EMi — Taylor & Francis
Taylor & Francis Group

Interferon-gamma-activated macrophages infected with Burkholderia cenocepacia process and present bacterial antigens to T-cells by class I and II major histocompatibility complex molecules

Roberto Rosales-Reyes [a], Paola Garza-Villafuerte [a], Daniela Vences-Vences [a*], Daniel F. Aubert [b‡], Rubi Aca-Teutle [a], Vianney F. Ortiz-Navarrete [c], Laura C. Bonifaz [d], Julio Cesar Carrero-Sánchez [e], Alfonso Olivos-García [a§], Miguel A. Valvano [b,f] and José Ignacio Santos-Preciado [a]

[a]Facultad de Medicina, Unidad de Investigación en Medicina Experimental, Universidad Nacional Autónoma de México, Mexico City, México; [b]Department of Microbiology and Immunology, The University of Western Ontario, London, Canada; [c]Departamento de Biomedicina Molecular, Centro de Investigación y de Estudios Avanzados del IPN, Mexico City, México; [d]Unidad de Investigación Médica en Inmunoquímica Hospital de Especialidades Centro Médico Nacional Siglo XXI, IMSS, Mexico City, Mexico; [e]Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Mexico City, Mexico; [f]The Wellcome-Wolfson Institute for Experimental Medicine, Queen's University Belfast, Belfast, UK

ABSTRACT
Burkholderia cenocepacia is an emerging opportunistic pathogen for people with cystic fibrosis and chronic granulomatous disease. Intracellular survival in macrophages within a membrane-bound vacuole (BcCV) that delays acidification and maturation into lysosomes is a hallmark of B. cenocepacia infection. Intracellular B. cenocepacia induce an inflammatory response leading to macrophage cell death by pyroptosis through the secretion of a bacterial deamidase that results in the activation of the pyrin inflammasome. However, how or whether infected macrophages can process and present B. cenocepacia antigens to activate T-cells has not been explored. Engulfed bacterial protein antigens are cleaved into small peptides in the late endosomal major histocompatibility class II complex (MHC) compartment (MIIC). Here, we demonstrate that BcCVs and MIICs have overlapping features and that interferon-gamma-activated macrophages infected with B. cenocepacia can process bacterial antigens for presentation by class II MHC molecules to CD4$^+$ T-cells and by class I MHC molecules to CD8$^+$ T-cells. Infected macrophages also release processed bacterial peptides into the extracellular medium, stabilizing empty class I MHC molecules of bystander cells. Together, we conclude that BcCVs acquire MIIC characteristics, supporting the notion that macrophages infected with B. cenocepacia contribute to establishing an adaptive immune response against the pathogen.

Introduction

The *Burkholderia cepacia* complex (Bcc) is a group of closely-related opportunistic bacteria associated with poor clinical outcomes in individuals with cystic fibrosis and chronic granulomatous disease [1]. In particular, *Burkholderia cenocepacia*, has become notorious for its ability to be transmitted across patients, often causing a fatal necrotizing pneumonia in people with cystic fibrosis [2-4].

Several studies have shown that *B. cenocepacia* can survive within macrophages in culture and in human lung mucosal tissue macrophages [5,6]. Survival occurs in a membrane-bound cytoplasmic vacuole (BcCV) [7]

that delays acidification and fusion with lysosomes [8], in part due to inactivation of Rab7 [9]. In addition, intracellular *B. cenocepacia* inactivate the small GTPases Rac1 [10,11] and RhoA in a Type 6-secretion system-dependent manner [12]. Rac1 inactivation delays the assembly of the NADPH oxidase complex onto the BcCV [11,13] and affects actin remodeling, which in turn compromises normal cell migration and phagocytosis [10,11], while RhoA dysfunction, sensed by the pyrin inflammasome pathway, leads to cell death by pyroptosis [12]. Intracellular *B. cenocepacia* can also induce necrosis of human neutrophils [14,15] and alter the normal functioning of

human dendritic cells by inhibiting upregulation of costimulatory molecules and inducing necrosis [16]. However, the mechanisms of cell death in both neutrophils and dendritic cells have not been investigated. *B. cenocepacia* can also mediate TNFα and IL-6 release through the stimulation of Toll-Like receptors (TLR)-4 and TLR-5 [17,18]. Macrophages are one of the first lines of defense against intracellular pathogens [19]; when activated, macrophages upregulate antimicrobial mechanisms causing bacterial lysis. Similar to dendritic cells, macrophages also act as antigen-presenting cells (APCs) by processing and displaying bacterial peptides at the cell surface, which prime T-cell immune responses [20]. Upon engulfment, bacterial antigens are processed into peptides in a late endosomal compartment (MIIC) and loaded onto newly-synthesized class II MHC molecules [21]. The class II MHC/peptide complexes assembled in the MIIC are transported to the plasma membrane, where they can be recognized by specific $CD4^+$ T-cells to initiate an adaptive immune response [22]. Further, antigens in the cytosol of an APC are processed into peptides by the proteasome [21]; these are subsequently translocated into the endoplasmic reticulum by the transporter antigen processing protein 1 and 2 (TAP1/TAP2). In the endoplasmic reticulum, the translocated peptides are loaded onto newly-synthesized class I MHC molecules. The class I MHC/peptide complex is transported to the plasma membrane and recognized by specific $CD8^+$ T-cells [21]. APCs could also use an alternative pathway to present exogenous antigens that reside in vacuoles, which involves the release of processed peptides from vacuoles into the extracellular medium where they can interact with empty class I MHC molecules of the same cell or bystander cells [23].

Although several mechanisms triggering innate immune responses upon *B. cenocepacia* infection have been described, whether infected macrophages prime the adaptive immune system has remained unexplored. The intracellular infection of macrophages with *B. cenocepacia* alters the normal functions of these cells [10,11]. Because the bacteria reside within a modified vacuole that delays the fusion with the lysosomes and the MIIC is an intracellular compartment that does not necessarily interact with lysosomes, we speculated that the BcCV shares features with the MIIC and wanted to investigate whether and how *B. cenocepacia*-infected macrophages can present antigens to T-cells. Here, we show that BcCVs and the MIIC acquire similar markers and *B. cenocepacia*-infected macrophages can present bacterial antigens by class I and class II MHC molecules to primed T-cells, suggesting that the infected macrophages, despite being functionally impaired in some respects, can still contribute to establish an adaptive immune response against *Burkholderia*.

Materials and methods

Ethics statement

Mice C57BL/6 and C3H/HeJ were obtained from Unidad de Investigación en Medicina Experimental and the Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, respectively. Animals were maintained under Institutional Animal Care and Use Committee guidelines described in the NOM-062-ZOO-1999. This protocol was approved by the Research and Ethics committee of the School of Medicine, National Autonomous University of Mexico # FMED/CI/JMO/008/2014.

Bacterial strains and growth conditions

Bacteria and plasmids used in this study are described in Supplementary Table 1. In this study, we employed the *B. cenocepacia* strain MH1 K (herein *B. cenocepacia*), which carries a deletion of an efflux pump that makes it sensitive to aminoglycoside antibiotics, enabling us to carry out accurate determinations of intracellular bacterial survival by differential killing of extracellular bacteria with gentamicin [24] (see below). *Escherichia coli* and *B. cenocepacia* were grown in Luria–Bertani broth (LB; Sigma-Aldrich) overnight at 37°C with shaking (180 rpm). Plasmids in *Escherichia coli* were mobilized into *B. cenocepacia* by triparental mating [25]. *B. cenocepacia*(pDSRedT3) was grown in LB supplemented with 30 μg/ml chloramphenicol, while *B. cenocepacia*(p*zmpA*-HEL$_{48-61}$) or *B. cenocepacia* (p*zmpA*-OVA$_{257-264}$) were grown in LB supplemented with 50 μg/ml trimethoprim. Once introduced in *B. cenocepacia* plasmid-encoded fusion proteins were expressed by induction with 0.2% (w/v) rhamnose (Sigma-Aldrich) during 4 h at 37°C with shaking.

Construction of recombinant plasmids

To determine antigen presentation to $CD4^+$ and $CD8^+$ T-cells, we constructed two plasmids (Supplementary Table 1). Briefly, we used pJLP1E (Crl-OVA) that encodes the *crl* gene fused to a DNA fragment encoding an ovalbumin peptide (OVA$_{254-267}$) and pJLP2H (Crl-HEL) encoding the *crl* gene fused to a DNA fragment encoding a hen egg lysozyme peptide of the (HEL$_{48-61}$). Both plasmids were kindly donated by Dr. Mary Jo Wick (University of Gothenburg, Sweden) and confirmed by DNA sequencing. The HEL$_{48-61}$ coding sequence was amplified with the HEL-Xba (Fw) 5'-TTTTTCTAGACGTAACACCGATGGGAGTAC-3' and HEL-HindIII (Rv) 5'-TTTTAAGCTTCCTGC-CATCGTTAACCCATCA-3' primer pairs, and the OVA$_{254-267}$ coding sequence was amplified with the OVA-Xba (Fw) 5'-TTTTTCTAGATCTATAAT-CAACTTTGAAAAAC-3' and OVA-HindIII (Rv) 5'-

TTTTAAGCTTTTTCACTCACGCCGTTATCAC-3'
primers. PCR products were cloned into pSCRha
behind the rhamnose-inducible promoter [26], resulting in pSCR-HEL$_{48-61}$ and pSCR-OVA$_{257-264}$. Next,
we amplified the *Burkholderia zmpA* gene using primers 5647 and 5648 [26] to construct plasmids encoding ZmpA protein fusions to HEL$_{48-61}$ and OVA$_{257-264}$.
A haemagglutinin tag was also incorporated into these
fusions to assess protein expression by western blot.
The resulting plasmids, pSCR-ha-ZmpAHEL$_{48-61}$
(pDA195) and pSCRha-ZmpAOVA$_{254-267}$ (pDA196)
were mobilized into *B. cenocepacia* as described above.

Antibodies

The rat monoclonal antibody anti-LAMP1 (clone
ID4B) was obtained from Beckton Dickinson, the rabbit anti-Rab7 (H-50) was from Santa Cruz Biotechnology, the mouse anti-H-2K^b clone Y3 was a gift from Dr.
Gunter J. Hammerling, German Cancer Research Center, Heidelberg, Germany. Armenian hamster anti-
CD80 (clone 16-10A1) conjugated to PE, rat anti-
CD86 (clone GL1) conjugated to FITC, rat anti-
MHC-II (I-A/I-E, clone M5/114), and mouse anti-H-
2Kb/pOVA$_{257-264}$ (SIINFEKL) conjugated to PE were
from Thermo Scientific. AF-488-conjugated chicken
anti-mouse and AF-488-conjugated goat anti-rat (Invitrogen) were used as secondary antibodies

Bone marrow-derived macrophages and infection conditions

Bone marrow-derived macrophages (BMDM) were
obtained from femurs of C3H/HeJ or C57Bl/6 mice, as
previously described [27]. Briefly, 3×10^5 macrophages
from C57Bl/6 were seeded on 12-well plates; cells were
activated with 300 U/ml of interferon gamma (IFNγ)
of 10 ng/ml of lipopolysaccharide (LPS) (both from
Sigma-Aldrich) for 24 h, as previously reported
[28,29]. *B. cenocepacia* was grown overnight in LB at
37°C with shaking. One millilitre of culture was washed
twice with PBS and resuspended in 1 ml of RPMI 1640.
On the day of infection, the medium was replaced with
fresh RMPI without antibiotics. *B. cenocepacia* were
added to macrophages and plates were centrifuged for
1 min at 1200 rpm, followed by incubation at 37°C
under 5% CO$_2$ and 5% humidity for the desired time.
At 1 h postinfection, cells were washed 3 times with
PBS to remove extracellular bacteria and placed in
fresh medium supplemented with 50 μg/ml gentamicin
(Sigma-Aldrich). To determine colony-forming units
(CFUs), cells were lysed with PBS-Triton X-100 2%
(Sigma-Aldrich). Aliquots of 10-fold serial dilutions of
cell lysates were plated on LB-agar to quantify the number of CFUs. The percent intracellular survival was calculated as % survival = (CFUs[24 h]×100)/CFUs[1 h].

Immunofluorescence and flow cytometry assays

For immunofluorescence, BMDM from C57Bl/6 mice
were seeded onto square coverslips in six-well tissue
culture plates. *B. cenocepacia*(pDSRedT3) was added
at a multiplicity of infection (MOI) of 50 and the
plates were centrifuged for 1 min at 1200 rpm.
Infected BMDM were washed 3 times with PBS
and incubated at 37°C under 5% CO$_2$ with fresh
RPMI 1640 supplemented with 50 μg/ml gentamicin
for the desired times. Infected macrophages were
washed three times with PBS and fixed for 30 min
with 2% paraformaldehyde at room temperature;
then, cells were washed twice with PBS and incubated
20 min with 100 mM glycine at room temperature.
After fixation, macrophages were permeabilized
with cold methanol for 1 min and incubated for 1 h
in blocking buffer (PBS with 2% goat serum and
3% of BSA). Samples were then incubated for an
additional hour at 37°C with the primary antibody,
washed three times, and incubated with the secondary AF488-conjugated antibody for 45 min at 4°C
in blocking buffer. Coverslips were mounted on
glass slides using fluorescent mounting medium
(Dako, Cytomation). For phagocytosis assays, macrophages were incubated with 1μg/ml of AF488-conjugated zymosan particles (Molecular Probes) for
15 min, washed three times, and then fixed and
mounted on glass slides with fluorescent mounting
medium. Acidification of BcCVs in *B. cenocepacia*-
RFP-infected macrophages was examined with 1μM
LysoTracker Green (Molecular Probes) at 30 min
post-infection. Fluorescence was assessed using a
Nikon NE300 inverted microscope; images were analysed by Metamorph® (Molecular Dynamics, Downington, PA, USA). The colocalization between
B. cenocepacia-RFP and cellular markers was evaluated by quantifying pixels in the red and green channels of every acquired image and determining the
Pearson's correlation coefficient (PCC) [30].

For flow cytometry, macrophages from C57Bl/6
mice incubated with zymosan particles or infected
with *B. cenocepacia* were harvested in cold PBS with
0.1 mg/ml EDTA. Infected cells were incubated in
blocking buffer in a 96-well plate for 45 min on ice.
The cells were centrifuged and incubated in blocking
buffer with anti-CD80, anti-CD86, anti-MHC-I (Y3),
anti-MHC-I (H-2K^b/pOVA) and anti-MHC-II,
respectively. Stained macrophages were analysed by
forward and side scatter; a region in which macrophages are located (excluding cell debris) was selected
and cells were analysed by detecting specific molecules
in green and red channels. The autofluorescence was
compensated in relation to unstained cells. Fluorescence readings were acquired in an LSRF-Fortessa
BD flow cytometer and samples analysed with the
FlowJo software.

Antigen presentation assays

Interferon-gamma (IFNγ)-activated macrophages from C3H/HeJ (haplotype I-A^k) mice (3 x10^5 cells) were seeded onto 12-well tissue culture plates one day before the infection. As a positive control, we incubated the cells with 250 μg/ml of HEL protein (Sigma-Aldrich). At the same time, cells were infected with live or heat-killed *B. cenocepacia*-p$zmpA$-HEL$_{48-61}$ at 37℃ with a MOI of 50 for 1 h and extracellular bacteria removed by washing twice with PBS. Infected cells and uninfected cells incubated with the HEL protein were co-cultured with C-10 hybridoma (1:2 ratio) [31] for 24 h. Cell supernatants were collected and analysed for interleukin-2 (IL-2) by ELISA. We quantified the IL-2 released by C-10 T hybridoma after the recognition of the MHC-II/peptide (I-A^k/HEL$_{48-61}$) complex to evaluate APC mediated T-cell activation [31,32]. For detection of antigen presentation by MHC-I, we infected 3 x10^5 IFNγ-activated macrophages from C57Bl/6 (haplotype H-2K^b) as described above. The infected cells were co-cultured with RF33.70 hybridoma (1:2 ratio) [33] for 24 h; then, supernatants were collected and analysed by IL-2 release by ELISA. CD8$^+$ T-cell activation was assessed by quantifying IL-2 release by RF33.70 after the recognition of MHC-I (H-2K^b/pOVA$_{257-264}$) complex.

Co-culture assays

Three x 10^5 IFNγ-activated macrophages from C3H/HeJ mice were seeded onto 12-well tissue culture plates one day before the infection. The cells were incubated with live or heat-killed *B. cenocepacia* at 37℃ at a MOI of 50 for 1 h and then washed twice with PBS. Infected macrophages were co-cultured with RMA-S cells (TAP 2$^{-/-}$ at a 1:1 ratio) [23] in RMPI supplemented with 100 μg/ml gentamicin for 24 h. RMA-S cells were subsequently harvested and stained with anti-K^b monoclonal antibody (Y3 antibody). Fluorescence readings were acquired in an LSRF-Fortessa BD flow cytometer and analysed with the FlowJo software.

Phagosome purification and biochemical analyses

For the purification of phagosomes from macrophages infected with *B. cenocepacia* we employed the peritoneal macrophage-like cell IC21 obtained from C57Bl/6 mice (TIB-186 from ATCC, haplotype H-2K^b) [34]. Phagosomes were purified as previously described [11] with minor modifications. Briefly, 1×10^8 macrophages were infected with *B. cenocepacia* at a MOI of 50 for 1 h and broken in a Dura-Grind stainless-steel homogenizer (Wheaton Scientific), as described [11]. Post-nuclear supernatants (PNS) obtained after centrifugation at 700 RPM for 2 min were adjusted to 39% (w/v) sucrose (2 ml of PNS plus 2.4 ml of 65% sucrose, from Sigma-Aldrich). A step-sucrose gradient was prepared (Beckman) and centrifuged for 1 h (28000 rpm at 4℃) using a SW41Ti rotor (Beckman-Coulter) [11]. Ten-μl of each fraction was mixed with 90 μl of PBS plus 1% Triton X-100, the lysates were plated on LB-agar. Colony-forming units were counted after 24 h incubation at 37℃. Lysosomal activity was estimated by measuring β-galactosidase. For this, 25 μl of each fraction was mixed with 125 μl of 10 μM of p-nitrophenyl-β-D-galactopyranoside/0.7% Triton X-100/150 mM citrate buffer (pH 3.5) and incubated for 2 h at 37℃. The reaction was stopped by addition of 150 μl of 0.5 M of sodium carbonate, and the absorbance at 405 nm was measured in a spectrophotometer. The protein concentration in each fraction was determined by the Bradford assay (Bio-Rad). To detect LAMP1, 15 μl of each fraction was loaded on a 14% SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were incubated overnight at 4℃ in 10% of blocking solution (Bio-Rad) made in PBS-Tween 20 (0.1%) (PBS-T) and were incubated with 1:100 of anti-LAMP1 overnight at 4℃ in 5% of blocking solution in PBS-T. After washing with PBS-T, the blots were incubated with goat anti-Rat conjugated to IRDye 800CW (LICOR, Odyssey) for 1 h at room temperature in 5% blocking solution in PBS-T. Unbound antibodies were removed by several washes with PBS-T, and the membranes were analysed by infrared imaging using an Odyssey imager (LICOR Odyssey).

Cytotoxicity assay

Supernatants of uninfected or infected macrophages from C57Bl/6 mice with *B. cenocepacia* were assayed for released lactate dehydrogenase (LDH; Promega, Madison WI) activity. The percent LDH release was determined as percent of release = [experimental LDH release – spontaneous LDH release]/[maximal LDH release – spontaneous LDH release]×100% [27].

Statistical analysis

Data are shown as mean ± the standard deviation (SD). Statistical analyses were conducted with Graph-Pad Prism 7.0. One-way analysis of variance (ANOVA) and Tukey test for multiple comparisons were performed. Data analyses were performed with a minimum of three biological repeats, each with at least two technical repeats. Asterisks indicate statistical significance and the p values are indicated as *, $p<0.05$; **, $p<0.01$ and ***, $p<0.001$; ns, non-significant.

The statistical analysis of colocalization between the BcCV and cellular markers was conducted by

quantification of the Pearson's correlation coefficient (PCC) with the following formula:

$$PCC = \frac{\Sigma_i (R_i - \bar{R}) \times (G_i - \bar{G})}{\sqrt{\Sigma_i (R_i - \bar{R})^2 \times (G_i - \bar{G})^2}}$$

in where, R_i and G_i indicates the intensity values in pixel of the signal in red and green channels respectively. $\bar{R}$ and $\bar{G}$ indicates the mean intensities of the red and green channels respectively across the entire image. A PCC of 1.0 (100% of colocalization) indicates a strong correlation and a PCC of 0.0 indicates non-correlation (0.0% of colocalization) [30].

Results

IFNγ-treated macrophages control B. cenocepacia infection

Efficient antigen processing and presentation to T-cells requires activation of APCs. Usually, APC activation with IFN-γ upregulates the expression of class I and class II MHC molecules, cathepsins, TAP1 and TAP2 chaperones, proteasomal proteins, and the CD80 (B7.1) and CD86 (B7.2) co-stimulatory molecules [35–37]. Intracellular *B. cenocepacia* causes defects in macrophage function affecting actin cytoskeleton remodeling, phagocytosis, Rho-GTPases functionality, delayed assembly of the NADPH dehydrogenase, and pyroptosis [8–13,38] However, the effect of intracellular *B. cenocepacia* on IFNγ-treated macrophages in relation to antigen processing and presentation has not been investigated. Resting and IFNγ-treated macrophages were infected with *B. cenocepacia* and the expression of class I and II MHC molecules, CD80, and CD86 was quantified at 24 h post-infection. LPS-treated macrophages infected with *B. cenocepacia* were used as a positive control for macrophage activation.

In LPS-treated macrophages from C57Bl/6 mice, the intracellular infection was associated with a modest but significant decrease in the expression of class I MHC molecules at 24 h post-infection (Figure 1(A)) but it did not significantly affect the expression of class II MHC, CD80 and CD86 costimulatory molecules (Figure 1(B–D)). In contrast, IFNγ-treated macrophages showed increased expression of class I and class II MHC molecules and CD86, while CD80 expression did not change (Figure 1). These differences were not related to variations in bacterial uptake since in resting and IFNγ-treated macrophages the uptake of *B. cenocepacia* was similar (Figure 2(A)). Further, resting or IFNγ-treated macrophages engulfed Zymosan particles in similar proportions (Figure 2(B,C)). Together, these results indicate that IFNγ-treated macrophages infected with *B. cenocepacia* are primed to process bacterial antigens.

We also evaluated whether IFNγ-activated macrophages clear the *B. cenocepacia* intracellular infection by determining bacterial colony forming units (CFUs) at 1 and 24 h post-infection. Given that *B. cenocepacia* replicates poorly in macrophages and its generation time is approximately 1 h in culture medium [24], the CFUs at 1 h post-infection denote bacterial uptake, while the values at 24 h post-infection denote bacterial survival. In contrast to untreated macrophages, which showed a 15 ± 9% reduction in the bacterial load over 24 h (Figure 2 (A)), IFNγ-activated infected macrophages had a 75 ± 6% reduction (Figure 2(A)). IFNγ-activated macrophages remained viable at 24 h post-infection (Figure 2(D)) and retained their ability to release pro-inflammatory cytokines to the culture supernatant (Figure 2 (E,F)).

Macrophages infected with B. cenocepacia process and present bacterial antigens to CD4+ T cells

Previous work demonstrated that intracellular *B. cenocepacia* reside in the BcCVs, which are late endosomal compartments that fuse partially with lysosomes [8]. Interestingly, the intracellular compartment in which antigenic peptides are generated from exogenous antigen processing (MIIC) has low lysosomal enzyme activity and is typically decorated with MHC class II molecules and Rab7 [39,40]. Therefore, we investigated if the BcCVs contain class II MHC molecules, LAMP1 and Rab7. IFNγ-treated macrophages were infected with *B. cenocepacia*(pDsRedT3) and bacterial intracellular localization was followed over time. The quantification of the colocalization between the BcCV and intracellular markers was performed by calculating the PCC as indicated in Materials and methods. As early as 3 h post-infection, 27% of the late endosome marker Rab7 (PCC: 0.268), 22% of the late endosome/lysosome LAMP1 (PCC: 0.2158), and the 35.5% of the class II MHC molecules (PCC: 0.3551) colocalized partially with the BcCVs (Figure 3). Therefore, the BcCVs can acquire class II molecules, suggesting they could have similar properties as MIIC where bacterial antigens are processed, which also include being a non-acidic intracellular compartment (Figure 4(A)). A biochemical experiment examining BcCVs purified by sucrose density gradient also showed that the majority of live *B. cenocepacia* is localized in fractions with low lysosomal activity (as defined by measuring the levels of β-galactosidase activity) and reduced LAMP1 expression (Fractions 10-12, Figure 4(B)). These data support the notion that BcCVs share properties of an MIIC compartment whereby bacterially-derived peptides are loaded into class II molecules and stimulate specific CD4+ T-cell responses.
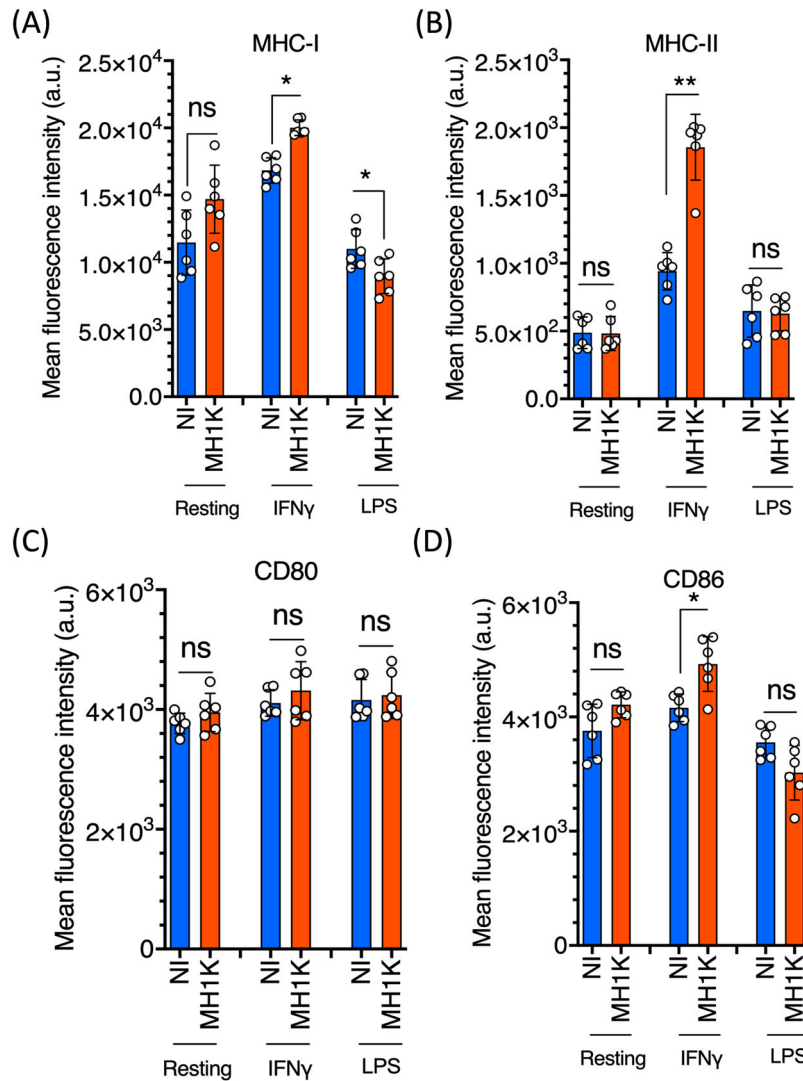
Figure 1. Macrophages infected with B. cenocepacia increased the expression of MHC and CD86 molecules. Resting macrophages or macrophages pre-treated with 300 U/ml of IFNγ or with 10 ng/ml of LPS from C57Bl/6 mice were infected with B. cenocepacia (MH1 K) at a MOI of 50 for 1 h. Cells were washed, incubated for 24 h and processed for flow cytometry analysis. Cell populations were analyzed by the fluorescence intensity of class I MHC (A), class II MHC (B), CD80 (C) and CD86 (D) molecules. Blue bars indicate expression in non-infected macrophages and the orange bars the level of expression in infected (MH1 K) macrophages. The results were obtained from 3 independent experiments, each one in duplicate ($n = 6$), and plotted as the mean fluorescence intensity ± SD and analyzed by the paired t-test. NI, non-infected; a.u., arbitrary units; *, $p<0.05$; ns, non-significant.

To further support this hypothesis, IFNγ-treated macrophages were infected with *B. cenocepacia* and assessed for their ability to present antigens to CD4+ T-cells. For specific-antigen recognition, we used a plasmid encoding the fusion protein ZmpA-HEL$_{48-61}$, the HEL-peptide is presented exclusively by class II MHC molecules of haplotype I-A^k, (hereafter, HEL) [41] to CD4+ T-cells. ZmpA is a secreted *Burkholderia* metalloprotease, presumably released into the BcCV by the Type II Secretion System (T2SS) [26,42]. If BcCVs share MIIC properties, we would expect that ZmpA could be processed by luminal resident proteases. This idea was tested by using IFN-γ-treated BMDM from C3H/HeJ (Haplotype I-A^k) mice that were infected with *B. cenocepacia*-HEL and co-cultured with C-10, a CD4+ T-cell hybridoma, which recognizes the HEL$_{48-61}$ peptide loaded by I-A^k class II molecules

[31]. IL-2 released by C-10 hybridoma was quantified in the co-culture supernatants. The results indicate that viable macrophages infected with live *B. cenocepacia*-HEL can present bacterial antigens to CD4+ T-cells in the context of class II MHC molecules (Figure 5). Indeed, macrophages infected with heat-killed *B. cenocepacia*-HEL (MH1K-HEL-HK) did not present the ZmpA-HEL antigen to CD4+ T-cells. Therefore, only macrophages containing viable bacteria can present the antigen since only viable bacteria produce and translocate the fusion protein outside the BcCV into the cytosol by the combined actions of the T2SS and T6SS, as previously described [26] where it may be processed and presented to CD8+ T-cells. Together, the experiments presented here indicate that BcCVs acquire the ability to process and present bacterial antigens to T-cells.
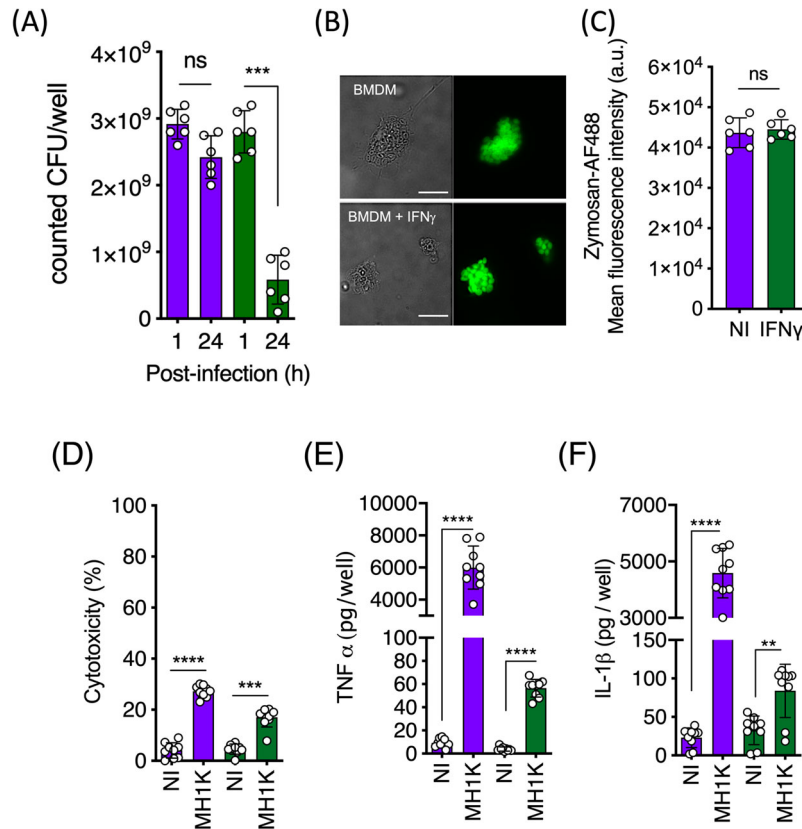
Figure 2. IFNγ-treated macrophages control intracellular *B. cenocepacia*. Three x 10^5 macrophages from C57Bl/6 mice were pre-treated with 300 U/ml of IFNγ and infected with *B. cenocepacia* at a MOI of 50. (A) Non-treated macrophages (purple bars) or pre-treated macrophages with IFNγ (green bars) were infected with *B. cenocepacia* MH1 K by 1 h, the cells were washed and lysed at 1 or 24 h post-infection to quantify the number of bacterial CFUs per well. (B) Non treated (BMDM) or pre-treated macrophages with IFNγ (BMDM + IFNγ) were incubated with 1μg/ml of zymosan particles conjugated to AF488 during 15 min. Cells were analyzed by immunofluorescence microscopy. Bars indicates 10 μm. One representative image of 15 is presented. (C) Cells were analyzed by flow cytometry. Non-treated macrophages (purple bar) or pre-treated macrophages with IFNγ (green bar), one representative experiment of three replicates (*n*=6) is presented. 20000 events were analyzed by each experiment. (D) Culture supernatants were used to quantify macrophage cell death (cytotoxicity) by the assaying released lactate dehydrogenase activity at 24 h post-infection. Released TNFα (E) and IL-1β (F) at 24 h post-infection were detected by ELISA on macrophages non-infected (NI) or infected with *B. cenocepacia* (MH1 K). Green bars (D, E and F) indicate IFNγ-treated macrophages and the purple bars indicate resting macrophages. The results were obtained from 3 independent experiments, each one in triplicate (*n* = 9), plotted as mean ± SD and analyzed by the paired test. **$p<0.01$; ****$p<0.0001$; ns, non-significant.

Macrophages infected with B. cenocepacia present bacterial antigens to CD8+ T cells

To investigate whether *B. cenocepacia*-infected macrophages can also present specific bacterial antigens by class I MHC molecules to CD8+ T-cells, we constructed a plasmid encoding the fusion protein ZmpA-OVA$_{257-264}$ (hereafter OVA). The OVA-peptide is specifically presented by MHC class I, haplotype H-2K^b [43]. The T6SS of *B. cenocepacia* affects the integrity of the BcCV membrane allowing ZmpA to gain access into the cytosol [26]. Cytosolic ZmpA could be processed by the proteasome and follow the endogenous pathway of antigen processing and presentation. To test this hypothesis, IFNγ-treated BMDM from C57Bl/6 (haplotype H-2K^b) were infected with *B. cenocepacia*-OVA, and the level of class I MHC molecules (Figure 6(A)) and the H-2K^b/pOVA$_{257-264}$ complex arising from *B. cenocepacia* processing (Figure 6(B)) were quantified

by flow cytometry. Expression of MHC class I molecules increased in macrophages infected with *B. cenocepacia* (Figure 6(A)). In contrast, specific H-2K^b/pOVA$_{257-264}$ complexes did not increase significantly at the cell surface of macrophages infected with *B. cenocepacia*-OVA (Figure 6(B)). We explored the ability of *B. cenocepacia*-OVA-infected macrophages to present specific-bacterial antigens to T-cells by co-culturing infected macrophages with RF33.70, a CD8+ T-cell hybridoma that specifically recognizes the OVA$_{257-264}$ peptide [33] loaded by H-2K^b molecules. IL-2 released by RF33-70 hybridoma in the supernatant indicates that *B. cenocepacia*-OVA-infected macrophages can present bacterial antigens by class I MHC molecules to CD8+ T-cells (Figure 6(C)). Together, these results suggest that *B. cenocepacia*-infected macrophages can also process and present bacterial antigens by class I MHC molecules to CD8+ T-cells.
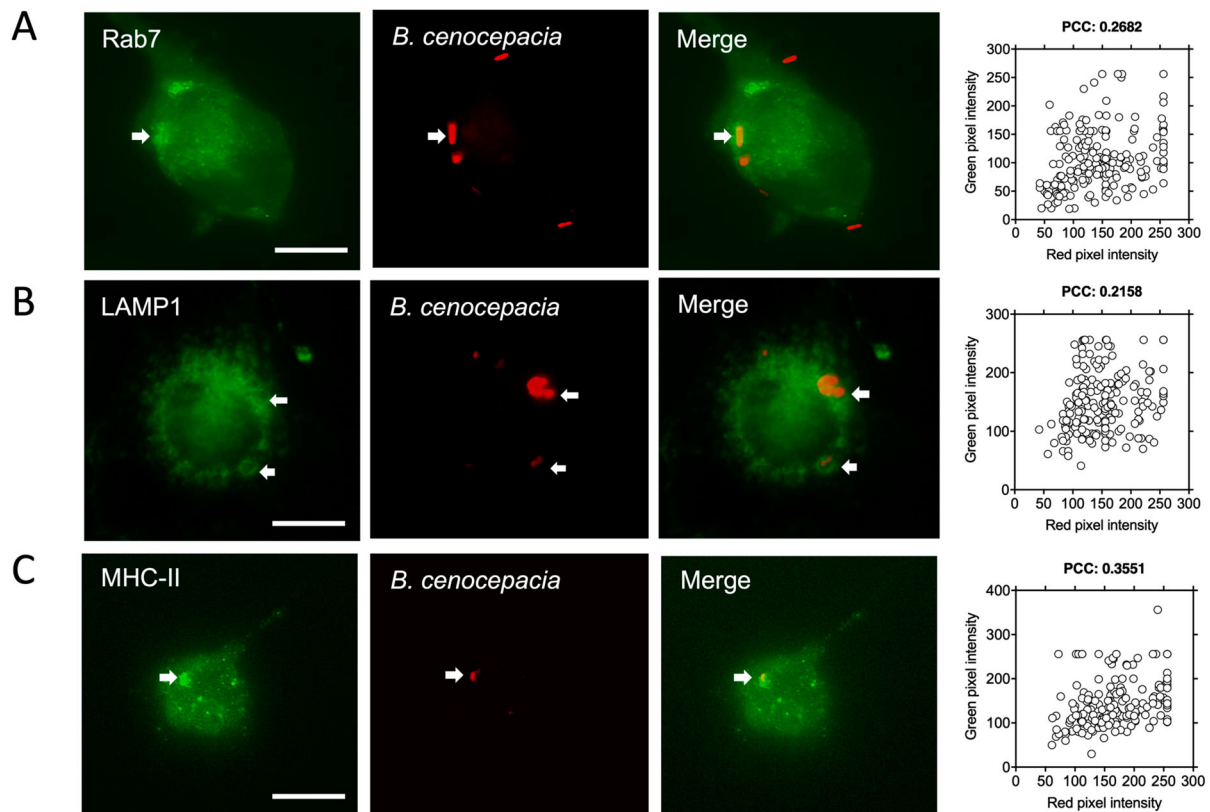
Figure 3. Intracellular *B. cenocepacia* is localized in the MHC class II compartment. IFNγ-treated (300 U/ml) macrophages from C57Bl/6 mice were incubated with *B. cenocepacia* (pDSRedT3) at a MOI of 50 during 1 h. At 3 h post-infection, infected macrophages were stained with anti-Rab7 (A), anti-LAMP1 (B) or anti-class II MHC (C). Images of infected macrophages were analyzed by immunofluorescence. Bars indicates 10 μm. For Rab7 we analyzed 202 BcCV in 141 cells; for LAMP1, 192 BcCV in 128 cells and for MHC class II, 181 BcCV in 132 cells. The co-occurrence of green and red pixels was determined by Pearson's correlation coefficient (PCC).

B. cenocepacia-infected macrophages release processed bacterial peptides into the extracellular medium that stabilize empty class I MHC molecules

When infected with *B. cenocepacia*, IFNγ-treated macrophages show reduced intracellular bacterial load (Figure 2(A)). Therefore, we also investigated if *B. cenocepacia*-infected macrophages could release peptides from processed bacterial antigens into the extracellular medium. For these experiments, *B. cenocepacia*-infected macrophages from C3H/HeJ mice were co-cultured with the TAP-2$^{-/-}$ RMA-S cells that express empty class I MHC molecules on their plasma membrane (Figure 7(A)) [44]. After co-culture of RMA-S cells with infected macrophages for 24 h, RMA-S cells were analysed by flow cytometry to determine the amount of MHC class I molecules with stable conformation [23]. The results indicated a higher level of class I MHC/peptide complexes on RMA-S cells' surface when co-cultured with infected macrophages (Figure 7(B)), suggesting that IFNγ-activated macrophages infected with viable bacteria can release bacterial peptides (derived from *B. cenocepacia* processing) that stabilize empty class I MHC molecules of bystander cells. In contrast, heat-killed *B. cenocepacia*-infected macrophages did not efficiently release bacterial peptides derived from antigen processing.

Discussion

In this study, we demonstrate that IFNγ-activated macrophages infected with *B. cenocepacia* can process and present bacterial antigens to T-cells by both class I and class II MHC molecules. Intracellular *B. cenocepacia* survive in a vacuole (BcCV) that delays fusion with lysosomes, preventing luminal acidification for at least 6 h post-infection [8] and also exhibiting proteolytic activity [26]. The MIIC compartment that processed antigens also contains low lysosomal activity and its membrane is decorated with class II MHC molecules, Rab7 and LAMP1 [39,40]. Our results indicate that the BcCV, containing live *B. cenocepacia*, acquires Rab7, LAMP1 and class II MHC molecules before lysosomal fusion. The BcCV is a not acidic compartment that also has low lysosomal activity, both features suggesting that the BcCV resembles the main features of MIIC before lysosomal fusion. Therefore, it is conceivable that bacterially-derived peptides within the BcCV are loaded into class II MHC molecules until
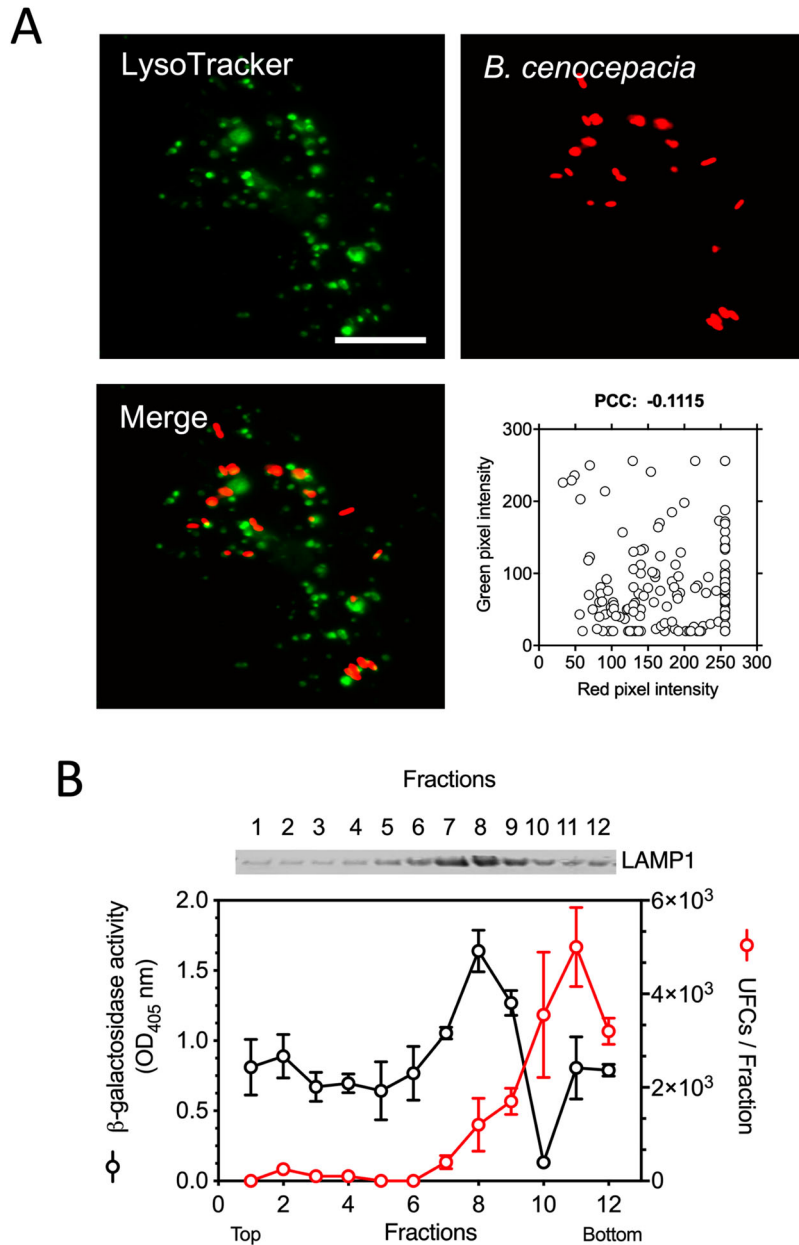
Figure 4. The BcCV is a non-acidic compartment. IFNγ-treated (300 U/ml) macrophages from C57Bl/6 mice were infected with *B. cenocepacia* (pDSRedT3) at a MOI of 50 during 1 h. At 3 h post-infection, infected macrophages were incubated with LysoTracker Green 1 μM (A). Images of infected macrophages were analyzed by immunofluorescence. Bars indicates 10 μm. The co-occurrence of green and red pixels was determined by Pearson's correlation coefficient (PCC). (B) Sucrose gradient purification of vacuoles from IC-21-like peritoneal macrophages from C57Bl/6 mice infected with *B. cenocepacia*. The CFUs (Black dots) and β-galactosidase activity (Red dots) were quantified from each gradient fraction. The expression of LAMP1 was determined by western blot. The average of two biological repeats are presented. Bottom correspond to the gradient fractions with high density and the Top to the gradient fractions with low density.

eventually the fusion with lysosomes leads to complete antigen degradation (Figure 8(A)). This model is consistent with our observation that compared to untreated cells, fewer bacteria (21 ± 17%) survive in IFNγ-treated macrophages. IFNγ pre-activated macrophages have an increased ability to process exogenous antigens due to increased expression of several IFNγ-induced endosomal proteases [36]. Likewise, early endosomes are also involved in exogenous antigen processing [45] due to the presence of IFNγ-induced lysosomal thiol reductase (GILT) [46]. In addition, IFNγ also promotes autophagy, a process by which macrophages control the proliferation of intracellular *B. cenocepacia* [47]. Indeed, autophagy increases the efficiency of antigen presentation by MHC class II molecules [48]. It has been reported that CD4 T-cells that recognize flagellin from *B. pseudomallei* can also recognize IFNγ-treated macrophages infected with viable *B. cenocepacia* J2315. Further, IFNγ-treated macrophages infected with heat-killed J2315 induce a stronger response than macrophages infected with live-J2315. This is expected since flagellin is an antigen present both in live and dead bacteria [49]. In contrast, our results show that IFNγ-treated macrophages infected
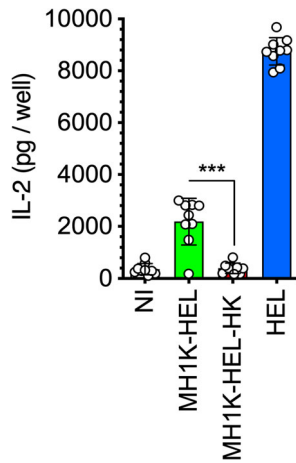
Figure 5. Macrophages infected with *B. cenocepacia* present bacterial antigens to CD4+ T-cells. Macrophages from C3H/HeJ mice were pre-treated with 300 U/ml of IFNγ prior to infection with *B. cenocepacia*-p*zmpA*-HEL (MH1K-HEL) at MOI of 50 during 1 h. Infected macrophages were co-cultured with C-10 cells, a CD4+ T-cell hybridoma during 24 h. Gray bar indicates the response of the C-10 hybridoma with non-infected cells (NI). Green bar indicates macrophages infected with MH1K-HEL, red bar indicates macrophages infected with heat-killed MH1K-HEL (MH1K-HEL-HK). Blue bar indicates the response of C-10 against macrophages pulsed with 250 μg/ml of HEL. The graph represents the results of 3 independent experiments in triplicate ($n = 9$), plotted as mean ±SD and analyzed by the paired t-test. **, $p=0.0038$; ns, non-significant.

with live *B. cenocepacia* present bacterial antigens more effectively than macrophages infected with dead bacteria, in agreement with the expression of ZmpA-pHEL$_{48-61}$ in live *B. cenocepacia*.

APCs can also process and present antigens by class I MHC molecules using alternative pathways. One of them, defined as cross-presentation [50,51], involves the transport of engulfed exogenous antigens from the vacuolar lumen into the cytosol (Figure 8(F)) and subsequently follows the classical pathway of antigen processing and presentation [21]. Another pathway involves the processing of the engulfed exogenous antigen within a vacuole [52]. We posit that the BcCV could be a vacuole for antigen processing. This is supported by our results demonstrating that macrophages infected with *B. cenocepacia* can present bacterial antigens by class I MHC molecules to CD8+ T-cells. The peptides generated by this mechanism could also stabilize post-Golgi class I MHC molecules (Figure 8(E)). As shown for other vacuoles, this pathway likely requires a peptide dissociation/exchange directly into the vacuole of antigen processing [53]. Further, peptides generated from the exogenous antigen processing could be released into the extracellular medium in which empty class I MHC molecules of self-cell or bystander-cells can be loaded by these peptides [23,34]. Our results indicate that *B. cenocepacia*-infected macrophages process the bacterium and the generated peptides are released into the extracellular media, stabilizing empty class I molecules of bystander-cells (Figure 8(D)).

In summary, this work illustrates the ability of *B. cenocepacia*-infected macrophages, when activated with IFNγ to process and present *B. cenocepacia* antigens by class I and class II MHC molecules to CD8+
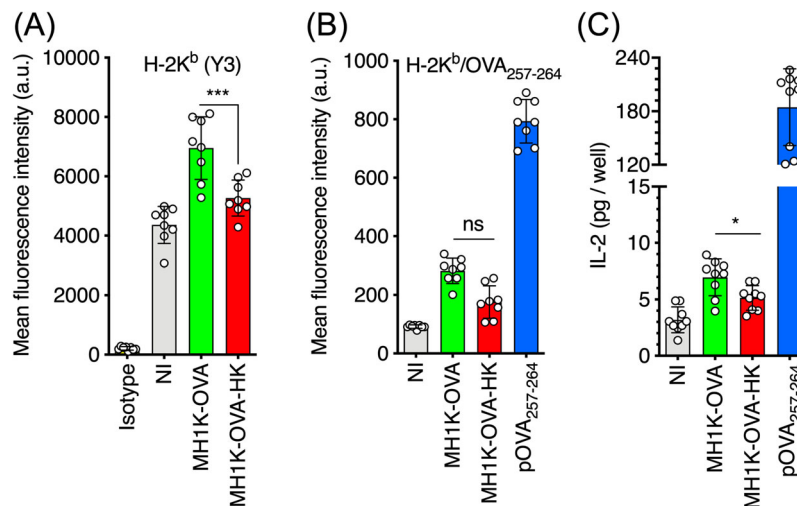

Figure 6. Macrophages infected with *B. cenocepacia* present bacterial antigens to CD8+ T-cells. Macrophages from C57Bl/6 mice were pre-treated with 300 U/ml of IFNγ and infected with *B. cenocepacia*-p*zmpA*-OVA (MH1K-OVA) or heat-killed (MH1K-OVA-HK) at MOI of 50 during 1 h. (A) Quantification of class I MHC H-2K^b/peptide complexes at 24 h post-infection. (B) Quantification of class I MHC molecules H-2K^b/pOVA$_{257-264}$ complexes at 24 h post-infection. (C) Infected macrophages were co-cultured with the RF33.70, a CD8+ T-cell hybridoma for 24 h. White bar indicates isotype antibody (A). Gray bars indicates non-infected cells (NI) (A, B), and response of RF33.70 hybridoma with non-infected cells (C). Green bars indicate macrophages infected with MH1K-OVA, red bars indicates macrophages infected with MH1K-OVA heat-killed (MH1K-OVA-HK). Blue bar indicates macrophages pulsed with100 ng/ml pOVA$_{257-264}$ (B) and the response of RF33.70 hybridoma against macrophages pulsed with 100 ng/ml of pOVA$_{257-264}$ (C). The results presented in panel A and B were obtained from 4 independent experiments, each one in duplicate ($n = 8$), and the result presented in panel C was obtained from 3 independent experiments, each one in triplicated ($n = 9$) and analyzed by one-way ANOVA with the Turkey multiple comparison test. The bars were plotted as mean ± SD. *, $p<0.05$; ns, non-significant.
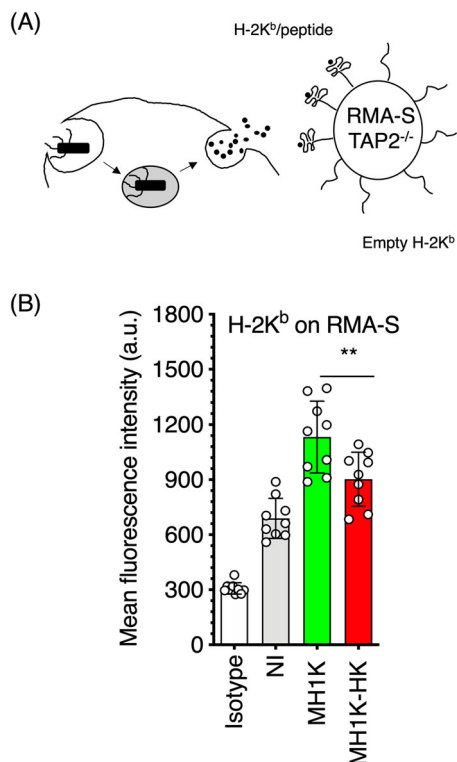
(A)

(B)

Figure 7. Macrophages infected with *B. cenocepacia* release products of bacterial processing into the extracellular media. Macrophages from C3H/HeJ mice (H-2K^k) were pre-treated with 300 U/ml of IFNγ and infected with *B. cenocepacia* (MH1 K) at a MOI of 50 during 1 h. (A) Infected macrophages were co-cultured with RMA-S (H-2K^b) cells at a 1:2 ratio during 24 h. (B) Class I MHC H-2K^b/peptide complexes were quantified by flow cytometry with Y3 (anti-H-2K^b/peptide) monoclonal antibody. White bar indicates isotype antibody (B). Gray bar indicates the quantification of class I MHC/peptides on RMA-S cells cocultured with non-infected macrophages (NI), green bars, RMA-S cells co-cultured with macrophages infected with live (MH1 K) and red bars, RMA-S cells co-cultured with macrophages infected with heat killed (MH1K-HK) bacteria. The results were obtained from 3 independent experiments, each one in triplicate ($n = 9$), plotted as the mean of fluorescence intensity ± SD, and analyzed by one-way ANOVA with the Turkey multiple comparison test. *, $p=0.0187$.

and CD4$^+$ T-cells, respectively. We also demonstrate that the BcCV plays a key role in this process by acquiring properties similar to the MIIC. The role of specific CD4$^+$ and CD8$^+$ T-cells during the induction of protective immunity against *B. cenocepacia* remains to be explored.

Acknowledgements

Disclosure statement

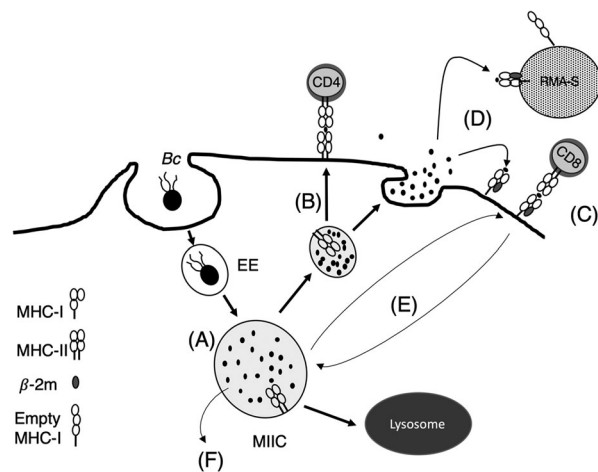No potential conflict of interest was reported by the author(s).

Figure 8. Model of antigen processing and presentation of *B. cenocepacia* by class I and class II MHC molecules to T-cells. (A) MIIC in which live *B. cenocepacia* is processed. (B) Bacterial antigens are recognized by CD4$^+$ T-cells or (C) CD8$^+$ T-cells. (D) Products of bacterial processing are released to the extracellular media. (E) Recycling of class I MHC from plasma membrane to the MIIC. (F) Translocation of bacterial antigens from the MIIC to the cytosol.

Funding

Author contributions

RRR conceived, designed and performed the experiments, analysed the data, and wrote the paper; PGV performed experiments and data analyses; DVV performed experiments and data analyses; RAT performed experiments and data analyses; DFA conceived, designed and performed the molecular cloning experiments; VON contributed reagents/materials/analysis tools and edited the manuscript; LCB contributed reagents/materials/analysis tools and edited the manuscript; JCCS contributed critical reagents/materials/analysis tools and provided advise; AOG contributed critical reagents/materials/analysis tools and provided advise; MAV analysed the data and critically revised and edited the manuscript; JISP conceived and designed the experiments, analysed the data, and edited the manuscript. All authors discussed ongoing results and commented on the manuscript.

ORCID

Roberto Rosales-Reyes http://orcid.org/0000-0001-9519-8766
Paola Garza-Villafuerte http://orcid.org/0000-0001-9102-2448
Daniela Vences-Vences http://orcid.org/0000-0003-1409-8646
Daniel F. Aubert http://orcid.org/0000-0003-2409-3654

Rubi Aca-Teutle 🆔 http://orcid.org/0000-0001-7833-4973
Vianney F. Ortiz-Navarrete 🆔 http://orcid.org/0000-0001-6546-9713
Laura C. Bonifaz 🆔 http://orcid.org/0000-0001-8482-5648
Julio Cesar Carrero-Sánchez 🆔 http://orcid.org/0000-0003-1055-5774
Alfonso Olivos-García 🆔 http://orcid.org/0000-0001-9864-2818
Miguel A. Valvano 🆔 http://orcid.org/0000-0001-8229-3641
José Ignacio Santos-Preciado 🆔 http://orcid.org/0000-0001-9298-2926

References

[1] Vandamme P, Dawyndt P. Classification and identification of the *Burkholderia cepacia* complex: past, present and future. Syst Appl Microbiol. 2011 Apr;34 (2):87–95.

[2] Blanchard AC, Tang L, Tadros M, et al. *Burkholderia cenocepacia* ET12 transmission in adults with cystic fibrosis. Thorax. 2020 Jan;75(1):88–90.

[3] Mahenthiralingam E, Vandamme P, Campbell ME, et al. Infection with *Burkholderia cepacia* complex genomovars in patients with cystic fibrosis: virulent transmissible strains of genomovar III can replace *Burkholderia multivorans*. Clin Infect Dis. 2001 Nov 1;33(9):1469–1475.

[4] Drevinek P, Mahenthiralingam E. *Burkholderia cenocepacia* in cystic fibrosis: epidemiology and molecular mechanisms of virulence. Clin Microbiol Infect. 2010 Jul;16(7):821–830.

[5] Valvano MA. Intracellular survival of *Burkholderia cepacia* complex in phagocytic cells. Can J Microbiol. 2015 Sep;61(9):607–615.

[6] Schwab U, Abdullah LH, Perlmutt OS, et al. Localization of *Burkholderia cepacia* complex bacteria in cystic fibrosis lungs and interactions with *Pseudomonas aeruginosa* in hypoxic mucus. Infect Immun. 2014 Nov;82(11):4729–4745.

[7] Saini L, Galsworthy S, John M, et al. Intracellular survival of *Burkholderia cepacia* complex isolates in the presence of macrophage cell activation. Microbiology. 1999;145:3465–3475.

[8] Lamothe J, Huynh KK, Grinstein S, et al. Intracellular survival of *Burkholderia cenocepacia* in macrophages is associated with a delay in the maturation of bacteria-containing vacuoles. Cell Microbiol. 2007 Jan;9 (1):40–53.

[9] Huynh KK, Plumb JD, Downey GP, et al. Inactivation of macrophage Rab7 by *Burkholderia cenocepacia*. J Innate Immun. 2010;2:522–533.

[10] Flannagan RS, Jaumouillé V, Huynh KK, et al. *Burkholderia cenocepacia* disrupts host cell actin cytoskeleton by inactivating Rac and Cdc42. Cell Microbiol. 2012;14:239–254.

[11] Rosales-Reyes R, Skeldon AM, Aubert DF, et al. The Type VI secretion system of *Burkholderia cenocepacia* targets multiple Rho family GTPases disrupting the actin cytoskeleton and the assembly of NADPH oxidase complex in macrophages. Cell Microbiol. 2012;14:255–273.

[12] Aubert DF, Xu H, Yang J, et al. A *Burkholderia* Type VI effector Deamidates Rho GTPases to activate the pyrin inflammasome and Triggers Inflammation. Cell Host Microbe. 2016;19:664–674.

[13] Keith KE, Hynes DW, Sholdice JE, et al. Delayed association of the NADPH oxidase complex with macrophage vacuoles containing the opportunistic pathogen *Burkholderia cenocepacia*. Microbiology. 2009;155:1004–1015.

[14] Bylund J, Burgess LA, Cescutti P, et al. Exopolysaccharides from *Burkholderia cenocepacia* inhibit neutrophil chemotaxis and scavenge reactive oxygen species. J Biol Chem. 2006 Feb 3;281 (5):2526–2532.

[15] Bylund J, Campsall PA, Ma RC, et al. *Burkholderia cenocepacia* induces neutrophil necrosis in chronic granulomatous disease. J Immunol. 2005 Mar 15;174 (6):3562–3569.

[16] Macdonald KL, Speert DP. Differential modulation of innate immune cell functions by the *Burkholderia cepacia* complex: *Burkholderia cenocepacia* but not *Burkholderia multivorans* disrupts maturation and induces necrosis in human dendritic cells. Cell Microbiol. 2008 Jul 3;10(10):2138–2149.

[17] Kotrange S, Kopp B, Akhter A, et al. *Burkholderia cenocepacia* O polysaccharide chain contributes to caspase-1-dependent IL-1beta production in macrophages. J Leukocyte Biol. 2011 Mar;89(3):481–488.

[18] Urban TA, Griffith A, Torok AM, et al. Contribution of *Burkholderia cenocepacia* flagella to infectivity and inflammation. Infect Immun. 2004 Sep;72(9):5126–5134.

[19] Weiss G, Schaible UE. Macrophage defense mechanisms against intracellular bacteria. Immunol Rev. 2015 Mar;264(1):182–203.

[20] Hume DA. Macrophages as APC and the dendritic cell myth. J Immunol. 2008 Nov 1;181(9):5829–5835.

[21] Neefjes J, Jongsma ML, Paul P, et al. Towards a systems understanding of MHC class I and MHC class II antigen presentation. Nat Rev Immunol. 2011 Nov 11;11 (12):823–836.

[22] Roche PA, Furuta K. The ins and outs of MHC class II-mediated antigen processing and presentation. Nat Rev Immunol. 2015 Apr;15(4):203–216.

[23] Martín-Orozco N, Isibasi A, Ortiz-Navarrete V. Macrophages present exogenous antigens by class I major histocompatibility complex molecules via a secretory pathway as a consequence of interferon-gamma activation. Immunology. 2001 May;103 (1):41–48.

[24] Hamad MA, Skeldon AM, Valvano MA. Construction of aminoglycoside-sensitive *Burkholderia cenocepacia* strains for use in studies of intracellular bacteria with the gentamicin protection assay. Appl Environ Microbiol. 2010 May;76(10):3170–3176.

[25] Craig FF, Coote JG, Parton R, et al. A plasmid which can be transferred between *Escherichia coli* and *Pasteurella haemolytica* by electroporation and conjugation. J Gen Microbiol. 1989 Nov;135:2885–2890.

[26] Rosales-Reyes R, Aubert DF, Tolman JS, et al. *Burkholderia cenocepacia* type VI secretion system mediates escape of type II secreted proteins into the cytoplasm of infected macrophages. PLoS ONE. 2012;7(7):e41726.

[27] Rosales-Reyes R, Sánchez-Gómez C, Ortiz-Navarrete V, et al. *Burkholderia cenocepacia* induces macropinocytosis to enter macrophages. Biomed Res Int. 2018 Apr 22;2018:4271560.

[28] Trost M, English L, Lemieux S, et al. The phagosomal proteome in interferon-γ-activated macrophages. Immunity. 2009 Jan 16;30(1):143–154.

[29] Herrero C, Marques L, Lloberas J, et al. IFN-γ-dependent transcription of MHC class II IA is impaired in macrophages from aged mice. J Clin Invest. 2001 Feb;107(4):485–493.

[30] Dunn KW, Kamocka MM, McDonald JH. A practical guide to evaluating colocalization in biological microscopy. Am J Physiol Cell Physiol. 2011 Apr;300 (4):C723–C742.

[31] Bonifaz L, Cervantes-Silva M, Ontiveros-Dotor E, et al. A role for mitochondria in antigen processing and presentation. Immunology. 2014 Sep 23;144(3):461–471.

[32] Allen PM, McKean DJ, Beck BN, et al. Direct evidence that a class II molecule and a simple globular protein generate multiple determinants. J Exp Med. 1985 Oct 1;162(4):1264–1274.

[33] De Haan L, Hearn AR, Rivett AJ, et al. Enhanced delivery of exogenous peptides into the class I antigen processing and presentation pathway. Infect Immun. 2002 Jun;70(6):3249–3258.

[34] Rosales-Reyes R, Alpuche-Aranda C, Ramirez-Aguilar Mde L, et al. Survival of *Salmonella enterica* serovar Typhimurium within late endosomal-lysosomal compartments of B lymphocytes is associated with the inability to use the vacuolar alternative major histocompatibility complex class I antigen-processing pathway. Infect Immun. 2005 Jul;73(7):3937–3944.

[35] Boehm U, Klamp T, Groot M, et al. Cellular responses to interferon-γ. Annu Rev Immunol. 1997;15:749–795.

[36] Zhou F. Molecular mechanisms of IFN-γ to up-regulate MHC class I antigen processing and presentation. Int Rev Immunol. 2009;28(3-4):239–260.

[37] Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. Nat Rev Immunol. 2013 Apr;13(4):227–242.

[38] Walpole GFW, Plumb JD, Chung D, et al. Inactivation of Rho GTPases by *Burkholderia cenocepacia* Induces a WASH-mediated actin Polymerization that delays Phagosome maturation. Cell Rep. 2020 Jun 2;31 (9):107721.

[39] Peters PJ, Neefjes JJ, Oorschot V, et al. Segregation of MHC class II molecules from MHC class I molecules in the Golgi complex for transport to lysosomal compartments. Nature. 1991 Feb 21;349(6311):669–676.

[40] Tulp A, Verwoerd D, Dobberstein B, et al. Isolation and characterization of the intracellular MHC class II compartment. Nature. 1994 May 12;369(6476):120–126.

[41] Viner NJ, Nelson CA, Deck B, et al. Complexes generated by the binding of free peptides to class II MHC molecules are antigenically diverse compared with those generated by intracellular processing. J Immunol. 1996 Apr 1;156(7):2365–2368.

[42] Kooi C, Corbett CR, Sokol PA. Functional analysis of the Burkholderia cenocepacia ZmpA metalloprotease [Research support, Non-U.S. Gov't]. J Bacteriol. 2005 Jul;187(13):4421–4429.

[43] Niedermann G, Butz S, Ihlenfeldt HG, et al. Contribution of proteasome-mediated proteolysis to the hierarchy of epitopes presented by major histocompatibility complex class I molecules. Immunity. 1995 Mar;2(3):289–299.

[44] Ortiz-Navarrete V, Hammerling GJ. Surface appearance and instability of empty H-2 class I molecules under physiological conditions. Proc Natl Acad Sci U S A. 1991 May 1;88(9):3594–3597.

[45] Guagliardi LE, Koppelman B, Blum JS, et al. Co-localization of molecules involved in antigen processing and presentation in an early endocytic compartment. Nature. 1990 Jan 11;343(6254):133–139.

[46] Phan UT, Arunachalam B, Cresswell P. Gamma-interferon-inducible lysosomal thiol reductase (GILT). maturation, activity, and mechanism of action. J Biol Chem. 2000 Aug 25;275(34):25907–25914.

[47] Assani K, Tazi MF, Amer AO, et al. IFN-γ stimulates autophagy-mediated clearance of *Burkholderia cenocepacia* in human cystic fibrosis macrophages. PLoS One. 2014;9(5):e96681.

[48] Crotzer VL, Blum JS. Autophagy and its role in MHC-mediated antigen presentation. J Immunol. 2009 Mar 15;182(6):3335–3341.

[49] Musson JA, Reynolds CJ, Rinchai D, et al. CD4+ t cell epitopes of FliC conserved between strains of *Burkholderia*: implications for vaccines against melioidosis and cepacia complex in cystic fibrosis. J Immunol. 2014 Dec 15;193(12):6041–6049.

[50] Guermonprez P, Saveanu L, Kleijmeer M, et al. ER-phagosome fusion defines an MHC class I cross-presentation compartment in dendritic cells. Nature. 2003 Sep 25;425(6956):397–402.

[51] Houde M, Bertholet S, Gagnon E, et al. Phagosomes are competent organelles for antigen cross-presentation. Nature. 2003 Sep 25;425(6956):402–406.

[52] Harding CV. Phagocytic processing of antigens for presentation by MHC molecules. Trends Cell Biol. 1995 Mar;5(3):105–109.

[53] Chefalo PJ, Harding CV. Processing of exogenous antigens for presentation by class I MHC molecules involves post-Golgi peptide exchange influenced by peptide-MHC complex stability and acidic pH. J Immunol. 2001 Aug 1;167(3):1274–1282.