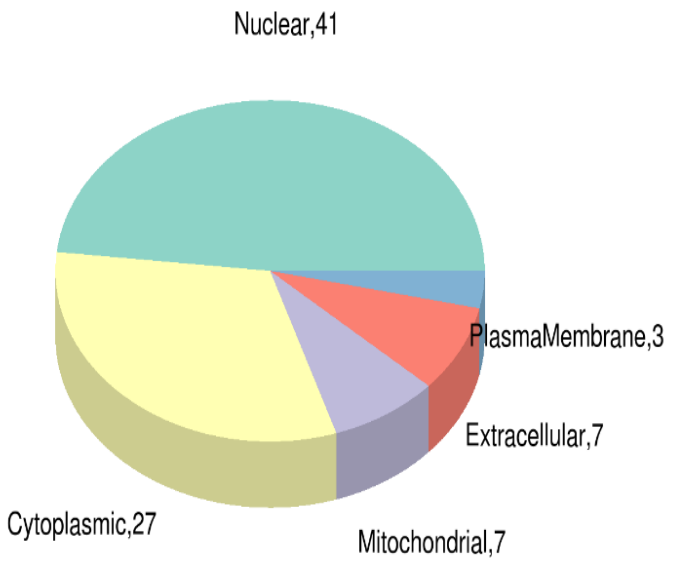
(H)


Fold of change
6
5
4
3
2
1.5
1.0
0.5
0.0
*
*
ns
*
*
ns
*
*
ns
H2Aa
H2Ab1
DC-SIGN
Control
E. hellem-infected
S.aureus-infected

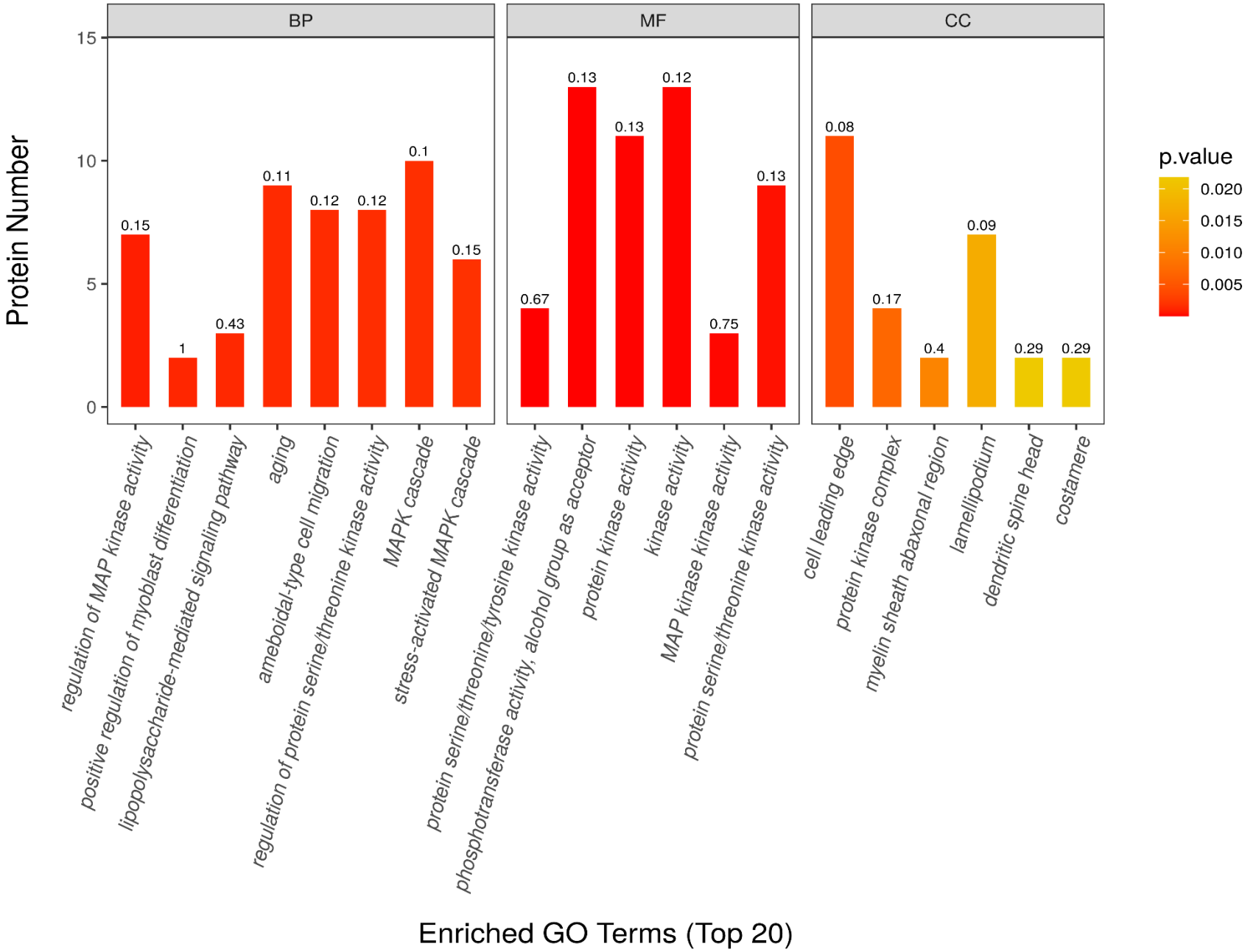
(I)


Fold of change
2.0
1.6
1.5
1.0
0.5
0.0
*
ns
ns
*
*
*
*
*
Ctla4
CD4
PD1
Control
E. hellem-infected
S.aureus-infected

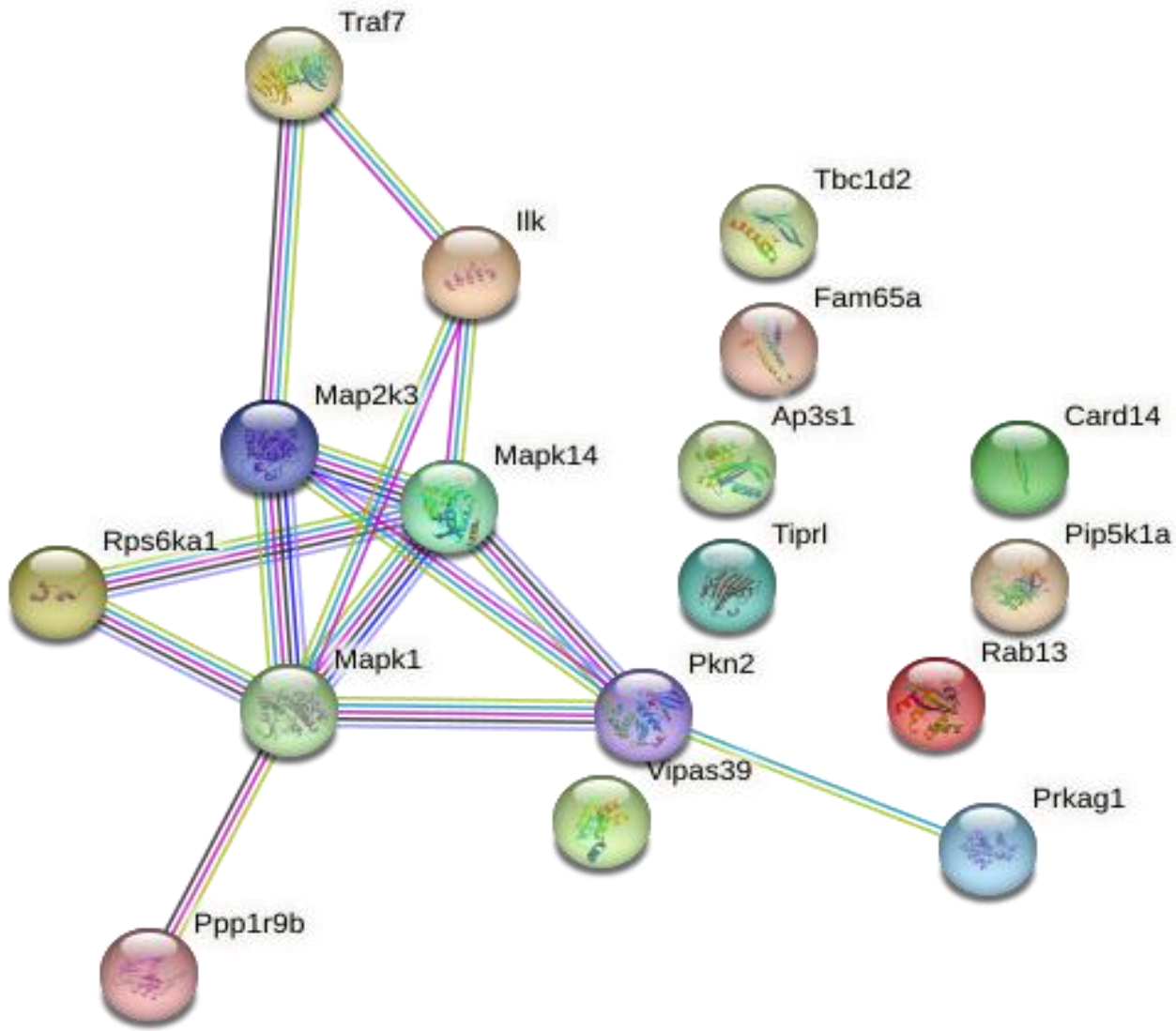
(A)


Nuclear,41
PlasmaMembrane,3
Extracellular,7
Mitochondrial,7
Cytoplasmic,27

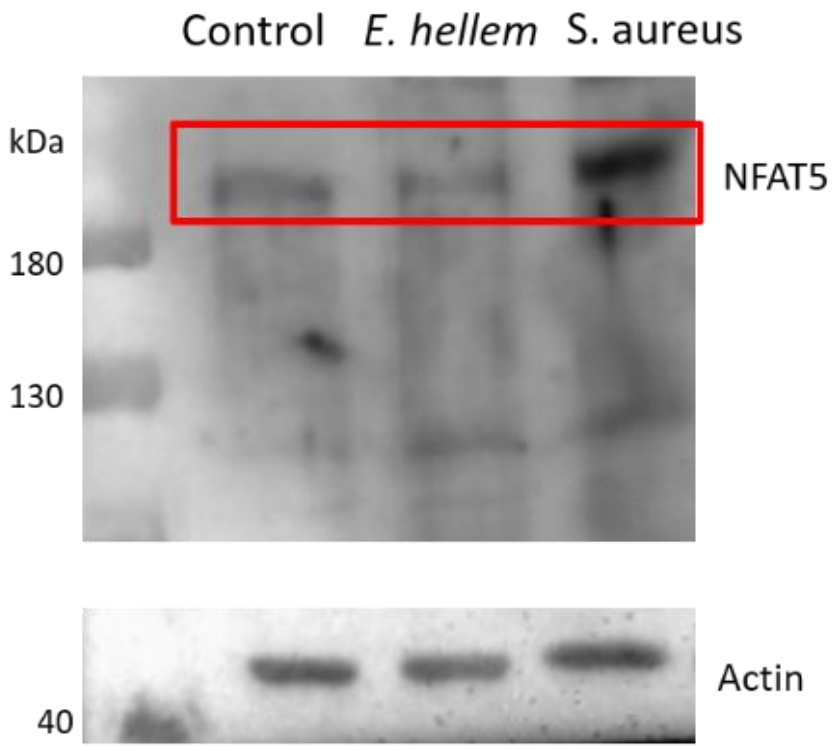
(B)

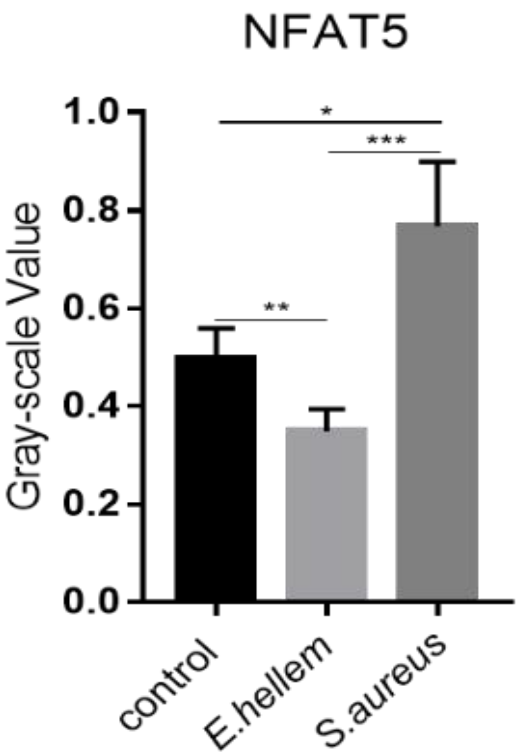

BP
MF
CC
Protein Number
0
5
10
15
0.15
1
0.43
0.11
0.12
0.12
0.1
0.15
0.67
0.13
0.13
0.12
0.75
0.13
0.08
0.17
0.4
0.09
0.29
0.29
regulation of MAP kinase activity
positive regulation of myoblast differentiation
lipopolysaccharide-mediated signaling pathway
aging
ameboidal-type cell migration
regulation of protein serine/threonine kinase activity
MAPK cascade
stress-activated MAPK cascade
protein serine/threonine/tyrosine kinase activity
phosphotransferase activity, alcohol group as acceptor
protein kinase activity
kinase activity
MAP kinase kinase activity
protein serine/threonine kinase activity
cell leading edge
protein kinase complex
myelin sheath abaxonal region
lamellipodium
dendritic spine head
costamere
p.value
0.020
0.015
0.010
0.005
Enriched GO Terms (Top 20)

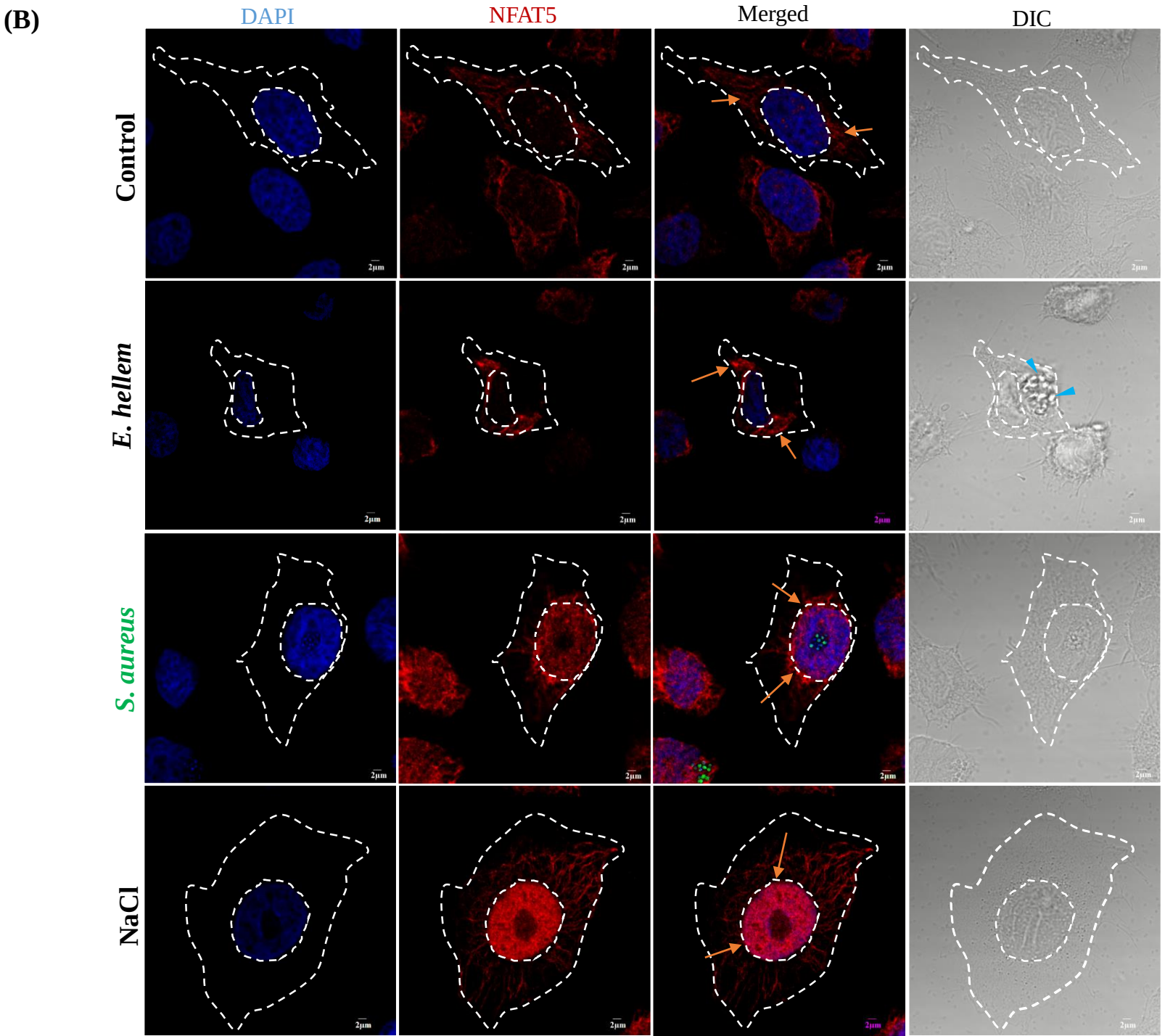
(C)


Traf7
Ilk
Tbc1d2
Fam65a
Map2k3
Ap3s1
Card14
Mapk14
Tiprl
Pip5k1a
Rps6ka1
Rab13
Mapk1
Pkn2
Vipas39
Prkag1
Ppp1r9b

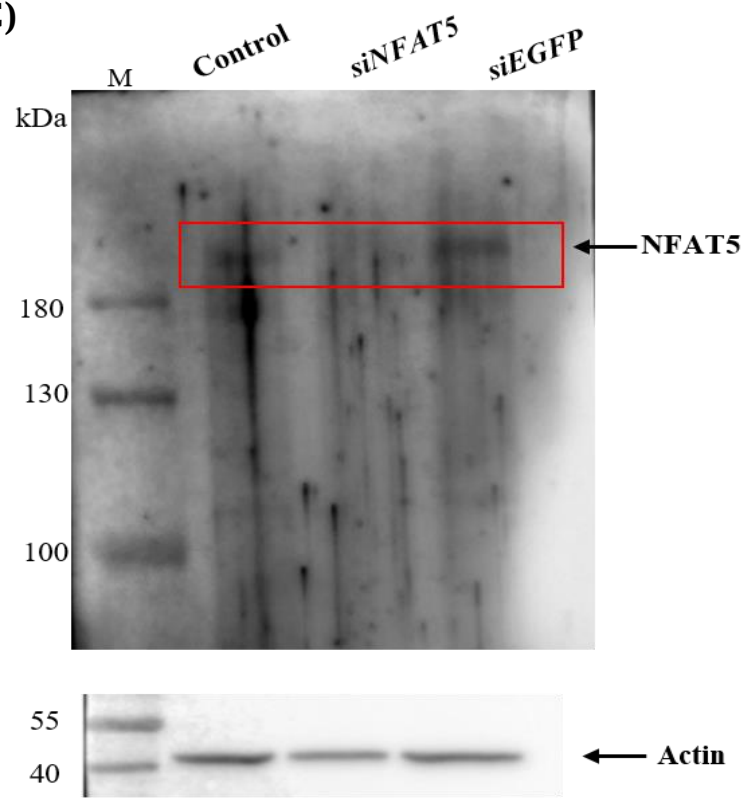
(A)


Control
E. hellem
S. aureus
kDa
180
130
40
NFAT5
Actin


NFAT5
Gray-scale Value
1.0
0.8
0.6
0.4
0.2
0.0
*
***
**
control
E.hellem
S.aureus

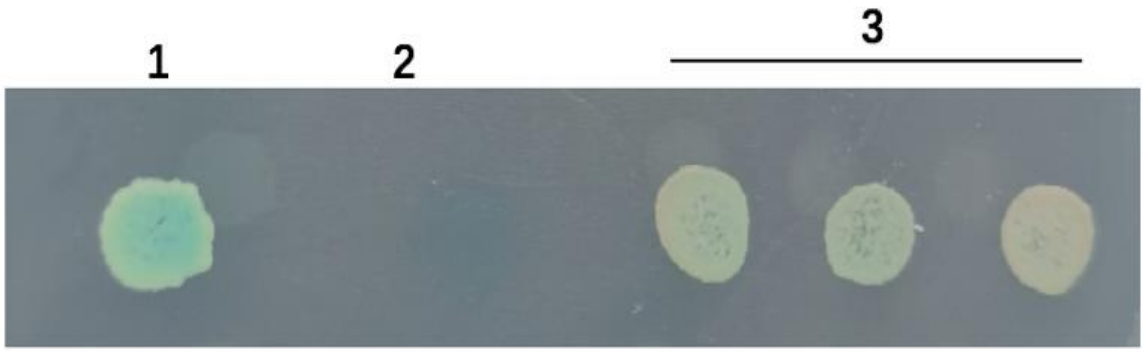

(B)
DAPI
NFAT5
Merged
DIC
Control
E. hellem
S. aureus
NaCl

(C)

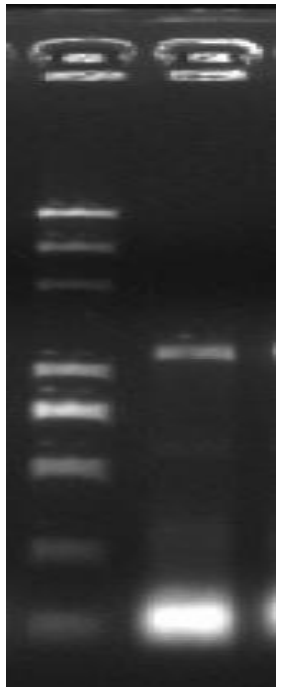

M
Control
siNFAT5
siEGFP
kDa
NFAT5
180
130
100
55
40
Actin

(D)

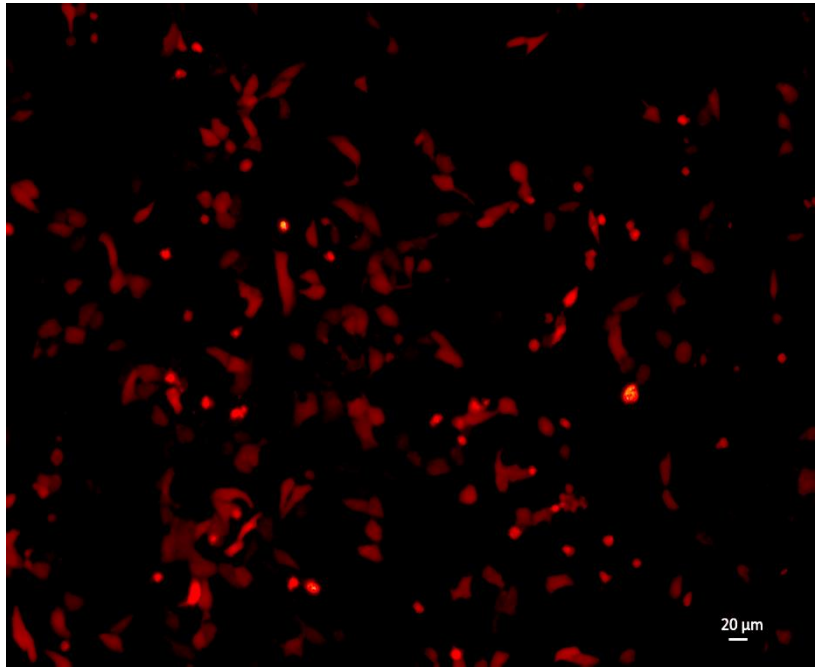
**
Copy number
100000
90000
80000
70000
60000
50000
10000
5000
0
Un-infected
E. hellem-infected
E. hellem-infected+siNFAT5

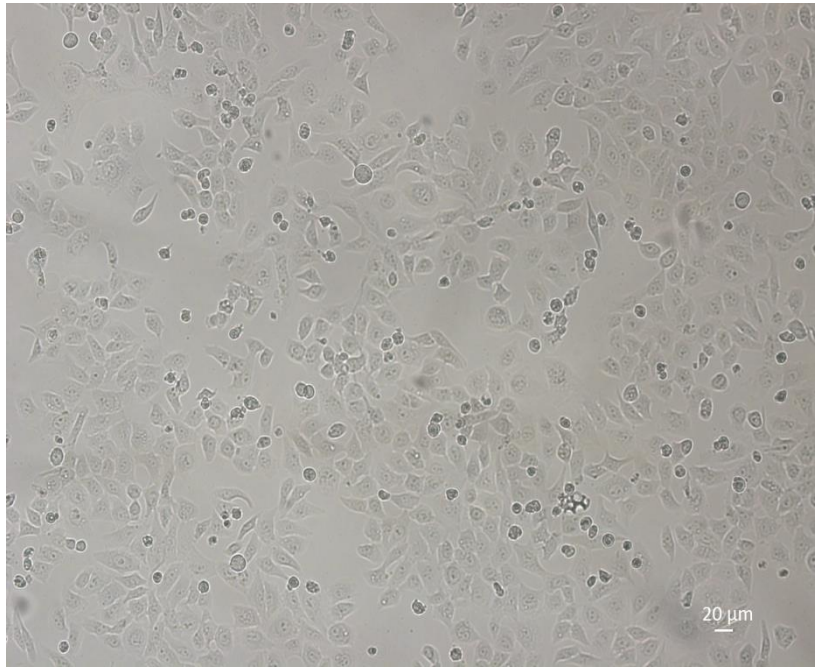


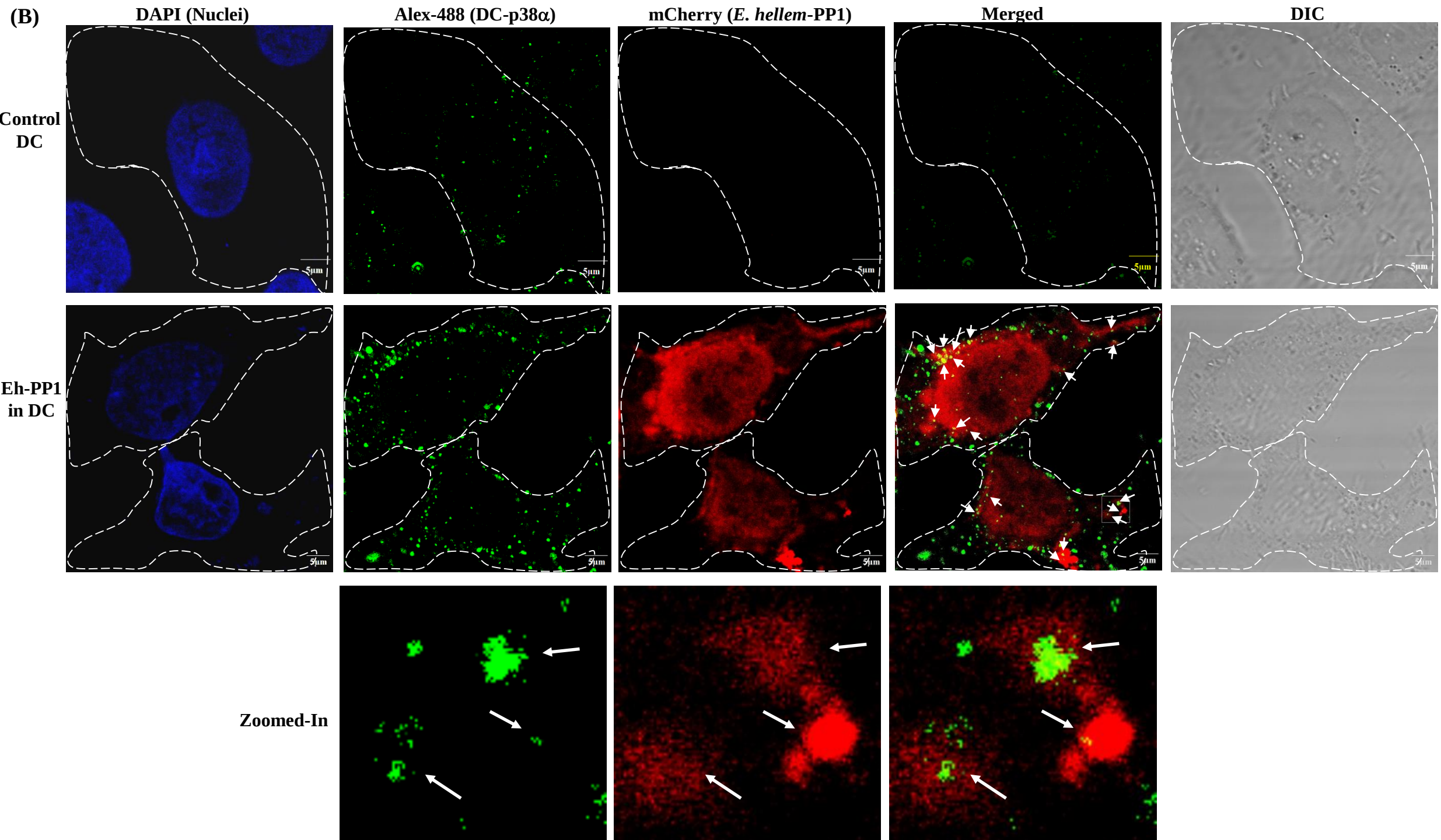
1: AD-T7-T X BD-53
2: AD-T7-7 X BDlam
3: (triplicates): AD-EhPP1 X BD-MAPK14



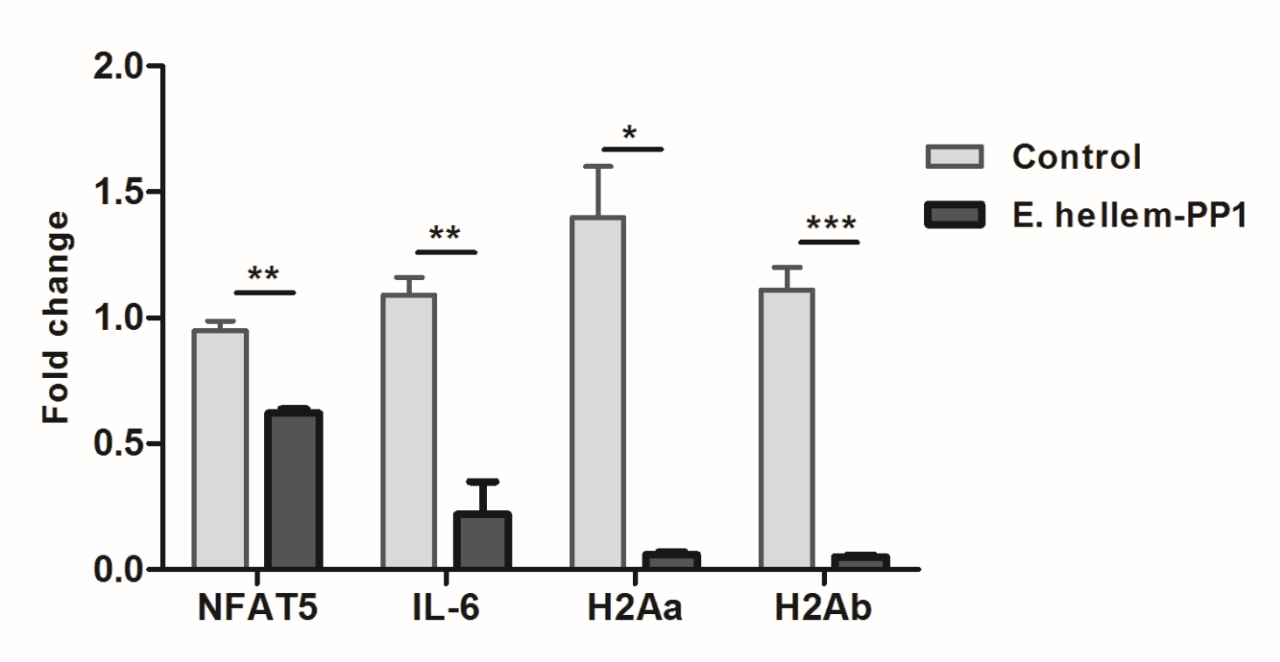
(A)






(B)
DAPI (Nuclei)
Alex-488 (DC-p38α)
mCherry (E. hellem-PP1)
Merged
DIC
Control DC
Eh-PP1 in DC
Zoomed-In
5μm

(C)


2.0
1.5
1.0
0.5
0.0
Fold change
**
**
*
***
NFAT5
IL-6
H2Aa
H2Ab
Control
E. hellem-PP1

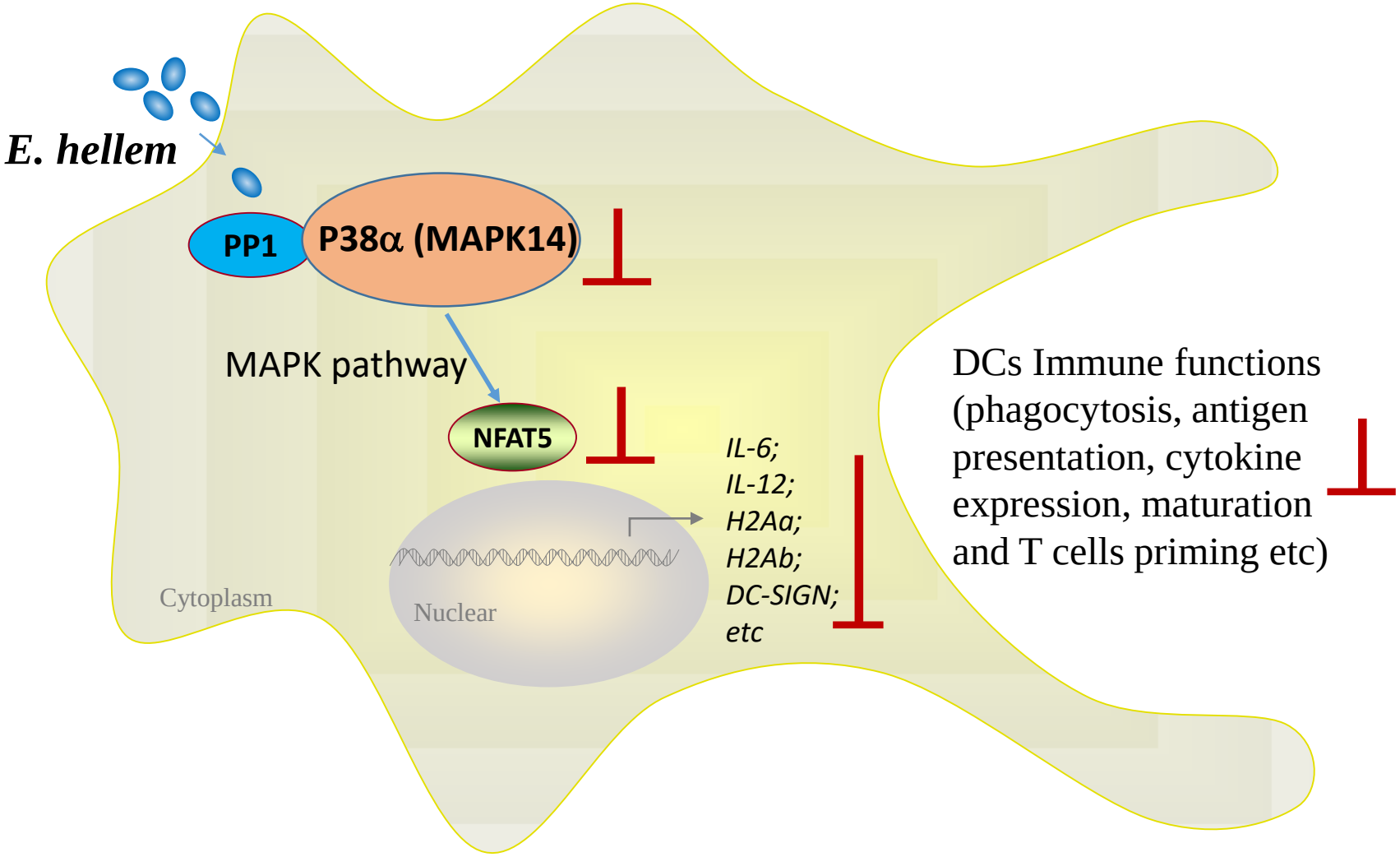

E. hellem
PP1
P38α (MAPK14)
MAPK pathway
NFAT5
IL-6;
IL-12;
H2Aa;
H2Ab;
DC-SIGN;
etc
Cytoplasm
Nuclear
DCs Immune functions (phagocytosis, antigen presentation, cytokine expression, maturation and T cells priming etc)

Table 1. Representative DCs proteins differentially expressed after *E. hellem* infection

Protein ID	Protein Name	Gene Name	Function	Alterations
P47811.3	Mitogen-activated protein kinase 14	Mapk14	MAP kinase p38. Essential component of the MAPK signal transduction pathway	Down
P63085	Mitogen-activated protein kinase 1	Mapk1	Serine/threonine kinase which acts as an essential component of the MAP kinase signal transduction pathway	Down
O09110	Dual specificity mitogen-activated protein kinase kinase 3	Map2k3	Essential components of MAP kinase signal pathway, catalyzes the concomitant phosphorylation of a threonine and a tyrosine residue in the MAP kinase p38	Down
P18653	Ribosomal protein S6 kinase alpha-1	Rps6ka1	Serine/threonine-protein kinase that acts downstream of ERK (MAPK1/ERK2 and MAPK3/ERK1) signaling and mediates activation of activation of the transcription factors CREB1, ETV1/ER81, NR4A1 and so on.	Down
Q922B6	E3 ubiquitin-protein ligase TRAF7	Traf7	Auto-ubiquitination regulated by MAP3K3. Potentiates MEKK3-mediated activation of the NF-kappa-B in signaling	Down
O55222	Integrin-linked protein kinase	Ilk	Act as a mediator of inside-out integrin signaling	Down
B1AVH7	TBC1 domain family member 2A	Tbc1d2	GTPase-activating protein for RAB7A, and signaling effector	Down
Q9DCR2	AP-3 complex subunit sigma-1	Ap3s1	Facilitates the budding of vesicles from the Golgi membrane and may be directly involved in trafficking to lysosomes	Down
Q8BH58	TIP41-like protein	Tiprl	Allosteric regulator of serine/threonine-protein phosphatase 2A (PP2A)	Down
Q8BGQ1	Spermatogenesis-defective protein 39 homolog	Vipas39	Involved in endosomal maturation, and lysosomal trafficking	Down
Q6R891	Neurabin-2	Ppp1r9b	Scaffold protein in multiple signaling pathways.	Up
Q99KF0	Caspase recruitment domain-containing protein 14	Card14	Scaffolding protein that can activate the inflammatory transcription factor NF-kappa-B and p38/JNK MAP kinase signaling pathways.	Up
Q68FE6	Rho family-interacting cell polarization regulator 1	Ripor1	Effector protein for Rho-type small GTPases that plays a role in cell polarity, signaling and directional migration	Up
O54950	5-AMP-activated protein kinase subunit	Prkag1	AMP/ATP-binding subunit of AMP-activated protein kinase (AMPK), a kinase that plays key	Up

	gamma-1		roles	
Q8BWW9	Serine/threonine-protei n kinase N2	Pkn2	PKC-related serine/threonine-protein kinase and Rho/Rac effector protein that participates in specific signal transduction during cellular singling	Up
P70182	Phosphatidylinositol 4-phosphate 5-kinase type-1 alpha	Pip5k1a	Catalyzes the phosphorylation of PtdIns4P to form PtdIns(4,5)P2, involves in cellular phagocytosis, migration, signaling.	Up
Q9DD03.1	Ras-related protein Rab-13	Rab13	Small GTPases key regulators of intracellular membrane trafficking	Up

Table 2. Representative *E. hellem* proteins identified in infected DCs

Protein ID	Gene ID	Protein Name/Function
XP_003888309.1	13466767	PP1 serine/threonine phosphatase
XP_003886772.1	13467540	Ras-like GTP binding protein
XP_003887182.1	13468215	GTP-binding nuclear protein
XP_003887892.1	13466917	Rab GTPase
XP_003886950.1	13467595	Beta-tubulin
XP_003886661.1	13466496	Eukaryotic translation initiation factor 2 subunit gamma
XP_003886837.1	13467379	RAD3-like DNA-binding helicase
XP_003887105.1	13467910	Nop56p-like protein
XP_003887061.1	13468065	DNA topoisomerase II
XP_003886739.1	13467235	Dihydrofolate reductase
XP_003887411.1	13467332	Histidyl-tRNA synthetase
XP_003887230.1	13468325	Hypothetical protein
XP_003887352.1	13467170	Hypothetical protein
XP_003887011.1	13467738	Hypothetical protein
XP_003887459.1	13467461	Hypothetical protein