TLR4-independent Upregulation of Activation Markers in Mouse B Lymphocytes Infected by HRSV

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TLR4-INDEPENDENT UPREGULATION OF ACTIVATION MARKERS IN MOUSE B LYMPHOCYTES INFECTED BY HRSV

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Short Running Title: TLR4-independent activation of B cells by HRSV

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ABSTRACT

Human respiratory syncytial virus (HRSV) is the most common cause of severe respiratory infections in infants and young children, often leading to hospitalization. In addition, HRSV poses a serious health risk in immunocompromised individuals and the elderly. It has been reported that this virus can infect mouse antigen-presenting cells, including B lymphocytes. In these B cells, HRSV infection upregulates the expression of activation markers, including MHC class II and CD86, but not MHC class I molecules. Here, we report that HRSV infection of spleen B lymphocytes downregulated TLR4. Either blocking with anti-TLR4 antibody or genetic deletion, but not functional deficiency of TLR4, moderately reduced the infectivity of HRSV in B lymphocytes. HRSV-infected B lymphocytes with deleted TLR4 upregulated MHC class II and CD86 molecules to the same levels as $TLR4^{+}$ wild type B cells. Since the activation of monocytes and macrophages by HRSV was previously reported to depend on TLR4, the current study indicates that these cells and B lymphocytes respond to HRSV infection with different activation pathways.

KEYWORDS

B lymphocyte

FACS

Respiratory models

Viral immunity

INTRODUCTION

Human respiratory syncytial virus (HRSV) (Collins et al. 2007), a *Pneumovirus* of the family *Paramyxoviridae*, is an enveloped virus that contains a negative-sense, single-stranded RNA genome encoding 11 proteins. This virus is the single most important cause of serious illnesses of the lower respiratory tract, such as bronchiolitis and pneumonia, in infants and young children (Hall 2001;Shay et al. 2001;Thompson et al. 2003). It infects people of all ages, but HRSV mainly poses a serious health risk in immunocompromised individuals (Wendt and Hertz 1995;Ison and Hayden 2002) and the elderly (Han et al. 1999;Falsey et al. 2005).

HRSV replicates primarily in the apical cells of the respiratory epithelium (Collins et al. 2007); these cells respond to HRSV infection with increased expression of MHC class I through the induction of IFN-β and IL-1α (Garofalo et al. 1996). In addition, HRSV can infect both human and murine immune system cells, mainly professional antigen-presenting cells (APCs). HRSV infection induces upregulation of maturation markers in human and murine monocytes and macrophages (Becker et al. 1991;Franke-Ullmann et al. 1995;Midulla et al. 1989;Panuska et al. 1990) and human plasmacytoid dendritic cells, but not myeloid dendritic cells (Hornung et al. 2004). Upregulation of activation markers such as MHC class II and CD86 upon infection of mouse spleen B lymphocytes was also previously reported (Rico et al. 2009).

During the first days of infection, the evolutionarily ancient and more universal innate immune system controls a large group of pathogens. In contrast to the clonotypic receptors of lymphocytes, the innate immune system uses nonclonal sets of recognition molecules called pattern recognition receptors. There are various

groups of these receptors and the Toll-like receptors (TLRs) are one of the most important families. TLRs are essential for initiating the innate response against different pathogens, such as Gram-negative and Gram-positive bacteria, mycoplasmas, spirochetes, fungi and viruses (reviewed in (Werling et al. 2009)).

Human and mouse monocytes, macrophages, and dendritic cells express TLR4. However, while naive murine B lymphocytes also express TLR4, human B cells seem to lack significant TLR4 expression (Peng 2005). Some studies (Kurt-Jones et al. 2000;Haynes et al. 2001) have shown that in human monocytes and mouse macrophages, HRSV infection induces cytokines, a response that is dependent on TLR4 expression. In addition, although the G glycoprotein was identified as the major HRSV attachment protein, the interaction between HRSV F protein and TLR4 (Kurt-Jones et al. 2000) suggests an alternative productive attachment and infection pathway (Techaarpornkul et al. 2002). Thus, the aim of this study was to investigate the potential involvement of this pattern recognition receptor in B lymphocyte activation by HRSV infection.

MATHERIALS AND METHODS

Mice and cells

$Tlr4^{lps\text{-}n}$ ($TLR4^{+}$), $Tlr4^{lps\text{-}del}$ ($TLR4^{-}$), and $Tlr4^{Lps\text{-}d}$ (mutant TLR4) mice were purchased from Charles River Laboratories (Lyon, France). $Tlr4^{lps\text{-}del}$ mice have a deletion of the gene encoding TLR4 (Poltorak et al. 1998) and a second genetic defect that implies loss-of-function mutation in the IL-12R β-chain (Merlin et al. 2001). $Tlr4^{Lps\text{-}d}$ mice have a spontaneous point mutation in the intracellular domain of TLR4, which results in a Pro712His substitution, yielding a signaling-defective TLR4 molecule with normal expression (Poltorak, et al. 1998).

Spleen cell suspensions were obtained from 8-12-week-old females by gently tearing the spleen. Erythrocytes were lysed with 0.15 M NH_4Cl lysis buffer and spleen cells were washed with α-MEM medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% FBS and $5x10^{-5}$ M β-mercaptoethanol (Sigma-Aldrich, St Louis, MO, USA). The human epithelial cell line HEp-2 was maintained in DMEM (Gibco BRL) supplemented with 10% FBS. All cells were cultured at 37ºC in a 5% CO_2 atmosphere.

Magnetic Antigen Cell Separation (MACS)

Mouse $B220^{+}$ B lymphocytes were isolated by depletion of non-B cells (negative selection) using the B Cell Isolation Kit (Miltenyi Biotec GmbH, Gladbach, Germany), according to the manufacturer's specifications. The purity of the cell preparations recovered after negative selection was verified by FACS and found to be higher than 95% for $B220^{+}$ B lymphocytes.

Preparation of HRSV stocks

Viruses used in this study were either the A2 strain of HRSV or a recombinant A2 virus, called rgHRSV (kindly supplied by M.E. Peeples) (Hallak et al. 2000). This is a recombinant HRSV that contains the green fluorescent protein (GFP) gene inserted immediately downstream of the viral promoter. GFP expression can be detected directly by FACS analysis of infected cells. Mycoplasma-free stocks of HRSV and rgHRSV were made in HEp-2 cells by infection at a multiplicity of infection (MOI) of 0.3 plaque-forming units (PFU)/cell. After 3 days, infected cells were harvested by scraping the monolayer with a rubber policeman and vortexing. Virus stocks were titrated by a plaque formation assay, as previously described (Streckert et al. 1996). Stocks were aliquoted and stored at -70ºC.

To inactivate the viruses, aliquots of HRSV or rgHRSV were irradiated with UV light, as previously described (UV-HRSV) (Thurau et al. 1998). After this treatment, no residual infectivity was detected by FACS analysis of GFP expression in cells incubated with rgHRSV. For HRSV, inactivation was confirmed by a lack of newly expressed F and G proteins, assayed by FACS.

Infection of B lymphocytes

Purified B lymphocytes were incubated in suspension with HRSV or rgHRSV at an MOI of 10 PFU/cell for 2 h at 37ºC to allow virus binding. An MOI of 10 was selected after performing experiments to examine the infection of B cells with different MOI of HRSV (Rico et al. 2009). Moreover, this is the MOI previously used with other cells such neutrophils (Jaovisidha et al. 1999) or APCs such dendritic cells (Bartz et al. 2003). The virus inoculum was then removed by centrifugation

and replaced with fresh culture medium. A mock-infected control culture was included. Aliquots of infected and non-infected cells were taken immediately for FACS analysis (t = 0 h). Otherwise, cells were further incubated for 24 h or 48 h and then harvested. In preliminary experiments (data not shown and (Rico et al. 2009)), these conditions proved to be optimal with regard to infection rate and spleen cell survival.

For TLR4 blocking assays, B cells were incubated for 30 minutes in the presence or absence of 10 μg/mL of purified anti-TLR4 (clone MTS 510, eBioscience, St Diego, CA, USA), washed in fresh medium and then infected with HRSV as described above.

Cell staining and FACS analysis

MACS-purified cells were monitored by flow cytometry. The antibodies used for staining were as follows: PE anti-TLR4 (clone MTS 510), FITC polyclonal anti-HRSV, which recognizes HRSV F and G proteins (Chemicon International, Single Oak Drive Temecula, CA, USA), allophycocyanin (AP) anti-B220 (CD45R) (clone RA3-GB2) (eBioscience, St Diego, CA, USA), FITC anti-CD86 (clone GL1), PE anti-MHC class I (H-2D^b) (clone KH95), PE anti-MHC II (H-2 I-A^b) (clone AF6-120.1), FITC goat polyclonal IgG isotype control, FITC rat IgG isotype control (clone r35-95), PE rat IgG isotype control (clone R35-95), and AP rat IgG isotype control (clone R35-95) (BD Pharmingen, San Diego, CA, USA). The 2F monoclonal Ab 2F-Cy5, which recognizes an epitope of the HRSV F protein, was previously described (García-Barreno et al. 1989;Rico et al. 2009) .

Cells were first incubated with Mouse SeroBlock FcR (1 μg/mL in FACS buffer) for 10 min at 4ºC to block the Fc-receptor expressed by B lymphocytes.

Afterwards, cells were stained with mAbs diluted in FACS buffer for 20 min at 4ºC. Propidium iodide (BD Pharmingen) was added to the samples (1 μl/sample) for dead cell exclusion. Cells were then washed three times in cold FACS buffer to eliminate excess propidium iodide and fixed with 1% paraformaldehyde in PBS. Data were acquired on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using CellQuest Pro 2.0 software (BD Bioscience). The results shown are means of 2-4 different experiments and are expressed both as mean fluorescence intensity (MFI) and as the percentage of inhibition, which was calculated as follows:

$100 - 100 \times (MFI^{HRSV+} - MFI^{Isotype})/(MFI^{No\ HRSV} - MFI^{Isotype})$ in Fig. 1;

$100 - 100 \times (MFI^{HRSV+\ \alpha\text{-TLR4}} - MFI^{No\ HRSV})/(MFI^{HRSV+} - MFI^{No\ HRSV})$ in Fig. 2;

and $100 - 100 \times [(MFI^{HRSV+} - MFI^{No\ HRSV})\ TLR4^{-}$ or mutant TLR4$]/[(MFI^{HRSV+} - MFI^{No\ HRSV})\ TLR4^{+}]$ in Figs. 3 and 4.

RESULTS

HRSV infection downregulates TLR4 expression in B lymphocytes.

Previously we reported (Rico et al. 2009) that mouse spleen B lymphocytes are susceptible to HRSV infection *in vitro*. To test the role of TLR4 in HRSV infection of these cells, splenocytes were isolated from naive mice. Next, the spleen $B220^{+}$ B lymphocyte subpopulation was purified, cultured and assayed 48 h later for the presence of TLR4 by flow cytometry. The results shown in Fig. 1A indicate that TLR4 was expressed in murine B lymphocytes as described previously for several APCs, including B cells (Peng 2005). Similar experiments with B lymphocytes infected with the A2 strain of HRSV and cultured for 48 h were carried out. Under these conditions, the HRSV-infected B lymphocytes decreased their TLR4 expression (Fig. 1A). Figure 1B summarizes the data of TLR4 expression levels obtained at different times. While TLR4 expression in B lymphocytes was not induced by culture (Fig. 1B), a reduction in membrane TLR4 levels was detected in infected B lymphocytes at 24 hours (44% ± 4%) (Fig. 1C). This decrease was more marked at 48 hours post-infection (62% ± 5%) (Fig. 1C). Thus, HRSV infection downregulates TLR4 expression in B lymphocytes.

Blockage of TLR4 interferes with HRSV infection of B lymphocytes.

To study the role of TLR4 in the infection of B lymphocytes by HRSV, infection in the presence of TLR4-blocking antibodies was carried out. HRSV F protein was detected in HRSV-infected cells by flow cytometry and served as a

marker of infection (Fig. 2A). Incubation of B lymphocytes with anti-TLR4 mAb prior to HRSV infection decreased the expression of the HRSV F protein by 38% ± 2% in infected cells (Fig. 2A and B). Using rgHRSV (Hallak et al. 2000), a GFP-expressing recombinant A2 strain HRSV, similar inhibition (43% ± 3%) of GFP expression was obtained in the presence of anti-TLR4 mAb (Fig. 2B). Thus, blockage of TLR4 reduces the infectivity of HRSV in B lymphocytes.

Deletion, but not functional deficiency, of TLR4 interferes with HRSV infection of B lymphocytes.

Incomplete blockage by anti-TLR4 mAb of surface TLR4 in B cells could explain the partial inhibition of HRSV infection shown in Fig. 2. Spleen $B220^+$ B lymphocytes from $TLR4^+$ (wild type), $TLR4^-$ (lacking the gene encoding TLR4 (Poltorak et al. 1998)) and mutant signaling-defective TLR4 mice (Poltorak et al. 1998) were purified. B lymphocytes from each strain were infected with HRSV and expression of the HRSV F protein was measured. A representative experiment is shown in Figure 3A, indicating reduced expression of the HRSV F protein in infected $TLR4^-$ B lymphocytes compared with $TLR4^+$ B cells. Figure 3B summarizes the data obtained from the HRSV infection of B lymphocytes of the three strains described above. The lack of TLR4 reduced HRSV F protein expression by 33% ± 5% (Fig. 3C). Comparable inhibition (40% ± 7%) of GFP expression was detected in similar experiments using rgHRSV to infect B lymphocytes of either mouse strain (Fig. 3C). These inhibitions are similar to the block with the anti-TLR4 mAb (Fig. 2) indicating a moderate role for TLR4 in the initial interaction with HRSV. This effect is independent of TLR4 signaling function because no differences between B

lymphocytes from TLR4^{+} and mutant TLR4 mice were found in Fig. 3B (4% ± 4% of inhibition).

Upregulation of activation markers in HRSV-infected B lymphocytes independent of TLR4 expression.

In our previous report, HRSV infection up-regulated MHC class II but not MHC class I molecules and induced the expression of the activation marker CD86 in B lymphocytes. The study of these markers in HRSV-infected B lymphocytes from TLR4^{+} and TLR4^{-} mice was carried out next. No differences in basal level or in up-regulation of CD86 molecule by HRSV infection were detected in B cells from either strain of mice (Fig. 6, upper panel). Identical results were obtained when the expression of MHC class II was measured Fig. 6, lower panel). Similar to TLR4^{+} mice (Rico et al. 2009), no difference in MHC class I expression between uninfected B cells and HRSV-infected B lymphocytes was detected in TLR4^{-} mice (data not shown). Lastly, this upregulation of CD86 and MHC class II activation markers did not take place when UV-inactivated HRSV, which presents an intact fusion protein, was used (Fig. 6). In summary, HRSV infection of murine B lymphocytes induced a TLR4-independent upregulation of MHC class II and CD86 activation markers.

DISCUSSION

The present report investigates the role of TLR4 in HRSV infection and activation of mouse B lymphocytes. We found that this protein was downregulated by HRSV infection in a time-dependent manner. In addition, the infectivity of HRSV in B lymphocytes was moderately decreased both by blocking and by deleting TLR4 but not by expressing non-functional TLR4. Finally, no differences were found in MHC class II and CD86 upregulation when HRSV-infected B lymphocytes from wild type and TLR4-deficient mice were compared.

Human monocytes stimulated with purified HRSV F protein or UV-inactivated HRSV induced an increase in cytokine secretion (Kurt-Jones et al. 2000). In this same study, Finberg et al. showed that the presence of TLR4 is required for an HRSV F protein-induced response in mouse macrophages and that TLR4-deficient mice, in contrast to control mice, are unable to clear HRSV from their lungs (Kurt-Jones et al. 2000). By contrast, another study showed that TLR4 had no impact on HRSV elimination and that activation of HRSV-specific T cell immunity was normal in TLR4-deficient mice (Ehl et al. 2004). A recent study published by Finberg et al. (Murawski et al. 2009) showed a moderate decrease in the production of inflammatory cytokines in TLR4-deficient mice. By contrast, our studies indicate that UV-inactivated HRSV did not increase the expression of their CD86 or MHC class II molecules in mouse B lymphocytes. Additionally, TLR4 is not necessary for the upregulation of activation markers. Thus, HRSV could use distinct activation pathways in different antigen-presenting cells.

Human papillomavirus type 16 (HPV16) virus-like particles bind to murine B lymphocytes, thereby inducing activation and also activating the production of

proinflammatory factors (Yang et al. 2005). These virus-like particles directly activated class switch recombination and costimulatory molecule expression by B cells from $TLR4^+$ mice, but not TLR4-deficient mice. Thus, HPV16 virus-like particles directly activated B cells to induce $CD4^+$ T cell-independent humoral immune responses via TLR4-dependent signaling. In addition, mouse mammary tumor virus caused B cell activation in $TLR4^+$ mice, but not in congenic mice that possess a mutant TLR4 gene (Rassa et al. 2002). This activation was independent of viral gene expression, because it occurred after treatment of the virus with UV light. In contrast to these two viruses, we found that HRSV infection activates both $TLR4^+$ and TLR4-deficient B lymphocytes. In addition, because UV-inactivated HRSV failed to activate $TLR4^+$ wild type B lymphocytes, TLR4-independent activation of B cells by HRSV requires virus replication.

Hepatitis C virus infection directly induced TLR4 expression, thereby activating human B lymphocytes (Machida et al. 2006). We showed that HRSV infection activates mouse B lymphocytes while downregulating TLR4 expression. The different viruses or different time points examined in each study, 12 days post-infection with Hepatitis C virus and 24-48 hours with HRSV, could help to explain the differences found. Microbial components such as LPS from Gram-negative bacteria also reduce the surface expression of TLR4 on peritoneal macrophages (Nomura et al. 2000) similar to the effect of HRSV infection of B cells reported here. LPS is a powerful initiator of the inflammatory response to infection by Gram-negative bacteria. Most of these bacteria produce a heterogeneous mixture of LPS molecules with different complexity of the polysaccharide component, defined as smooth-form and rough-form LPS (Huber et al. 2006). In macrophages, recognition of LPS depends on the interaction of at least three molecules forming the LPS-

receptor complex: CD14, MD2 and TLR4 (Wright 1995). Smooth-form LPS is known to bind to LBP and interacts with CD14 (Jiang et al. 2005) (Huber et al. 2006). Subsequently, LPS is believed to interact with TLR4 to trigger a stimulation pathway. In contrast to macrophages, in B lymphocytes, which lack CD14 expression, RP105 and MD1 (the homolog of MD2) appear to cooperate with TLR4 in the activation by rough-form, but not by smooth-form LPS (Huber et al. 2006). Thus, LPS could activate macrophages and B cells through different pathways. As we found here, HRSV could also infect and activate diverse antigen-presenting cells using different pathways.

In addition to TLR4, recent studies indicate that HRSV induces inflammatory mediators through both TLR2 and TLR6 proteins (Murawski et al. 2009). Also, lung expression of TLR7 and TLR3 mRNA was detected in mice intranasally inoculated with HRSV (Huang et al. 2009). Together, these data indicate that HRSV can initiate a proinflammatory response via multiple TLRs. As murine B lymphocytes also express all of these molecules except TLR3 (Barton and Medzhitov 2002), future studies will allow comparisons of these activation signals between different antigen-processing cells.

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The abbreviations used are:

APC, Antigen presenting cells

CTL, Cytotoxic T lymphocytes

GFP, green fluorescent protein

HRSV, Human respiratory syncytial virus

LPS, lipopolisaccharide

MACS, Magnetic antigen cell separation

MHC, Major Histocompatibility Complex

MOI, multiplicity of infection

PFU, plaque-forming unit

rgHRSV, Human respiratory syncytial virus that encodes GFP protein

TLR, Toll-like receptor

UV-HRSV, Human respiratory syncytial virus inactivated with ultraviolet light

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FIGURE LEGENDS

Figure 1.

Effect of HRSV on TLR4 expression by B lymphocytes.

Purified B lymphocytes were infected with the A2 strain of HRSV, cultured at the indicated time points and stained with PE-labeled anti-TLR4 Ab. A mock-infected control was included as a negative control. Samples were analyzed by FACS. The code used in panel A is as follows: isotypic control (shaded histogram), no HRSV (thick line), and HRSV (thin line). The code used in panel B is as follows: isotypic control (single line), no HRSV (squares), and HRSV (circles). The data are expressed as MFI ± SD (panel B) and percentage of inhibition of TLR4 surface expression ± SD at each time point (panel C).

Figure 2.

Effect of anti-TLR4 Ab on the efficiency of HRSV infection.

Purified B lymphocytes were incubated with anti-TLR4 Ab or medium for 30 min. The cells were subsequently infected, cultured, and stained with 2F-Cy5 Ab, which recognizes the HRSV F protein. The data are expressed as MFI ± SD of 2F-Cy5 Ab staining (panel A) or as the percentage of inhibition ± SD of 2F-Cy5 Ab staining (open bar) or GFP expression (filled bar) (panel B).

Figure 3.

Effect of TLR4 expression on the efficiency of HRSV infection.

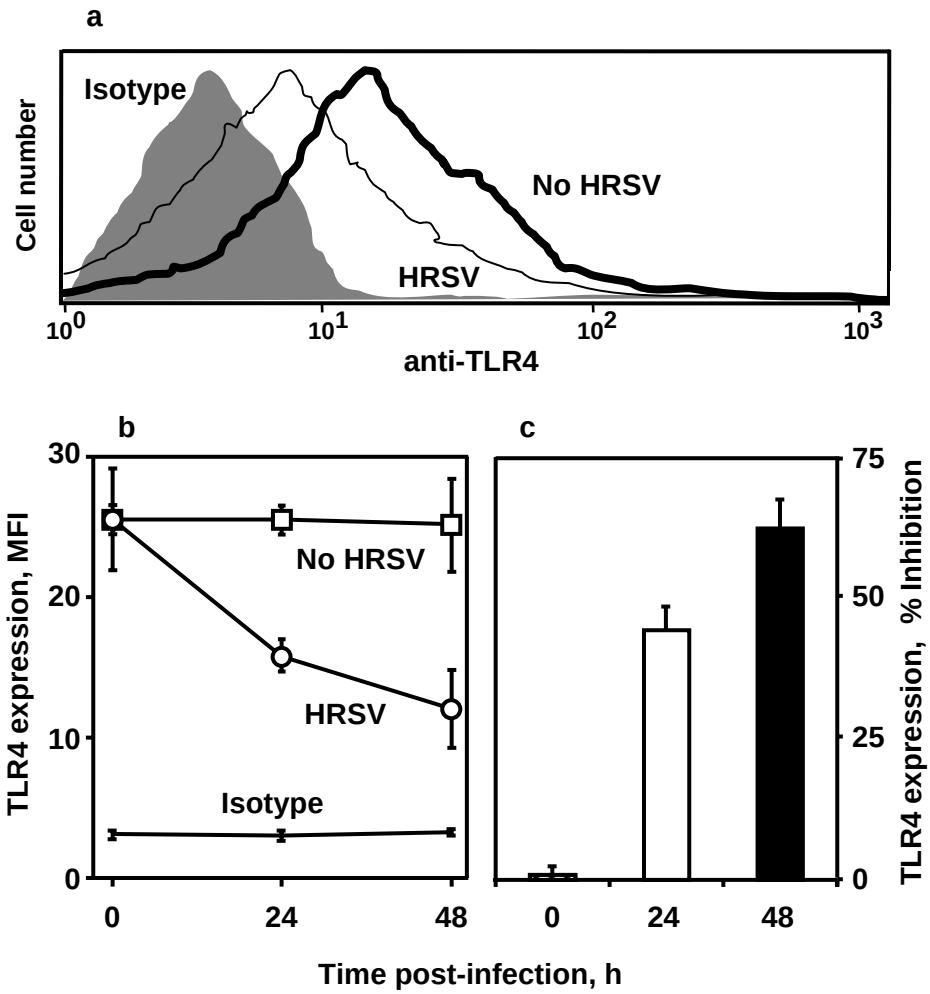
Purified B lymphocytes from $TLR4^{+}$, $TLR4^{-}$, and mutant TLR4 mice were infected, cultured, and stained as indicated in the legend to Figure 2. Conditions represented

in panel A: isotypic control (shaded histogram), $TLR4^-$ (thin line) and $TLR4^+$ (thick line). The data of 2F-Cy5 staining are expressed as MFI ± SD (panel B) or as the percentage of inhibition ± SD of 2F-Cy5 Ab staining (open bar) or GFP expression (filled bar) of $TLR4^-$ versus $TLR4^+$ B lymphocytes (panel C).

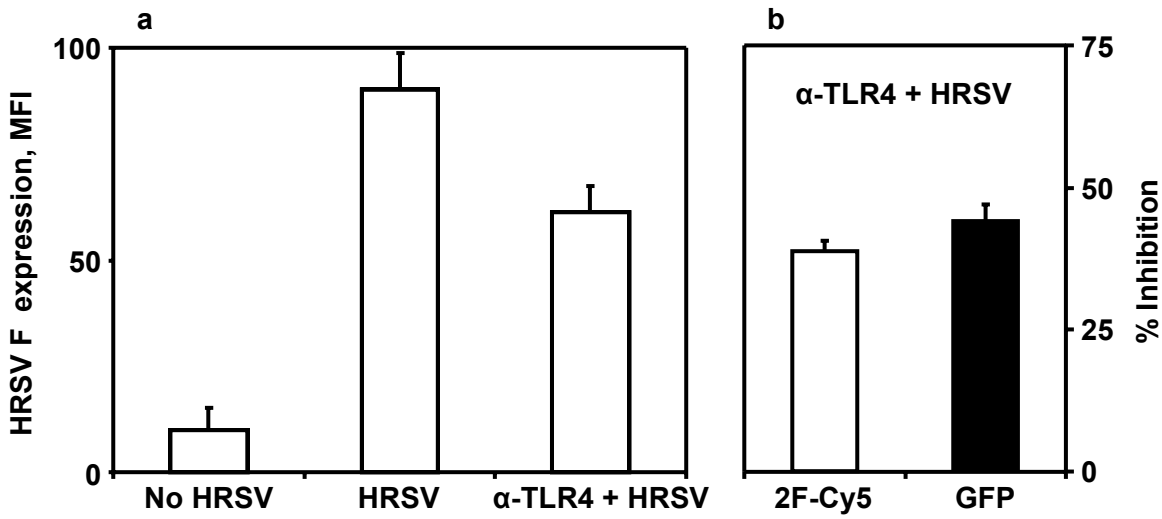
Figure 4.

Surface expression of CD86 and MHC class II in HRSV-infected B lymphocytes.

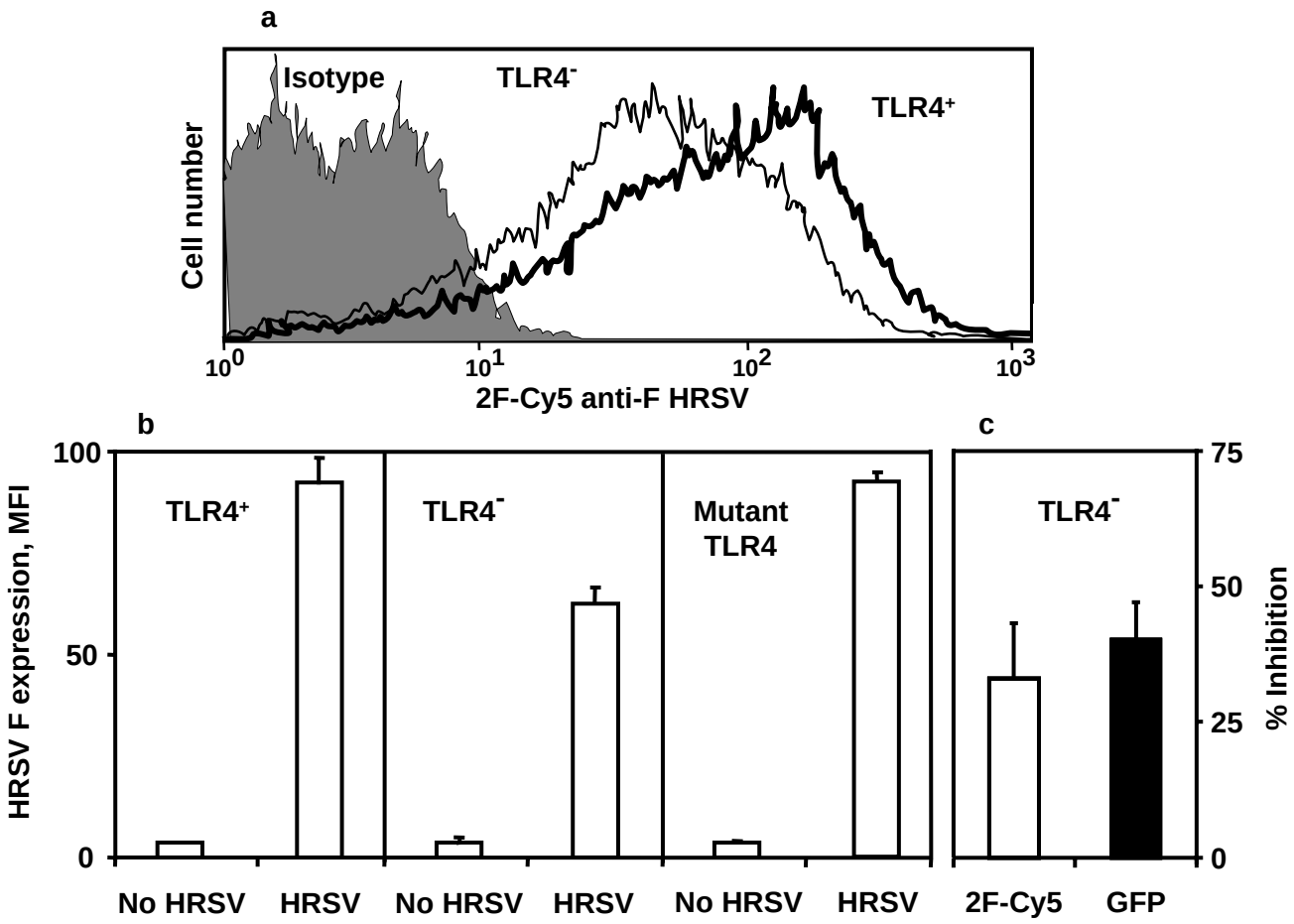
Purified B lymphocytes from $TLR4^+$ (open bars), and $TLR4^-$ (filled bars) mice were infected and cultured for 48 h, and CD86 (upper panel) or MHC class II (lower panel) expression was assessed by FACS. The data are expressed as MFI ± SD. ND, not done.



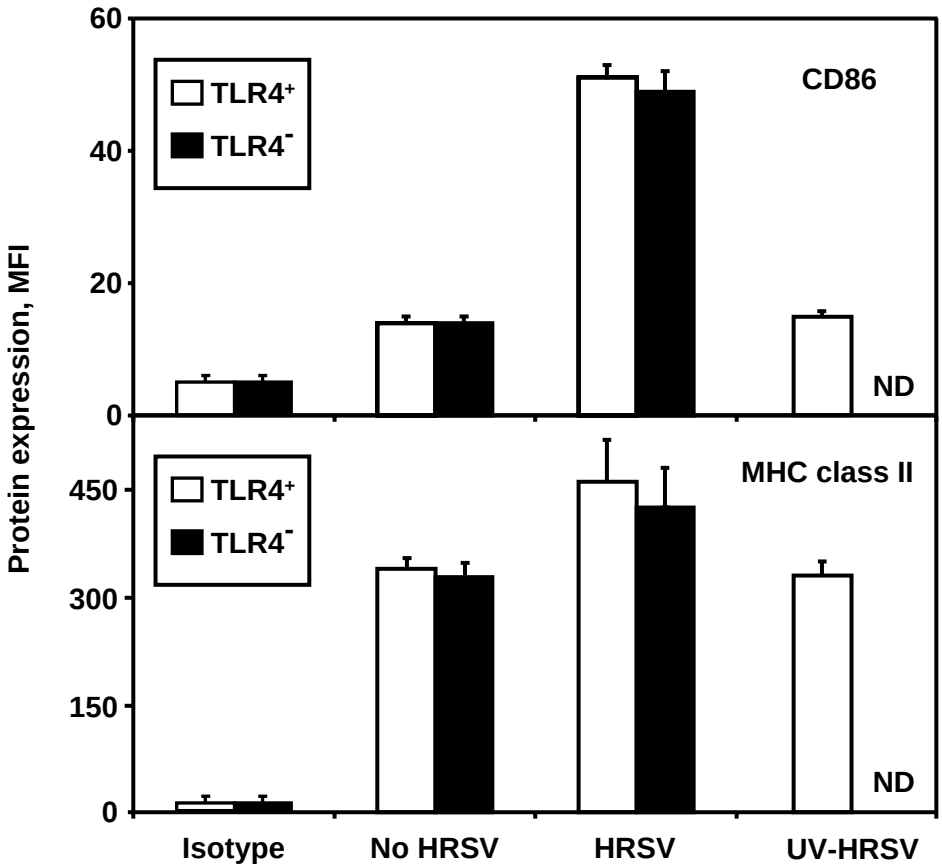
Rico et al. Figure 1



Rico et al. Figure 2



Rico et al. Figure 3



Rico et al. Figure 4