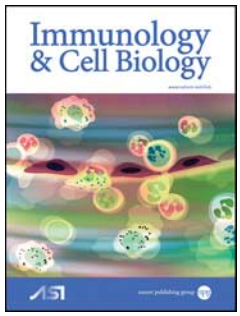

Inhibition of MHC-I by Brucella abortus is an early event during infection and involves EGFR pathway

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Cite this article as: Lis N Velásquez, M Ayelén Milillo, M Victoria Delpino, Aldana Trotta, M Florencia Mercogliano, Roberto G Pozner, Roxana Schillaci, Patricia V Elizalde, Guillermo H Giambartolomei, Paula Barrionuevo, Inhibition of MHC-I by Brucella abortus is an early event during infection and involves EGFR pathway, *Immunology and Cell Biology* accepted article preview 4 November 2016; doi: 10.1038/icb.2016.111.

Received 16 August 2016; revised 24 October 2016; accepted 25 October 2016; Accepted article preview online 4 November 2016

Inhibition of MHC-I by *Brucella abortus* is an early event during infection and involves EGFR pathway

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Running title: *B. abortus* inhibits MHC-I through EGFR pathway

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The authors declare no conflict of interest.

ABSTRACT

Brucella abortus is able to persist inside the host despite the development of potent $CD8^+$ T cell responses. We have recently reported the ability of *B. abortus* to inhibit the IFN-γ-induced MHC-I cell surface expression on human monocytes. This phenomenon was due to the *B. abortus*-mediated retention of MHC-I molecules within the Golgi apparatus and was dependent on bacterial viability. However, the implications of bacterial virulence or replicative capacity and the signaling pathways remained unknown. Here, we demonstrated that the *B. abortus* mutant strains RB51 and *virB10*$^-$ are able to inhibit MHC-I expression in the same manner as wild type *B. abortus*, even though they are unable to persist inside human monocytes for a long period of time. Consistent with this, the phenomenon was triggered early in time and could be observed at 8 h post-infection. At 24 h and 48 h it was even stronger. Regarding the signaling pathway, targeting EGF receptor (EGFR), ErbB2 (HER2) or inhibition of TACE, one of the enzymes which generates soluble EGF-like ligands, resulted in partial recovery of MHC-I surface expression. Moreover, recombinant EGF and TGF-α as well as the combination of both were also able to reproduce the *B. abortus*-induced MHC-I down-modulation. Finally, when infection was performed in the presence of an Erk1/2 inhibitor, MHC-I surface expression was significantly recovered. Overall, these results describe how *B. abortus* evades $CD8^+$ T cell responses early during infection and exploits the EGFR-ERK signaling pathway to escape from the immune system and favor chronicity.

INTRODUCTION

Host control of brucellosis requires a set of cells and components of the immune system which together promote a complex response against *Brucella* spp.[1]. Yet, from the many defensive resources that adaptive immunity brings into play, cytotoxic $CD8^+$ T cells are determinant to restrain *Brucella* infection. The importance of these cells resides in their capacity to eliminate *Brucella*-infected target cells [2, 3] . Previous studies in humans, mice and bovines have shown that specific $CD8^+$ T cells are developed during *Brucella* infection [1, 4], confirming the ability of *Brucella*-infected macrophages to present bacterial antigens on MHC-I molecules and to activate cytotoxic $CD8^+$ T cell responses. Despite this immune response *Brucella* can persist inside these cells establishing a chronic infection. Therefore, as a successful chronic and persistent pathogen *Brucella* must own an effective strategy to subvert the obnoxious challenge of highly outfitted $CD8^+$ T cells. In this regard, we have recently reported the ability of *B. abortus* to inhibit the IFN-γ-induced cell surface expression of MHC-I molecules on human macrophages [5]. As a consequence, *B. abortus* infected macrophages have diminished capacity of antigen presentation to $CD8^+$ T cells [5]. Furthermore, our results demonstrate that the inhibition of MHC-I molecules resides on the ability of *B. abortus* to prevent the transit of these molecules to the cell surface by retaining them within the Golgi apparatus [5]. Interestingly, MHC-I down-modulation mediated by *B. abortus* was dependent on bacterial viability as was demonstrated by the inability of heat-killed *B. abortus* to inhibit the expression of such molecules [5]. This last result led us to think that some aspects of bacterial metabolism, such as their virulence, could be involved in MHC-I inhibition. *B. abortus* virulence relies on the ability of this organism to enter, survive and replicate within vacuolar phagocytic compartments of macrophages [6]. Once inside the macrophage, *Brucella* dwells in an acidified compartment which fuses with components of

the early and late endosomal/lysosomes pathway [7, 8]. There, the vast majority of the ingested bacteria are rapidly killed. However, the establishment of a persistent infection depends on the ability of the bacterium to form a *Brucella*-containing vacuole (BCV), which traffics from the endocytic compartment to the endoplasmic reticulum (ER) [6, 7, 9]. Once inside the replicative BCV, bacteria are resistant to further attack and begin to multiply dramatically [7, 9]. *Brucella* also inhibits macrophage apoptosis [10], which in turn favors the pathogen's survival and replication.

Several key virulence factors have been described in various stages of the *Brucella* intracellular cycle among which LPS and the VirB type IV secretion system are included [11, 12]. In an attempt to assign functions to these virulence factors, it has been demonstrated that the LPS O-polysaccharide chain inhibits phagocytosis, protecting bacteria from the phagolysosome and inhibiting host cell apoptosis [10, 13] and that the VirB type IV secretion system is involved in controlling the maturation of the BCV into a replication permissive organelle [14]. As a consequence, it has been reported that rough strains of *Brucella* (which have LPS lacking the O-chain) and VirB-deficient *Brucella* have affected virulence compared to virulent smooth *Brucella* strains [7, 13, 14].

On the other hand, although our previous results showed that MHC-I down-modulation seems to occur in the whole cell population [5] it is well-known that *B. abortus*-infected monocytes constitute only 5-10% of all cells [7]. These results prompted us to think about a possible indirect mechanism taking place on the population and to investigate the potential factors or pathways involved in the phenomenon. ErbB receptors belong to a transmembrane tyrosine kinases receptor family which includes 4 members: the epidermal growth factor (EGF) receptor (EGFR; ErbB1), ErbB2 (HER2), ErbB3 (HER3) and ErbB4 (HER4) [15]. One possible mechanism which has been described to explain MHC-I inhibition is the induction of the EGFR pathway [16, 17]. This mechanism has been widely investigated in

several infectious pathologies, autoimmunity and tumors [16, 17]. Particularly with regard to viruses, in which the inhibition of MHC-I is a widely exploited immune subversion strategy [18], the activation of the EGFR pathway has been intimately linked to replicative and metabolically active microorganisms [19].

According to the above, there are a lot of unexplored aspects in the mechanism of MHC-I inhibition elicited by *B. abortus*. In this study we explore some features related to this phenomenon (implications of virulence or replicative capacity of the bacteria, kinetics and participation of the EGFR pathway) to elucidate more deeply how this key immune evasion strategy occurs. Our results indicate that *B. abortus* inhibits MHC-I independently of its virulence and its ability to persist inside the host cells. We also show that the phenomenon is an early event during infection and that the EGFR-ERK pathway is most likely involved.

RESULTS

Mutant strains of *B. abortus* incapable of persisting inside monocytes, down-modulate the IFN-γ-induced surface expression of MHC-I molecules by retaining them within the Golgi apparatus

It is a well-known fact that *B. abortus* mutant strains in key virulence factors, such as the LPS O-antigen or the VirB type IV secretion system, are incapable of surviving intracellularly despite their capacity of infecting cells [7, 13, 14]. We have recently reported that both *B. abortus* rough LPS mutant RB51 and a mutant in the *B. abortus* type IV secretion system VirB were capable of inhibiting the IFN-γ-induced MHC-I surface expression to the same extent as wild-type *B. abortus* [5]. In turn, we demonstrated that the inhibition of MHC-I resides in the ability of *B. abortus* to retain these molecules within the Golgi apparatus [5]. However, whether mutant strains RB51 and *virB10*$^{-}$ were also capable of reproducing this phenomenon remained to be elucidated. To evaluate this, the subcellular localization of MHC-I molecules was determined by confocal microscopy in THP-1 cells treated with IFN-γ and infected with *B. abortus* S2308 and the mutant strains RB51 and *virB10*$^{-}$. As expected, and even in the presence of IFN-γ, the 3 strains of *B. abortus* were able to infect human monocytes but only *B. abortus* S2308 was able to survive intracellularly and generate a stable infection (Figure 1a). Yet, confirming and extending our previous results, the 3 *B. abortus* strains were able to diminish the IFN-γ-induced surface expression of MHC-I molecules in a dose-dependent manner after 48 h (Figure 1b-d). Inhibition of IFN-γ-induced MHC-I expression was not due to a loss of cell viability in infected cultures (Supplementary Figure 1). Also, the phenomenon was reproducible in both human monocytes purified from peripheral blood and murine bone marrow-derived macrophages (BMM) (Supplementary Figure 3). Confocal microscopy experiments indicated that IFN-γ-treated THP-1 cells only showed MHC-I expression almost

exclusively confined to the cellular surface and no particular colocalization was detected with any of the subcellular compartments (Figure 1e). Meanwhile, cells infected with either *B. abortus* RB51 or *B. abortus virB10* mutant showed MHC-I expression restricted to the cell interior and colocalizing with the GA marker GM130 (Figure 1g and h). Therefore, both strains of *B. abortus* reproduce the intracellular retention of MHC-I molecules within the Golgi apparatus observed with the virulent strain *B. abortus* S2308 (Figure 1f-h). This MHC-I retention was observed in healthy cells with intact nucleus (Supplementary Figure 2). Overall, these results indicate that even though *B. abortus* mutant strains are unable to persist inside monocytes for a long period of time, they are still able to retain MHC-I molecules within the Golgi apparatus and inhibit their surface expression in the same manner as wild type *B. abortus*. This also suggests that MHC-I retention is triggered early during infection.

MHC-I surface down-regulation occurs at early time points during infection

Taking into account that the down-regulation of MHC-I expression seemed to be triggered at early points during infection, we then evaluated the kinetics of MHC-I down-modulation. For this, THP-1 cells were infected with different MOI of the 3 bacterial strains in the presence of IFN-γ and the surface expression of MHC-I was evaluated by flow cytometry at different times post-infection. In the 3 cases the down-regulation of IFN-γ-induced MHC-I expression begins to be observed at 8 h post-infection, especially at the higher MOI (Figure 2a-c). At 24 h the phenomenon becomes more evident and it even increases at 48 h post-infection (Figure 2a-c). These results corroborate that the inhibition of MHC-I surface expression mediated by *B. abortus* is a phenomenon which occurs early during infection.

The EGF receptor pathway is involved in the inhibition of MHC-I surface expression mediated by *B. abortus*

Our previous results had demonstrated that *B. abortus*-mediated MHC-I inhibition required bacteria to be metabolically active [5]. This fact had to be specially taken into account in the determination of the pathway involved in the phenomenon. Therefore, as mentioned before we focused on studying the epidermal growth factor (EGF) receptor (EGFR) pathway. The activation of this pathway results in the inhibition of MHC-I expression in several infectious pathologies [16, 17] and most importantly, it is triggered by metabolically active pathogens [19]. Thus, we first tested the presence of the ErbB family receptors in THP-1 cells and evaluated whether *B. abortus* infection was capable of modulating their expression. For this, THP-1 cells were infected with *B. abortus* S2308 in the presence of IFN-γ and the expression of EGFR, ErbB2, ErbB3 and ErbB4 was evaluated by Western Blot. We observed a slight expression of EGFR and ErbB2 in untreated or IFN-γ-treated cells which increased in the presence of *B. abortus* (Figure 3a and b). However, ErbB3 and ErbB4 expression was not detected in any of the experimental groups studied (data not shown). We also evaluated the phosporylation of ErbB receptors as a measure of their activation. Both EGFR and ErbB2 were phosphorylated in cells infected with *B. abortus* (Figure 3a and b), indicating that activation of these receptors occurs in the presence of infection. To evaluate if the EGF receptor pathway could be implicated in the MHC-I down-modulation, THP-1 cells were infected with *B. abortus* in the presence of a ligand-blocking antibody of the EGF receptor (Cetuximab). After that, the surface expression of MHC-I and subcellular localization of these molecules were evaluated by flow cytometry and confocal microscopy respectively. Neutralization of the EGF receptor, partially but significantly reversed the inhibition of MHC-I surface expression mediated by *B. abortus* (Figure 3c). Moreover, the treatment with Cetuximab reversed the retention inside the Golgi apparatus observed in *B. abortus*-infected cells (Figure 3f and g). While ErbB2 does not bind any of the ErbB ligands directly (unlike EGFR, ErbB3 and ErbB4), it is able to form heterodimers with EGFR and its catalytic activity

can potently amplify the signaling by these heterodimers via increasing ligand binding affinity and/or receptor recycling and stability [20, 21]. To determine the role of ErbB2 in the MHC-I down-modulation, we performed experiments in the presence of the ErbB2-targeted antibody Trastuzumab. Treatment with Trastuzumab of *B. abortus*-infected THP-1 cells also partially reversed, although to a lesser extent than Cetuximab, the inhibition of MHC-I surface expression and Golgi apparatus-retention mediated by *B. abortus* (Figure 3d, f and g). On the other hand, it has been well established that EGF-like ligands (i.e., TGF-α, EGF, amphiregulin) are synthesized as type I transmembrane proteins that are at first inserted into the plasma membrane. In order to be transformed into their soluble form they are cleaved by cell surface proteases, among which TNF-α-converting enzyme (TACE/ADAM17) is one of the most studied [22, 23]. Therefore, another approach to confirm the participation of the EGFR pathway is to inhibit this enzyme. For this, THP-1 cells were infected with *B. abortus* in the presence of the TACE inhibitor GM6001. The inhibition of TACE significantly reversed both the inhibition of MHC-I surface expression and the retention in Golgi apparatus mediated by *B. abortus* (Figure 3e-g). Overall, these results demonstrate that the EGF receptor pathway is involved in the intracellular retention of MHC-I mediated by *B. abortus*.

EGF-like ligands are soluble mediators involved in the MHC-I surface down-regulation mediated by *B. abortus*

EGF-like ligands can either be released to the extracellular milieu and act in an autocrine or paracrine manner or they can active their receptors directly when bound to the plasma membrane in a juxtacrine manner [22]. Our results observed with the TACE inhibitor suggest that EGF-like ligands can be effectively cleaved during *B. abortus* infection. Thus, we decided to evaluate whether soluble EGF and TGF-α could reproduce the *B. abortus*-induced MHC-I down-modulation. Confirming our results, exposure of THP-1 cells to recombinant

EGF and TGF-α also inhibited the IFN-γ-induced MHC-I surface expression (Figure 4a and b) by retaining these molecules within the Golgi apparatus (Figure 4c and d), although to a lesser extent than *B. abotus* infection. Furthermore, the combination of EGF and TGF-α was even more potent in inducing both phenomena (Figure 4a-d). We next evaluated whether supernatants from *B. abortus*-infected cells were able to inhibit MHC-I expression in non-infected cells. Corroborating our results, supernatants from THP-1 cells infected with the 3 *Brucella* strains (*B. abortus* S2308, RB51 and *virB10*$^{-}$) had this capacity and could even inhibit MHC-I expression more efficiently than cells treated with recombinant EGF-like ligands, indicating that they could probably contain a combination of these ligands/agonists (Figure 4e). Moreover, the effect of these supernatants on MHC-I surface expression was significantly reverted in the presence of Cetuximab or Trastuzumab (Figure 4f). Overall, these results demonstrate that supernatants from infected cells inhibit MHC-I expression in non-infected cells and EGF-like ligands secreted by *B. abortus* could be soluble mediators implicated in this phenomenon.

The MHC-I surface down-regulation mediated by *B. abortus* is dependent on EGFR-ERK signaling pathway

MAPK play a key role in the modulatory effects mediated by the EGFR [24, 25]. In particular, the ERK pathway was well established in several EGFR-induced phenomena [19]. Recently, the ERK pathway has been described as a predominant regulator of MHC-I expression, indeed a strong inverse correlation was found between p-Erk and expression of class I molecules [26]. Thus, to gain insight into the signaling pathways downstream of EGFR we investigated the role of Erk1/2, p38 and JNK MAPK in the inhibition of MHC-I mediated by *B. abortus*. For this, infection of THP-1 cells was performed in the presence of specific inhibitors of MAPK signaling pathways. We used the inhibitors PD98059, SB203580 and SP600125, to inhibit

Erk1/2, p38 and Jnk1/2 respectively. The inhibition of MHC-I surface expression mediated by *B. abortus* was significantly reversed by the Erk1/2 inhibitor (Figure 5). Conversely, inhibition of p38 or Jnk1/2 had no effect on *B. abortus*-induced MHC-I surface down-modulation (Figure 5). These results indicate that the inhibition of MHC-I mediated by *B. abortus* depends on the activation of the EGFR-ERK signaling pathway.

DISCUSSION

Intracellular bacteria can display a number of strategies to evade or counteract the immune response of the host, in order to succeed in the generation of a long-lasting infection. For this, they require a battery of virulence factors capable of modulating in a very precise manner the immune system of the host, primarily avoiding the innate immunity, preventing the appropriate mounting of adaptive immunity and finally establishing a chronic phase of infection [27]. An important member of this group of organisms is *Brucella* spp. Although information about *Brucellae* intracellular replication and trafficking has notably increased in the last years, little is known about how *Brucella* achieves a chronic state of infection. Recently, we demonstrated that *B. abortus* is capable of down-modulating the IFN-γ-induced surface expression of MHC-I on human monocytes. Moreover, *B. abortus* is able to inhibit antigen presentation to $CD8^+$ T cells [5]. This mechanism could explain how *Brucella*-containing macrophages can avoid the immunological surveillance of MHC-I restricted cytotoxic $CD8^+$ T cells, thus promoting a prolonged infection. However, several relevant aspects from the *B. abortus*-mediated MHC-I inhibition, such as the requirement of bacterial virulence, the time during infection at which the phenomenon occurs and the possible factors or pathways involved, remained to be elucidated. In this study our findings demonstrate that *B. abortus* early during infection and independently of its virulence or replicative capacity generates the activation of the EGFR-ERK pathway. This in turn leads to the retention of MHC-I molecules within the Golgi apparatus and the consequent inhibition of the surface expression of these molecules (Figure 6).

With regard to the physiological mechanisms of MHC-I retention within the Golgi apparatus, we could mention some points. The detection of endogenous MHC-I within the Golgi apparatus with mAb W6/32, which recognizes properly folded MHC-I molecules in

association with b2-microglobulin [28], indicates that MHC-I molecules are properly assembled. This suggests that the arrest of MHC-I transport occurs following MHC-I complex formation in the ER and is instead a fault in cellular transport across the Golgi compartments. In fact, Sohn *et al* described that CD99, a transmembrane protein expressed on all leukocytes, endothelial cells but mainly in thymocytes may be associated with the post-Golgi trafficking machinery by regulating the transport to the plasma membrane of surface MHC-I molecules [29], which provides a novel mechanism for immune escape. Moreover, it is widely known that Rab GTPases are a large family of small GTP-binding proteins that are essential for controlling and coordinating intracellular traffic [30], so it could be possible that *B. abortus* might affect the activities of these GTPases during its lifecycle. On the other hand, we previously demonstrated that treatment of IFN-γ-stimulated cells with the ionophore monensin (an inhibitor of Golgi acidification) resulted in the retention of MHC-I in the Golgi apparatus, with a concordant down-regulation of surface MHC-I [5]. Taking into account the similar functional effect of monensin and *B. abortus*, we speculate that *B. abortus* causes distress and retention of the MHC-I complex within the Golgi apparatus by altering its pH. Although *B. abortus* never traffics through the Golgi apparatus in its life cycle, we speculate that when present in the ER in the replicative phase of the cycle, the bacterium could release proteins that by interacting with nascent MHC-I molecules could transit through the endomembrane compartments of the ER and Golgi apparatus and inhibit the vacuolar ATPase [31], causing in this way an alteration on the acidification of the Golgi apparatus and the retention of MHC-I molecules, as has been described for other pathogens [32, 33].

Over the years, several *Brucella* mutant strains in key virulence factors necessary for the survival in host cells have been described. Among them RB51 and *virB10*$^{-}$ strains, LPS and VirB type IV secretion system mutants respectively, have been extensively studied [11, 12]. RB51 strain is a rough O-polysaccharide chain-deficient mutant derived from the S2308 strain

and it is employed as a vaccine for cattle [34]. The LPS O-polysaccharide chain is a key virulence factor for these bacteria because it plays a role in the lipid-rafts-dependent entry into macrophages and also in early bacterial survival because it delays vacuole fusion with lysosomes [13]. Meanwhile, the *virB10*$^{-}$ mutant is unable to assemble the type IV secretion system into the bacterial membrane, which is absolutely necessary for the efficient evasion of the BCV from the phagocytic route [35, 36]. One important question that arises from our results is why the lack of these two key virulence factors does not affect MHC-I inhibition. The answer still eludes us but we can attempt a couple of hypothesis. On the one hand, the absence of a certain virulence factor does not necessarily imply that all other evasion strategies are also eliminated. In fact, evasion strategies are known to be abundant and in some cases even redundant for several organisms [37, 38]. Therefore, a mutant strain in a key virulence factor might elicit other immune evasion mechanisms in order to compensate and have a better chance of surviving, even when in the end it is eliminated [39]. Another possibility could be that MHC-I down-modulation is provoked either by a bacterial component shared by all *Brucella* strains or by a host cellular factor/pathway triggered by bacterial infection, a so called collateral effect.

Two striking features of this phenomenon allow us to shed light on which of these explanations is better suited. The first, which is derived from a previous publication, is that heat-killed *B. abortus* is incapable of inhibiting MHC-I expression [5]. The second is that the phenomenon is triggered early during infection. Together, these results indicate that only metabolically active viable bacteria can inhibit MHC-I expression and that it must occur during the time span before the bacteria are removed, and/or mediated by a product generated early in response to infection. Furthermore, we previously demonstrated that the inhibition of MHC-I molecules induced by *B. abortus* occurs in the whole cell population [5] although the level of monocyte infectivity by these bacteria is only of about 5-10% [7]. Thus, this led us to

think about a possible indirect mechanism shared by all 3 *Brucella* strains and to investigate the potential factors or pathways involved in the phenomenon.

Intracellular bacteria share multiple immune evasion strategies with several viruses and tumors. Particularly inhibition of MHC-I expression is a strategy greatly exploited in those types of pathologies [18, 40, 41]. The mechanisms described so far to achieve this goal are diverse, however there are several evidences demonstrating the importance of the EGFR and ErbB2 (HER2) signaling pathway in MHC-I inhibition. It was recently reported that the EGFR pathway is involved in the expression of class II transactivator (CIITA), MHC-I and MHC-II molecules [16, 17]. Pollack *et al*. described that the use of EGFR inhibitors is capable of increasing the basal and IFN-γ-induced expression of MHC-I and MHC-II molecules in primary and malignant human keratinocytes [16]. The association between EGFR and the expression of these molecules was based on the ability of IFN-γ to induce the transactivation of EGFR [42]. While IFN-γ could be in part responsible for the activation of this pathway, multiple receptors belonging to the TLR family are also able to generate the same effect [43]. Particularly in the case of rhinoviruses, activation of the EGFR pathway has been linked to signaling through TLR3, specifically by metabolically active microorganisms with the ability to replicate [19]. On the other hand, exists an inverse correlation between MHC-I expression and HER2 signaling, and the overexpression of functional HER2 severely impairs MHC-I expression and CTL-mediated recognition of several MHC-I-restricted tumor antigens [44]. In line with these evidences, we hypothesized that the EGFR pathway could be implicated in the inhibition of MHC-I mediated by *B. abortus*. Consistent with this, expression and phosphorylation of EGFR and ErbB2 receptors was increased in cells infected with *B. abortus*. More importantly, both Cetuximab and Trastuzumab were able to reverse the *B. abortus*-mediated inhibition of MHC-I surface expression and its intracellular retention within the Golgi apparatus. Moreover, the use of an inhibitor for the TACE enzyme was also able to

reverse both phenomena. This latter result reinforces the participation of the EGFR pathway given that TACE is a metalloprotease present in the cell surface and responsible for cleaving EGFR ligands, which are first synthesized as transmembrane proteins [22, 23].

Treatment with exogenous recombinant EGF and TGF-α also inhibited the surface expression of MHC-I molecules and induced their intracellular retention within the Golgi apparatus similar to *B. abortus*. The attenuating ability of these two EGFR ligands in IFN-γ-mediated responses has been previously reported [45]. For its part, TACE has been implicated in the shedding of epiregulin, TGF-α, amphiregulin, and heparin-binding EGF-like growth factor [46]. Thus, the observation that the combination of EGF plus TGF-α potentiates both phenomena (MHC-I surface expression inhibition and intracellular retention of these molecules) is in agreement with these evidences and allowed us to explain the increased efficacy of the supernatants of infection to inhibit MHC-I expression, since these supernatants could contain a combination of EGFR ligands. There at least 11 known members of the ErbB family ligands, 7 of which (among them EGF and TGF-α) can bind to the EGFR. Given the number of important cellular processes in which these receptors are involved, the complexity of the network is not surprising nor the fact that redundancy among them is very common [47].

There are several signal transduction pathways that can be induced downstream from EGFR and ErbB2 activation. Among them MAPK, PI3K, phospholipase C and STAT1 are the best known [24, 25]. Particularly, ERK activation was associated with MHC-I inhibition [26]. Mimura *et al* reported that inhibition of the MAPK pathway induced up-regulation of MHC-I expression in parallel with an enhanced target sensitivity to tumor Ag-specific CTL lysis [26]. Moreover, a strong inverse correlation between p-Erk expression and MHC-I expression was observed in clinical tumor samples [26]. On the other hand, it was recently demonstrated that TLR2-dependent ERK signaling in *Mycobacterium tuberculosis*-infected macrophages drives the inhibition of MHC-II expression and antigen presentation [48]. This led us to investigate

whether ERK signaling pathway could be involved in the *B. abortus*-mediated MHC-I inhibition through EGFR. Consistent with the mentioned evidences, a specific inhibitor of Erk1/2 pathway, but not p38 and JNK inhibitors, reversed the inhibition of MHC-I mediated by *B. abortus*.

To our knowledge, this is the first report describing that a bacterium makes use of the EGFR pathway to inhibit MHC-I expression. Overall, the results obtained in this study show that *B. abortus* inhibits MHC-I expression early during infection employing an EGFR-ERK-dependent mechanism, even in the absence of key virulence factors. Thus, here we provide new insights about how *B. abortus* is able to avoid being recognized and eliminated by $CD8^+$ T cells and therefore persist in its host, allowing the establishment of a chronic infection.

METHODS

Bacteria

B. abortus S2308, *B. abortus* RB51 and *B. abortus virB10* mutant were cultured in tryptose-soy agar supplemented with yeast extract (Merck). The number of bacteria on stationary-phase cultures was determined by comparing the OD at 600 nm with a standard curve. All live *Brucella* manipulations were performed in biosafety level 3 facilities, located at the Instituto de Investigaciones Biomédicas en Retrovirus y SIDA (Buenos Aires, Argentina).

Reagents

Antibodies targeting EGFR (Cetuximab) or ErbB2 (Trastuzumab) were purchased from Merck Serono and Hoffmann-La Roche Ltd, respectively. TNF-α-converting enzyme (TACE) inhibitor (GM6001) and MAPK inhibitors (PD98059: Erk1/2 inhibitor; SB203580: p38 inhibitor; SP600125: Jnk1/2 inhibitor) were obtained from Calbiochem. Recombinant EGF and TGF-α were purchased from PeproTech.

Cells and media

All experiments were performed at 37°C in 5% CO_2 atmosphere and standard medium composed of RPMI-1640 supplemented with 25 mM Hepes, 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum (Gibco), 100 U of penicillin/ml and 100 µg of streptomycin/ml. THP-1 cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured as previously described [49]. To induce maturation, cells were cultured in 0.05 µM 1,25-dihydroxyvitamin D_3 (Calbiochem) for 72 h. Peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque (GE Healthcare) gradient centrifugation from human blood collected from healthy adult individuals. Monocytes were then purified from

PBMCs by Percoll gradient (GE Healthcare) and resuspended in standard medium. Purity of the isolated $CD14^+$ monocytes was more than 80% as determined by flow cytometry. Viability of cells was more than 95% in all the experiments as measured by trypan blue exclusion test. Mouse bone marrow-derived macrophages (BMM) were generated by differentiation of bone marrow progenitors from C57BL/6 mice with rM-CSF (PeproTech), as previously described [50].

***In vitro* infection**

THP-1 cells at a concentration of 0.5 x 10^6/ml were infected in round-bottom polypropylene tubes (Falcon) with different multiplicities of infection (MOI) of *B. abortus* in the presence of 150 U/ml IFN-γ (Endogen) for 2 h in standard medium containing no antibiotics. Then cells were extensively washed to remove uninternalized bacteria and infected cells were maintained in culture in the presence of IFN-γ, 100 µg/ml gentamicin and 50 µg/ml streptomycin for an additional 8, 24 or 48 h. At different times post-infection (8, 24 or 48 h), the supernatants from infected cells were harvested and sterilized by filtration through a 0.22 µm nitrocellulose filter, and used to stimulate non-infected THP-1 cells. Supernatants were diluted 1/5 or 1/2 in complete medium.

***Brucella* intracellular survival**

After *B. abortus* infection, cells were washed three times with sterile PBS and lysed with a sterile solution of 0.1% (vol/vol) Triton X-100 in H_2O. Serial dilutions of lysates were rapidly plated on tryptic soy agar plates to enumerate CFU.

Apoptosis assay

THP-1 cells, at a concentration of 0.5 x 10^6/ml, were infected with different MOI of *B. abortus* in the presence of IFN-γ for 48 h. THP-1 cells treated with 2% paraformaldehyde (PFA) were also included as positive control. Cells were washed and incubated with Annexin V-FITC and Propidium Iodide (BD Biosciences) for 10 min on ice in darkness. Cells were evaluated in the quadrants of Annexin V^+/PI^- (early apoptosis), Annexin V^+/PI^+ (late apoptosis) and Annexin V^-/PI^+ (necrosis). In another set of experiments, cells were stained with FITC-labelled anti-human HLA-ABC (clone G46-2.6; BD Pharmingen), washed and incubated with 7-Amino-Actimycin D (7-AAD; BD Biosciences) for 10 min on ice in darkness. MHC-I expression was evaluated gating on viable cells (7-AAD negative cells). In both cases, cells were analyzed on a FACSCalibur® flow cytometer (BD Biosciences) and data were processed using CellQuest software (BD Biosciences).

Flow cytometry

After *B. abortus* infection, or treatment with EGF-like ligands or supernatants from *B. abortus*-infected cells; THP-1 cells or human primary monocytes were stained with FITC-labelled anti-human HLA-ABC (clone G46-2.6; BD Pharmingen) or isotype-matched control mAbs. In the experiments with murine macrophages, BMM were infected with *B. abortus* in the presence of 10 ng/ml recombinant murine IFN-γ (PeproTech) for 48 h. To determine MHC-I surface expression, cells were stained with FITC-labelled anti-mouse $H\text{-}2K^d/H\text{-}2D^d$ (clone 34-1-2S; BioLegend). After labelling, cells were analyzed on a FACSCalibur® flow cytometer (BD Biosciences) and data were processed using CellQuest software (BD Bioscience).

Confocal microscopy

THP-1 cells were incubated in chamber-slides (2 x 10^5 cells/well) with 10 ng/ml PMA (Sigma-Aldrich) for 24 h to promote adherence. Then, cells were infected with *B. abortus* S2308, RB51 or *virB10*$^-$ (MOI 100:1) in the presence of IFN-γ for 48 h, fixed with 2% paraformaldehyde, permeabilized with 0.1% saponin and incubated with anti- HLA class I mAb W6/32 (purified from murine hybridoma culture supernatants) and Alexa 546-labelled secondary Ab (Invitrogen). Subcellular compartments were detected using mAbs specific for EEA1 (early endosomes), LAMP-2 (late endosomes/lysosomes), GM130 (Golgi) and calnexin (ER) (BD Biosciences) following Alexa 488-labelled secondary Ab (Invitrogen). For nuclear staining, TO-PRO®-3 (Invitrogen) was used. Slides were mounted with PolyMount (Polysciences) and analyzed using FV-1000 confocal microscope with an oil-immersion Plan Apochromatic 60X NA1.42 objective (Olympus).

Western Blot

THP-1 cells at a concentration of 1 x 10^6 cells/ml were used. Cells were left untreated, treated with IFN-γ or they were infected with different MOI of *B. abortus* in the presence of IFN-γ as described before. Following the different experimental treatments, cells were lysed in an ice-cold buffer composed of 150 mM NaCl, 10 mM Tris, 5 mM EDTA, 1% SDS, 1% Triton X-100, 1% sodium deoxycholate, gentamicin/streptomycin, 0.2% azide plus a protease inhibitor cocktail (Sigma-Aldrich). Lysates were incubated on ice for 2 h and cleared by centrifugation for 15 min at 14,000 rpm at 4°C. Protein concentrations were determined using the BCA protein assay (Pierce). Equal amounts of protein (20 µg) were then resolved on a 7.5% SDS-PAGE. After electrophoresis, proteins were transferred to Hybond-ECL nitrocellulose membranes (Amersham) for 1 h at 300 mA and blocked with 5% milk protein-0.1% Tween 20 overnight at 4°C. Membranes were then probed with primary anti-human EGFR Ab (1:1000 dilution) (clone 1005; Santa Cruz Biotechnology), anti-human ErbB2 Ab (1:1000

dilution) (clone C-18; Santa Cruz Biotechnology), anti-human Phospho-EGFR (Tyr1068) Ab (1:1000 dilution) (clone D7A5; Cell Signaling Technology), or anti-human Phospho-ErbB2 (Tyr877) Ab (1:1000 dilution) (Polyclonal antibodies; Cell Signaling Technology) overnight. After washing three times with TBS-0.05% Tween 20, blots were incubated for 1 h with a HRP-conjugated goat anti-rabbit IgG Ab (Santa Cruz Biotechnology). Immunoreactivity was detected using ECL Western Blotting Substrate (Pierce). Protein bands were visualized using Kodak Medical X-Ray General Purpose Film. For internal loading controls, membranes were stripped by incubating for 10 min twice in buffer consisting of 1.5% Glycine, 0.1% SDS, 1% Tween 20, pH 2.2 and then reprobed with anti-β-actin Ab (1:2000 dilution) (clone AC-15; Ambion). Results from Western Blot were analyzed by densitometric analysis (Image J software).

Statistical analysis

Results were analyzed with one-way ANOVA followed by *post hoc* Tukey test using the GraphPad Prism software.

ACKNOWLEDGEMENTS

We thank Horacio Salomón and the staff at the Instituto de Investigaciones Biomédicas en Retrovirus y SIDA (Universidad de Buenos Aires) for allowing us the use of the biosafety level 3 laboratory facilities. We thank Dr. Comerci for the cession of the *Brucella virB* mutant. This work was supported by grants PICT 2011-1420, 2011-1200, 2012-2252, 2013-0162 and 2014-1925 from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT-Argentina), by grants PIP 1390 and 0373 from CONICET (Argentina), by grants UBACYT 20020130200030 and 20020120100128, and by grant from Fundación Alberto J. Roemmers (2012-2014) (Argentina).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. *B. abortus* S2308, RB51 and *virB10*$^{-}$ inhibit the IFN-γ-induced expression of MHC-I and retain MHC-I molecules inside the Golgi apparatus even though *B. abortus* S2308 is the only able to persist intracellularly. (**a**) THP-1 cells were infected with *B. abortus* S2308, RB51 and *virB10*$^{-}$ (MOI 100:1) in the presence or not of IFN-γ. At different times post-infection, cells were lysed to determine the number of viable intracellular bacteria by plating lysates on tryptic soy agar. (**b**-**d**) THP-1 cells were infected with *B. abortus* S2308 (**b**), RB51 (**c**) and *virB10*$^{-}$ (**d**) at different MOI in the presence of IFN-γ for 2 h, washed and cultured in the presence of IFN-γ for 48 h. MHC-I expression was assessed by flow cytometry. (**e**-**h**) Confocal micrographs of THP-1 cells treated with IFN-γ (**e**) or infected with *B. abortus* S2308 (**f**), RB51 (**g**) or *virB10*$^{-}$ (**h**) (MOI 100:1) in the presence of IFN-γ for 48 h and then stained with a primary anti-human MHC-I Ab (W6/32) and Alexa 546-labelled secondary Ab (red). Subcellular localization markers were detected using mAbs specific for EEA1 (early endosomes), LAMP-2 (late endosomes/lysosomes), GM130 (Golgi) and calnexin (ER) followed by Alexa 488-labelled secondary Ab (green). White arrows show colocalization (yellow staining). Results are representative of three independent experiments.

Figure 2. Inhibition of MHC-I expression occurs early on during infection. (**a**-**c**) THP-1 cells were infected with *B. abortus* S2308 (**a**), RB51 (**b**) and *virB10*$^{-}$ (**c**) at different MOI in the presence of IFN-γ for 2 h, washed and cultured in the presence of IFN-γ for 8, 24 and 48 h. MHC-I expression was assessed by flow cytometry. Bars indicate the arithmetic means ± SEM of three experiments. MFI, mean fluorescence intensity. **P*<0.05; ***P*<0.01; ****P*<0.001 *vs*. IFN-γ.

Figure 3. The EGF receptor pathway is involved in the *B. abortus*-mediated inhibition of MHC-I expression. (**a** and **b**) THP-1 cells were infected with *B. abortus* S2308 at different MOI in the presence of IFN-γ for 2 h, washed and cultured in the presence of IFN-γ for 48 h. Total-cell extracts were obtained and EGFR (**a**), ErbB2 (**b**) and their phosphorylated versions (p-EGFR and p-ErbB2 respectively) expression was determined by Western Blot. β-actin was determined as loading control. Bars correspond to the densitometric analysis ± SEM from three independent experiments performed. (**c**-**e**) THP-1 cells were infected with *B. abortus* S2308 in the presence of antibodies targeting EGFR (Cetuximab) (**c**) or ErbB2 (Trastuzumab) (**d**), the TACE inhibitor (GM6001) (**e**), an isotype-matched control or the corresponding negative control (Nc) for 48 h. MHC-I expression was assessed by flow cytometry. Bars indicate the arithmetic means ± SEM of three experiments. MFI, mean fluorescence intensity. (**f**) Confocal micrographs of THP-1 cells infected with *B. abortus* S2308 (MOI 100:1) in the presence of IFN-γ and Cetuximab, Trastuzumab or GM6001 for 48 h. As negative control representative micrographs with Cetuximab isotype are shown. MHC-I expression was determined with a primary anti-human MHC-I Ab (W6/32) and Alexa 546-labelled secondary Ab (red). Golgi apparatus was detected using a mAb specific for GM130 followed by Alexa 488-labelled secondary Ab (green). White arrow shows colocalization (yellow staining). Results are representative of three independent experiments. (**g**) Quantification of MHC-I retention. Data are expressed as percentage of cells with MHC-I retained ± SEM of three independent experiments. The number of cells counted per experimental group was 200. $^{\Delta}P<0.05$; $^{\Delta\Delta}P<0.01$ *vs.* Untreated; ***$P<0.001$ *vs.* IFN-γ. $^{\#}P<0.05$; $^{\#\#}P<0.01$; $^{\#\#\#}P<0.001$ *vs. B. abortus* + IFN-γ + Isotype or Nc accordingly.

Figure 4. EGF-like ligands are soluble mediators involved in the MHC-I inhibition induced by *B. abortus*. (**a** and **b**) THP-1 cells were cultured in the presence of IFN-γ and EGF (25

ng/ml), TGF-α (50 ng/ml) or the combination of both for 48 h. MHC-I expression was assessed by flow cytometry. Bars indicate the arithmetic means ± SEM of three experiments. (**c**) Confocal micrographs of THP-1 cells incubated in the presence of IFN-γ and EGF, TGF-α or both for 48 h. MHC-I expression was determined with a primary anti-human MHC-I Ab (W6/32) and Alexa 546-labelled secondary Ab (red). Golgi apparatus was detected using a mAb specific for GM130 followed by Alexa 488-labelled secondary Ab (green). White arrows show colocalization (yellow staining). Results are representative of three independent experiments. (**d**) Quantification of MHC-I retention. Data are expressed as percentage of cells with MHC-I retained ± SEM of three independent experiments. The number of cells counted per experimental group was 200. (**e** and **f**) THP-1 cells were treated with supernatants from *B. abortus* S2308, RB51 or *virB10*⁻-infected cells (MOI 100:1) for 48 h, in the absence (**e**) or presence of Cetuximab or Trastuzumab (**f**). MHC-I expression was assessed by flow cytometry. Bars indicate the arithmetic means ± SEM of three experiments. MFI, mean fluorescence intensity. $^{**}P<0.01$; $^{***}P<0.001$ *vs*. IFN-γ. $^{\#}P<0.05$; $^{\#\#}P<0.01$ *vs*. EGF or TGF-α + IFN-γ accordingly. $^{\nabla}P<0.05$; $^{\nabla\nabla}P<0.01$; $^{\nabla\nabla\nabla}P<0.001$ *vs*. SN + IFN-γ + Isotype.

Figure 5. MHC-I down-regulation mediated by *B. abortus* is dependent on the EGFR-ERK signaling pathway. THP-1 cells were infected with different MOI of *B. abortus* S2308 in the presence of IFN-γ and inhibitors of MAPK pathways (PD98059: Erk1/2 inhibitor; SB203580: p38 inhibitor; SP600125: Jnk1/2 inhibitor) or vehicle [dimethyl sulfoxide (DMSO)]. MHC-I expression was assessed by flow cytometry. Bars indicate the arithmetic means ± SEM of three experiments. MFI, mean fluorescence intensity. $^{**}P<0.01$; $^{***}P<0.001$ *vs*. IFN-γ. $^{\#\#\#}P<0.001$ *vs. B. abortus* + IFN-γ + DMSO.

Figure 6. Proposed model for the MHC-I surface down-regulation mechanism mediated by *B. abortus*. 1. Infection with *B. abortus* induces the secretion of EGF-like ligands. 2. EGF-like ligands such as EGF and TGF-α bind ErbB receptors on the cell surface and cause their activation. 3. ErbB receptors then signal through the ERK1/2 pathway. 4. These effects finally cause the retention of MHC-I molecules within the Golgi apparatus. 5. MHC-I molecules are therefore unable to reach the cell surface and present bacterial Ags to $CD8^+$ T cells. 6. Inhibition of Ag presentation enables the bacteria to hide inside macrophages and avoid the cytotoxic function of $CD8^+$ T cells.

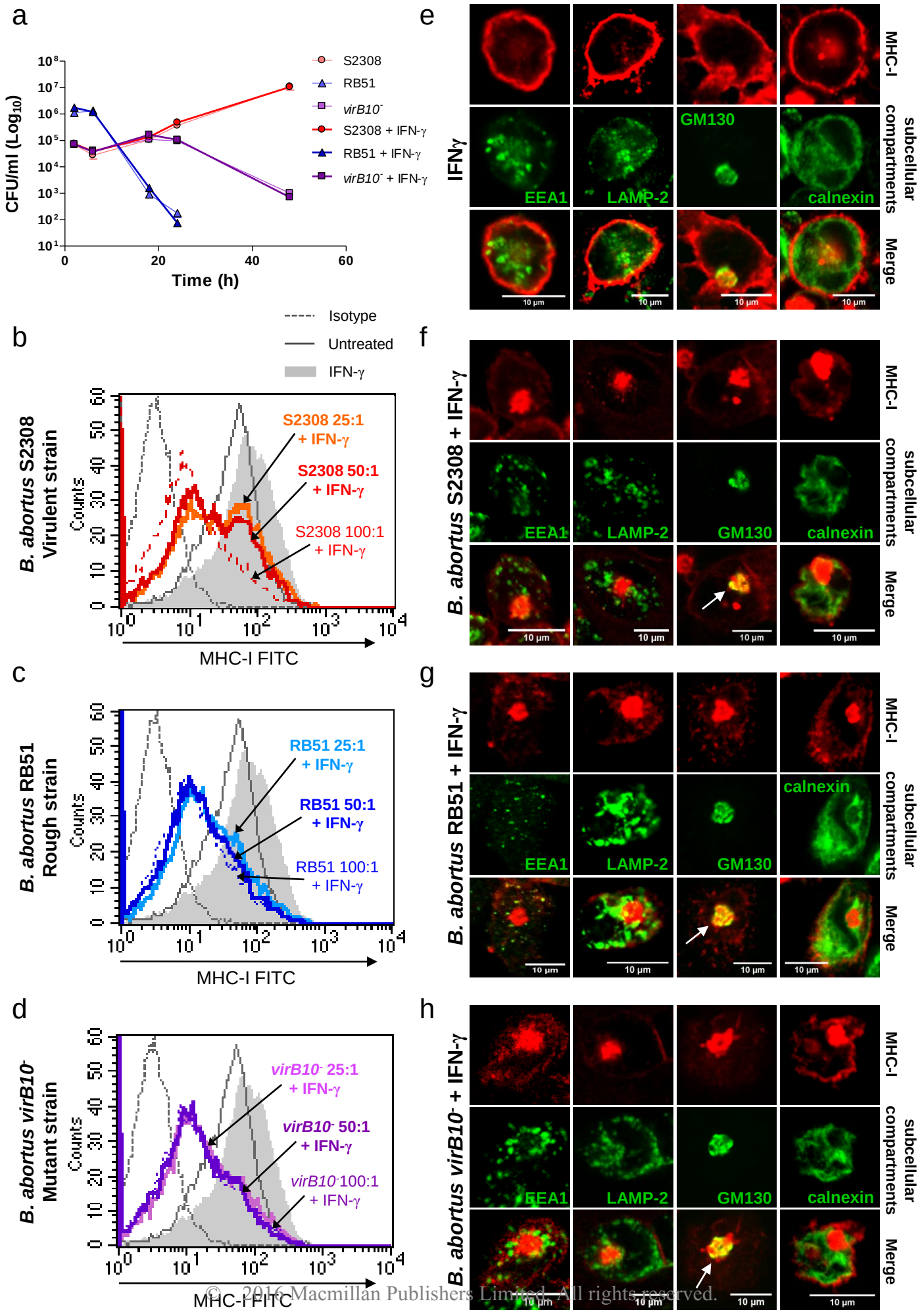

Figure 1

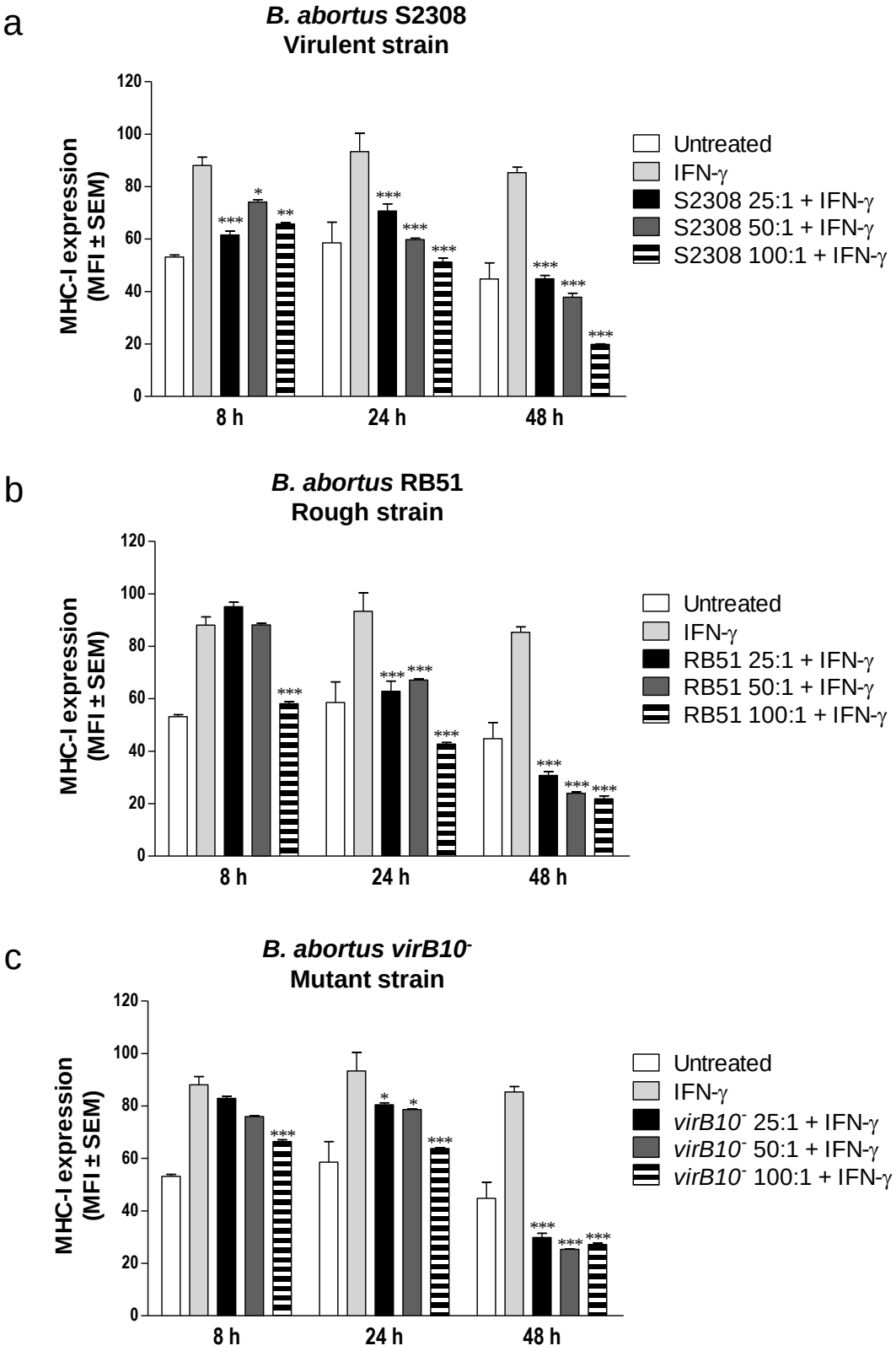

Figure 2

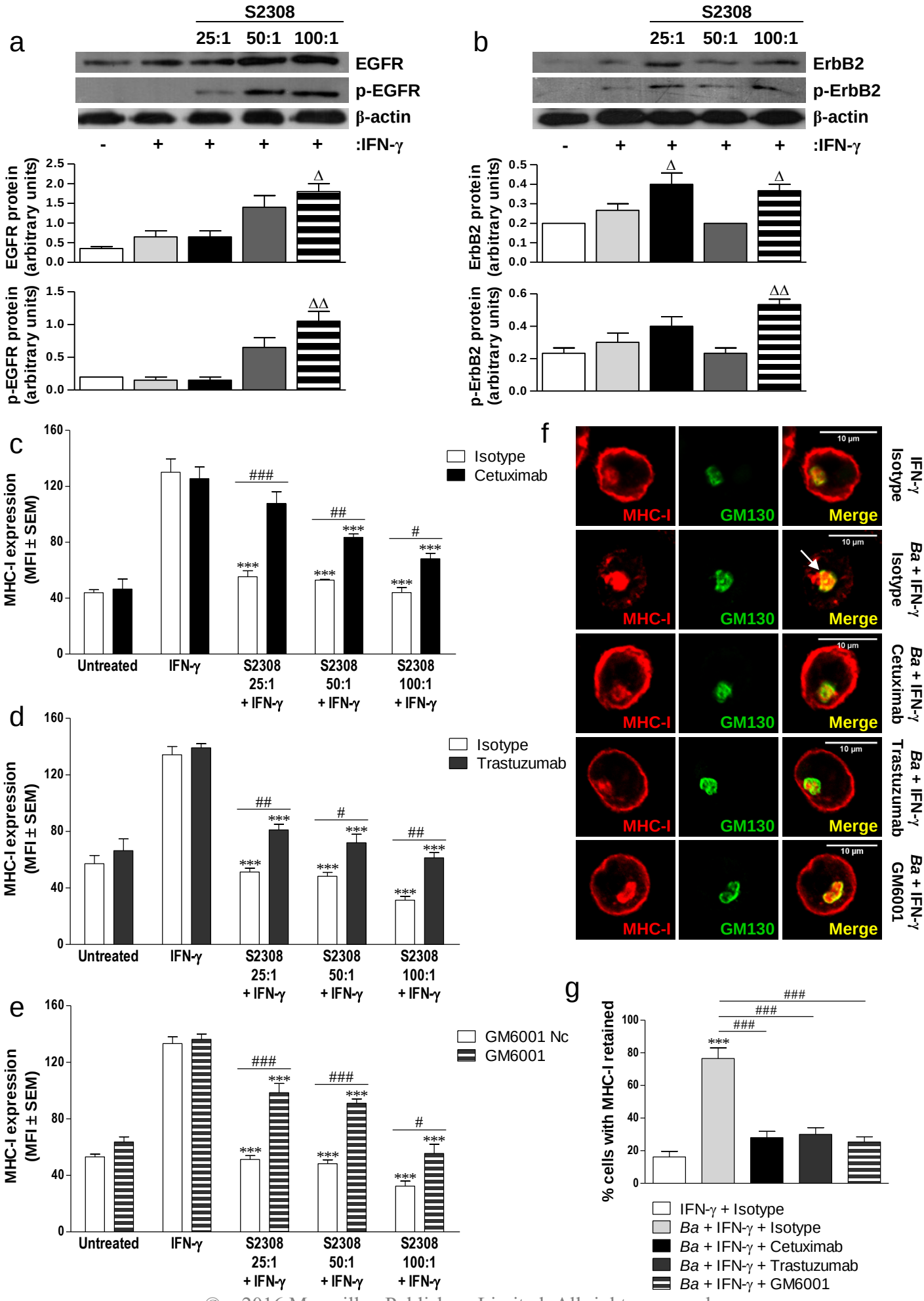

Figure 3

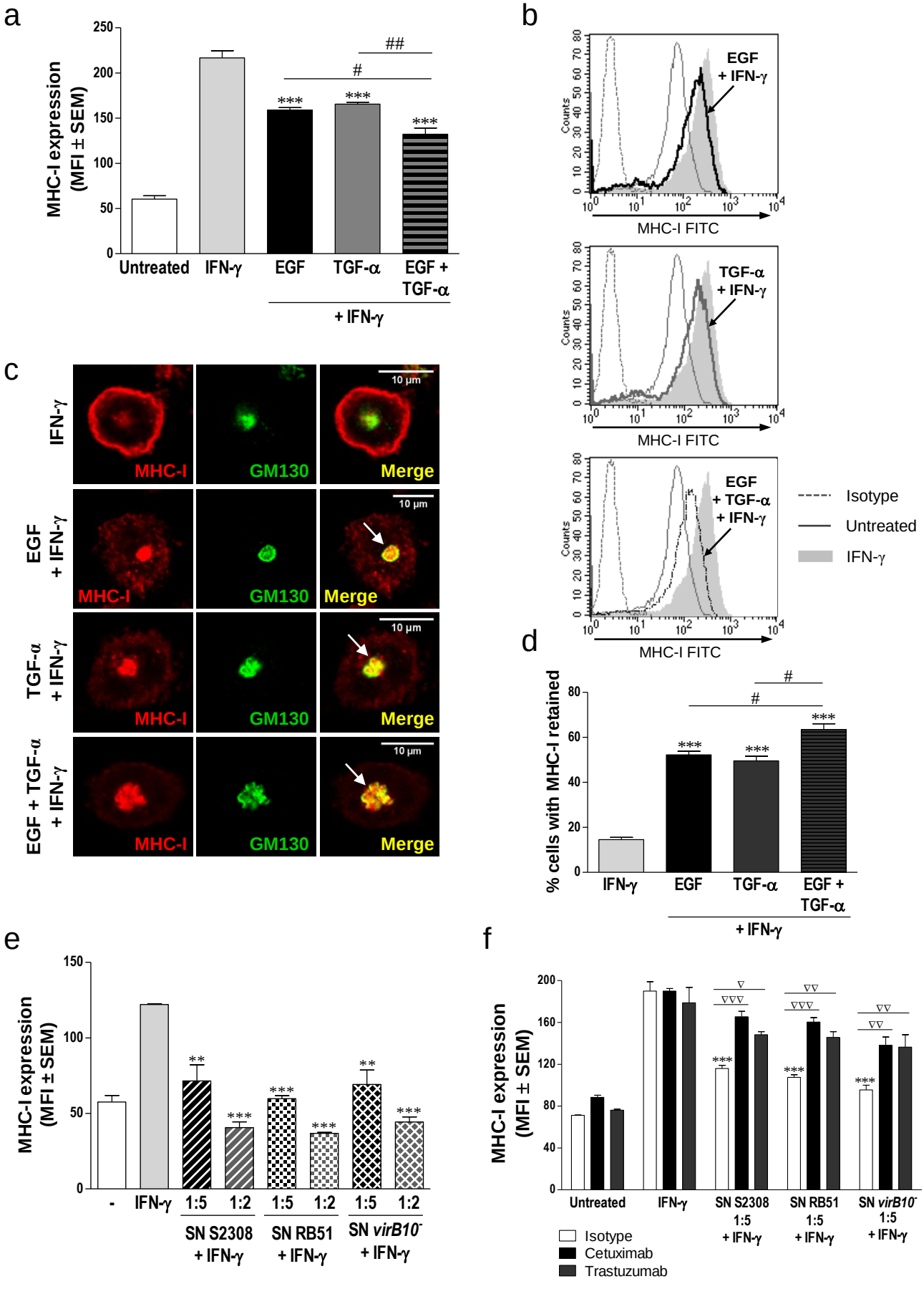

Figure 4

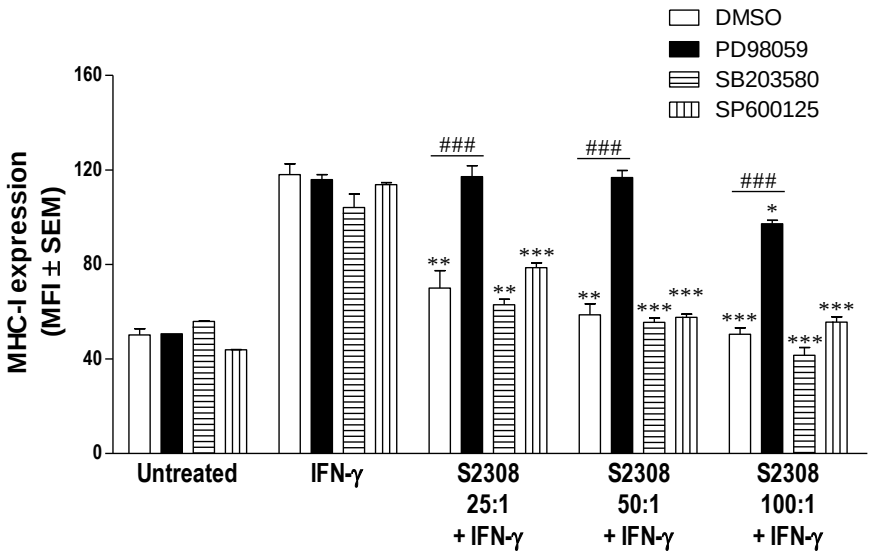

Figure 5

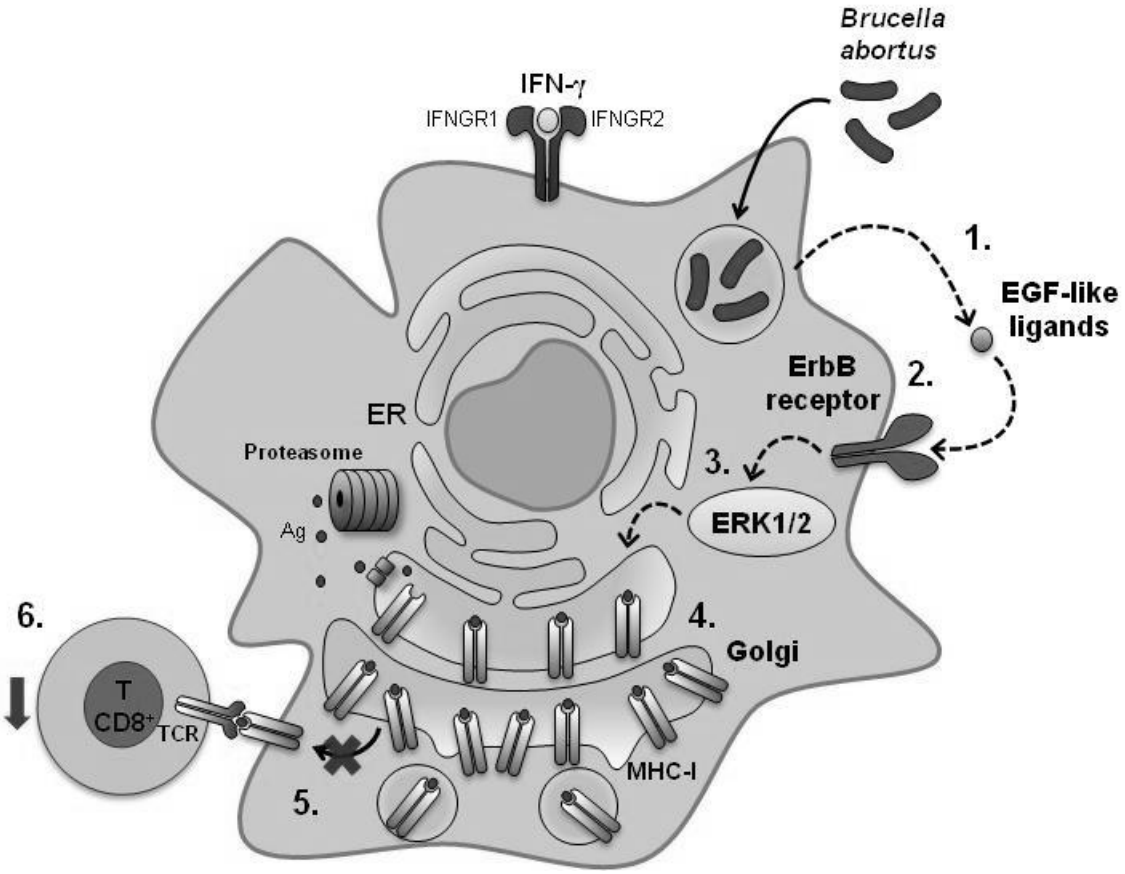

Figure 6