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**HUMAN RESPIRATORY SYNCYTIAL VIRUS INFECTS AND INDUCES  
ACTIVATION MARKERS IN MOUSE B LYMPHOCYTES**

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Short Running Title: HRSV infects murine B but not T splenocytes

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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. Although human airway epithelial cells are the main target of HRSV, it has been reported that this virus can also infect professional antigen-presenting cells such as macrophages and dendritic cells, promoting up-regulation of maturation markers. Here, we report that mouse spleen B220<sup>+</sup> B lymphocytes were susceptible to HRSV infection *in vitro*, probably involving a glycosaminoglycan-dependent mechanism. In contrast, neither CD4<sup>+</sup> nor CD8<sup>+</sup> T lymphocytes were infected. In B lymphocytes, HRSV infection up-regulated MHC class II but not MHC class I molecules and induced the expression of the activation marker CD86.

Keywords: B cells, FACS, Respiratory models, Viral Immunity.

## INTRODUCTION

Different viruses can infect certain cells of the immune system, including T lymphocytes and antigen presenting cells (APCs) such as dendritic cells, macrophages, or B lymphocytes (reviewed in <sup>1</sup>). Infection can alter the specific antiviral response that leads to activation and/or maturation of infected cells, and sometimes to deregulated production of cytokines and chemokines <sup>1</sup>. Indeed, these mediator molecules can modulate the responses of other immune cells in a variety of ways: e.g., altering the expression of Major Histocompatibility Complex (MHC) class I and/or class II molecules, inducing the production of growth or differentiation factors <sup>1</sup>.

Human respiratory syncytial virus (HRSV), a *Pneumovirus* of the family *Paramyxoviridae* <sup>2</sup> is an enveloped, non-segmented negative-stranded RNA virus. This virus is an important pathogen that infects people of all ages but, whereas usually mild infections are reported in healthy adults, serious illnesses such as bronchiolitis and pneumonia are frequent in infants <sup>3-5</sup>. In addition, HRSV poses a serious health risk in immunocompromised individuals <sup>6,7</sup> and in the elderly <sup>8,9</sup>.

Although the epithelial cells of the respiratory tract are the major sites of HRSV replication *in vivo*, several reports have shown that this virus can infect human monocytes and macrophages and modulate their activities <sup>10-12</sup>. In addition, HRSV enhances the expression of MHC class I and II molecules in human APCs, such as macrophages and dendritic cells <sup>13</sup>. Murine models of

HRSV infection (reviewed in <sup>14</sup>) have been effectively used to study pathogenesis of acute self-limited respiratory virus infection, CD8<sup>+</sup> and CD4<sup>+</sup> T-cell biology, and virus-induced innate immune responses. Most important, this murine model of HRSV allows the study of basic aspects of the interaction between allergic inflammation and virus-induced immune responses. The major disadvantage of studying HRSV in rodent models is the limited extent to which this host-restricted human pneumovirus replicates in mouse lung tissue. However, the interaction of HRSV with human APCs has been mimicked by infecting with HRSV certain mouse immune cells, such as macrophages <sup>15</sup>, and plasmacytoid dendritic cells <sup>16</sup>. Since HRSV infection modifies the activity of these professional APCs, it is important to know if this virus can infect other cells of the immune system and alter their immunological properties. In addition, murine HRSV-infected spleen cells are routinely used as APCs in Cytotoxic T Lymphocytes (CTL) restimulation protocols, but the susceptibility of splenic subpopulations to HRSV infection remains undefined. Thus, the aim of this study was to investigate the effect of HRSV infection on other cells of the murine immune system, in order to contribute to a better understanding of the mouse model of HRSV infection.

In this study, HRSV was found to infect B220<sup>+</sup> B but not T lymphocytes. Both MHC class II and CD86 expression levels were increased in infected B lymphocytes. It's well documented that the humoral immune response is critical to control HRSV infection (reviewed in <sup>17</sup>): e.g., production of HRSV-specific antibodies are detected in both serum and airway secretions and participate in

the elimination of infection but do not protect against subsequent infections. Newborns carry maternal antibodies which appear to decrease the likelihood of infection in the first month of life and administration to infants of sera with high HRSV-antibody titers reduces both the incidence and severity of HRSV infections. Thus, the alterations produced by HRSV in B lymphocytes could be physiologically relevant.

## RESULTS

### **HRSV infects in vitro murine spleen B but not T lymphocytes.**

In order to test the susceptibility of murine splenocytes to HRSV infection, splenocytes were isolated from naive C57BL/6 mice, incubated with the A2 strain of HRSV and assayed 48 h later for the presence of HRSV antigens by flow cytometry. The results shown in Fig. 1 indicate that a significant fraction of the spleen cells incubated with the virus, but not the mock infected controls, were expressing HRSV F and/or G proteins at that time ( $41\% \pm 11\%$  HRSV infected cells vs  $4\% \pm 2\%$  without virus,  $n=5$ ). Since no significant signal was observed in the infected cultures just after virus adsorption ( $t = 0$  h), the results of Fig. 1 are indicative of active virus replication in the splenocyte cultures.

Next, flow cytometry analyses were performed gating on different subpopulations of infected splenic cells and the results are shown in Fig. 2. HRSV antigens were only marginally detected in  $CD8^+$  and  $CD4^+$  T cells. In contrast, most cells in both  $CD11b^+$  macrophage/monocyte and  $B220^+$  B cell populations were positive for HRSV antigens after 48 hours of incubation with the virus. Table I summarizes the data obtained at different times. No significant differences were found both  $CD8^+$  and  $CD4^+$  T cells at 24/48 hours post-infection versus time zero indicating no or undetectable HRSV infection in these cell types. Similar results were obtained 72 h post-infection (data not shown). Infection of B cells by bovine RSV has previously been demonstrated in cattle<sup>18</sup>. In addition, previous studies have reported infection of alveolar macrophages

by HRSV <sup>11,12</sup>, but to the best of our knowledge, infection of mouse spleen macrophage/monocytes and B cells by HRSV have never been reported before. Next, spleen subpopulations were purified and infected with rgHRSV <sup>19</sup>, a GFP-expressing recombinant A2 strain HRSV. GFP expression can be detected directly by FACS analyses of infected cells. Fig. 3 shows the results. Purified B220<sup>+</sup> spleen cells infected with this recombinant virus expressed significant GFP protein levels only 48 h after infection. In contrast, no detection of GFP was found when purified CD8<sup>+</sup> and CD4<sup>+</sup> T cells were incubated with rgHRSV in parallel experiments. GFP expression was negative in either splenocytes or B cells incubated with non-infectious UV-irradiated or heat-inactivated rgHRSV (data not shown). Similar results to those shown in panels “spleen” and “B220<sup>+</sup>” of Fig. 3 were obtained when the cells were stained with the F protein specific mAb 2F-Cy5 (data not shown). In summary, the results of Fig. 3 with purified lymphocytes confirm the data presented in Fig. 2 with gated populations and indicate that HRSV can infect mouse B cells *in vitro* but not T lymphocytes.

#### **Glycosaminoglycan requirement for HRSV infection of B lymphocytes.**

Previous studies <sup>20,21</sup> have implicated glycosaminoglycans (GAGs) of the surface of cultured cells as a first step for efficient HRSV infection, probably by direct binding of the HRSV G glycoprotein to GAGs. Heparan sulphate appears to be the most important GAG for HRSV infection <sup>20,21</sup>. This GAG is found on the surface of almost all animal cells, including murine B cells, as a component of proteoglycans. Therefore, the role of heparan sulphate in the sensitivity of B



lymphocytes to HRSV infection was tested by preincubation of the virus with increasing amounts of heparin before being used to infect the cells. As previously reported <sup>22</sup>, preincubation of HRSV with soluble heparin inhibited virus infectivity of HEp-2 cells, showing an IC<sub>50</sub> of 0.7 µg/ml (Figure 4, left panel). In parallel, heparin efficiently inhibited HRSV infection of murine B cells (Figure 4, right panel) with an IC<sub>50</sub> of 9 µg/ml. For inhibition of HRSV infection of murine B cells (Figure 4, right panel). The ten-fold difference between both cell types is in accordance with the different MOI used for each cell type (MOI of 1 PFU/cell in HEp-2 cells and MOI of 10 PFU/cell in B cells), although the cell receptor density and/or type on these two different cell type could be a contributing factors to different IC<sub>50</sub> observed. As heparan sulphate is the analogue of heparin in cell surface proteoglycans, and not dismissing the contribution of other mechanism, the data are compatible with the involvement of this polysaccharide in promoting an efficient HRSV infection of B lymphocytes, as has been found for other cell types.

### **B lymphocytes are activated by HRSV infection.**

B lymphocytes constitute one of the major arms of the immune system because they are responsible for humoral immunity. Since it is feasible that HRSV infection may alter B lymphocyte function, changes in the expression of activation markers were explored. First, and as a control of infection, the expression of HRSV F protein on the membrane of infected B220<sup>+</sup> B cells was

checked by flow cytometry. As shown in Fig. 5a, the infected B cells expressed high amounts of F protein 48 h after infection. Lower but significant F protein levels were detected when inactivated HRSV viruses were used. As this level was the same already at 0 h (data not shown), the staining detected with inactivated viruses is probably due to particles of the initial viral inoculum that remained bound to the cellular membrane.

Subsequently, MHC expression was analyzed by FACS. Fig. 5b shows no difference in MHC class I expression between uninfected B cells and HRSV-infected B lymphocytes at 48 h after infection, or when the cells were incubated with inactivated virus (by irradiation or heat treatment). Similar experiments analysing MHC class II expression were carried out. As with MHC class I expression, no differences in MHC class II expression between uninfected B cells and B cells cultured in the presence of inactivated virus (Fig. 5c) were perceived. In contrast, HRSV infection significantly increased MHC class II expression in B cells, as shown in Fig. 5c. Thus, HRSV infection up-regulates MHC class II but not MHC class I expression in B cells.

The expression of MHC class II genes is increased by several inflammatory or immune stimuli and this enhancement is an activation landmark of professional antigen-presenting cells<sup>23</sup>. Accordingly, the increase in MHC class II expression after HRSV infection may indicate activation of infected B lymphocytes. To address this hypothesis, the expression of CD86 (B7.2) was checked in HRSV-infected B lymphocytes. CD86 is a transmembrane protein that is constitutively expressed at low levels and is rapidly up-regulated on

APCs (including B cells) upon activation. It is worth stressing that CD86 induction marks the initiation and amplification of immune responses <sup>24</sup>.

Uninfected B cells cultured for 48 h showed basal levels of CD86 expression as measured by flow cytometry (Fig. 5d). That level was unaffected when inactivated viruses (Fig. 5d) were present in the culture. As a positive control of B cell activation LPS was added to cultures, since it is a powerful B cell activator that signals through toll-like receptor 4. Accordingly, CD86 expression was increased to a great extent when B cells were cultured in the presence of LPS (Fig. 5d). In parallel cultures, HRSV infection of B lymphocytes also up-regulated CD86 expression (Fig. 5D), although at more moderate levels than LPS. Unexpectedly, UV-HRSV that presents intact fusion protein does not induce upregulation of either MHC class II nor CD86 molecules in contrast to previously reported effect of soluble HRSV F protein in human monocytes <sup>25</sup>. In summary, HRSV infection of murine B lymphocytes induced the activation markers MHC class II and CD86.

## DISCUSSION

The susceptibility of mouse spleen cells to HRSV infection was tested *in vitro*. The spleen CD11b<sup>+</sup> macrophage/monocyte subpopulation was infected, and this fact correlates with the previously reported susceptibility to HRSV of alveolar macrophages<sup>11,12</sup>. We found that spleen B220<sup>+</sup> B lymphocytes can be infected with HRSV. In contrast, no infection of CD8<sup>+</sup> or CD4<sup>+</sup> T lymphocytes was detected. Furthermore, heparan sulphate is important for efficient HRSV infection of B lymphocytes. Finally, it was found that HRSV infection up-regulates MHC class II but not MHC class I molecules in B cells and that infected spleen B220<sup>+</sup> cells express the activation marker CD86.

Routinely, spleen cells from uninfected mice are incubated with HRSV and then used as APCs to restimulate specific CTLs against HRSV *in vitro*<sup>26</sup>. The efficiency of this protocol suggests that a significant and functionally relevant subpopulation of these spleen cells is infected by HRSV. In a naive spleen, most of the splenocytes are lymphocytes, and our data demonstrate that HRSV infects murine naive B but not T lymphocytes. In addition, a contribution of HRSV-infected monocytes/macrophages to restimulate CTL cultures is also likely.

T and B lymphocytes derive from a common lineage and share biological features. In addition, in both cell types similar heparan sulfate GAGs are found on the plasma membrane<sup>27</sup>. Despite all these similarities, only murine B lymphocytes but not T lymphocytes are infected by HRSV. Therefore, it is likely

that the infection of T cells is blocked after initial HRSV-GAG interaction. Future analysis of requirements for productive HRSV infection should be aided by the comparison of murine T and B lymphocytes.

HRSV replicates primarily in the apical cells of the respiratory epithelium <sup>2</sup>. Studies in respiratory epithelial cell lines and bronchial epithelial cells from normal human tissue indicate that these cells respond to HRSV infection with an increased expression of MHC class I through the induction of IFN- $\beta$  and IL-1 $\alpha$  <sup>28</sup>. In addition, previous studies have reported HRSV infection of both human and mouse immune system cells, mainly professional antigen-presenting cells. HRSV infection induces up-regulation of maturation markers in human and murine monocytes and macrophages <sup>10-12,15</sup>, and human plasmacytoid dendritic cells, but not in myeloid dendritic cells <sup>29</sup>. In human monocytes, soluble fusion protein of HRSV induces cytokines, and this response is dependent on expression both CD14 and TLR4 <sup>25</sup>. In these cells, recognition of LPS depends on the interaction of at least three molecules forming the LPS-receptor complex: CD14, MD2 and TLR4 <sup>30</sup>. LPS is known to bind to LPS-binding protein and interacts with CD14. Subsequently, LPS is believed to interact with TLR4 to trigger LPS-dependent stimulation pathway. Thus, in these cells, both lipopolysaccharide and viral protein seem to use the same pathway. In B cells, that do not express CD14, RP105 and MD1 (the homologue to MD2) appear to cooperate with TLR4 in LPS recognition by an unknown mechanism <sup>31</sup>. Since LPS, but not UV-HRSV induces activation markers in murine B lymphocytes, the functional requirements of this alternative pathway may be different and

HRSV F protein may not suffice to stimulate B cells. In addition, HRSV infection and activation of dendritic cells caused impaired CD4<sup>+</sup> T cell activation characterized by a lower T cell proliferation and ablation of cytokine production in activated T cells<sup>32</sup>. The present study demonstrates that B lymphocytes can efficiently be infected *in vitro* by HRSV, and that this infection also up-regulates the activation marker CD86, selectively inducing MHC class II but not class I molecules. This MHC class II increase is probably related with the cathepsin S activity. Generally in resting APCs, surface expression of class II MHC molecules is low, while the their level of intracellular class II is high<sup>33,34</sup>. Cathepsin S activity in these cells is also low, and this limits invariant chain degradation, reduces generation of MHC class II  $\alpha\beta$  dimers, and prolongs the retention of MHC class II molecules. After maturation signals, cathepsin S activity is enhanced, and MHC class II molecules are released<sup>35</sup>. Up regulation both MHC class II molecules and CD86 require intact and infectious viral particles. Thus, murine spleen B cells behave like both human and mouse professional antigen-presenting cells after HRSV infection.

Studies of HRSV infection in human B lymphocytes are very scarce. Infection of Epstein-Barr virus-transformed human B-cell lines with HRSV virus *in vitro* readily caused a persistent infection<sup>36</sup>. In another study the frequency of HRSV-infected cells in different human cell populations present in peripheral blood mononuclear (PBMC) cells was studied by flow cytometry<sup>37</sup>. Around 23% of monocytes were infected, and HRSV binding to around 10% of CD8<sup>+</sup> T (but not CD4<sup>+</sup>) cells was also detected, leading to an increase in IL-5 production by

these cells. However, no simultaneous expression of HRSV antigens and IL-5 was detected in CD8<sup>+</sup> T cells and it was concluded that up-regulation of IL-5 production in CD8<sup>+</sup> cells required an active HRSV infection of other cell types present in PBMC. Although the same authors found HRSV binding to only about 13% of the peripheral human B lymphocytes, our results should stimulate future work to find out the relevance of B lymphocytes infection by HRSV and its effects when interacting with other cells of the immune system, especially when also Bovine RSV was detected in B lymphocytes of calf pulmonary-draining lymph nodes of infected animals and causes persistent infection of B cell lines *in vitro*<sup>18</sup>.

The level of CD86 expression on APCs exerts multiple influences on both T and B cell function because it regulates the level of T cell-dependent Ab production by the B cell and of cytokine production by the T cell (reviewed in<sup>38</sup>). While CD86 is expressed at very low levels on resting B cells, specific (B cell receptor-induced) or unspecific (LPS-induced) activation increases the level of CD86 mRNA and protein expression in the B cell<sup>39</sup>. However, signals originating outside the immune system as infection by viruses also regulate the level of B cell-associated CD86 expression<sup>40</sup>, and alter their function. We describe here the up-regulation of the activation marker CD86 in HRSV-infected murine B cells. However, HRSV inhibits mitogen-induced human T-cell proliferation, even though T-cell activation markers such as IL-2 receptor are induced<sup>41</sup>. Therefore, the activation effects of HRSV on murine B cells do not rule out functional alterations that must be evaluated in future studies.

## **METHODS**

### **Mice and cells**

C57BL/6 mice were bred in our animal facilities in accordance with national regulations (accreditation number 28079-34A). Spleen cell suspensions were obtained of 8-12 week-old female by gently tearing the spleen. Erythrocytes were lysed with 0.15 M  $\text{NH}_4\text{Cl}$  lysis buffer and spleen cells were washed with  $\alpha$ -MEM medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% FBS and  $5 \times 10^{-5}$  M  $\beta$ -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 37°C in a 5%  $\text{CO}_2$  atmosphere.

### **Magnetic Antigen Cell Separation (MACS)**

Mouse B220<sup>+</sup> B lymphocytes or CD3<sup>+</sup> T cells were isolated by depletion of non-B or non-T cells (negative selection) using the B Cell Isolation Kit or the Pan T Cell Isolation Kit (Miltenyi Biotec GmbH, Gladbach, Germany), respectively, according to the manufacturer's specifications. Briefly, non-B cells or non-T cells were indirectly magnetically labelled with a cocktail of biotin-conjugated monoclonal antibodies, as primary labelling reagent, and anti-biotin monoclonal antibodies conjugated to magnetic MicroBeads, as secondary labelling reagent, in MACS buffer (PBS containing 2 mM EDTA and 5% BSA). Cells were then applied onto magnetic columns (Miltenyi Biotec). The magnetically labelled non-



B cells or non-T cells were depleted by retaining them on the column while the unlabeled B or T cells passed through. Unbound cells were collected, washed and resuspended in fresh medium. Viable cells were scored by trypan blue staining using a Neubauer haemocytometer. The purity of the cell preparations recovered after negative selection was verified by FACS analyses and found higher than 95%, both for CD19<sup>+</sup>/B220<sup>+</sup> B cells and for CD3<sup>+</sup> T lymphocytes.

### **Preparation of HRSV stocks**

Viruses used in this study were either the A2 strain of HRSV or a recombinant A2 virus, named rgHRSV (kindly supplied by M.E. Peeples)<sup>19</sup>. This is a recombinant HRSV that contains the green fluorescent protein (GFP) gene inserted as an extra gene immediately downstream of the viral promoter. GFP expression can be detected directly by FACS analyses 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 unit (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<sup>42</sup>. Stocks were aliquoted and stored at -70°C.

To inactivate the viruses, aliquots of HRSV or rgHRSV were either irradiated with ultraviolet light, as previously described (UV-HRSV)<sup>37</sup>, or heated at 56°C for 30 min (heat-HRSV)<sup>43</sup>. After these treatments no residual infectivity could be detected by FACS analysis of GFP expression in cells incubated with

rgHRSV. For HRSV, inactivation was confirmed by lack of newly-expressed F and G proteins in FACS.

### **Infection of immune cells**

Either unseparated spleen cells or purified B and T lymphocytes were incubated in suspension with HRSV or rgHRSV at a MOI of 10 PFU/cell for 2 h at 37°C, to allow virus binding. MOI of 10 was selected after experiments of infection of B cells with increasing MOI of HRSV (data not shown). Moreover, this is the moi previously used with other naive cells as neutrophils<sup>44</sup> or APCs as dendritic cells<sup>45</sup>. The virus inoculum was then removed by centrifugation and replaced by 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 for FACS analysis. In preliminary experiments, these conditions proved to be optimal with regard to infection rate and spleen cell survival. HEp-2 cells were infected under the same conditions but using a MOI of 1 PFU/cell.

For B lymphocyte activation experiments, lipopolysaccharide (LPS) (Sigma-Aldrich) was used as a positive control (10 µg/mL).

### **Soluble heparin blocking assay**

Bovine intestinal mucosal heparin (Sigma-Aldrich) was serially diluted in medium. rgHRSV was then added, and the mixtures were incubated at 37°C for 30-60 min. These mixtures were used to infect HEp-2 cells at a MOI of 1

PFU/cell or purified B cells at a MOI of 10 PFU/cell. The inoculum was removed after the adsorption period by washing the cells, and fresh medium was added. After 24 h the cells were analyzed by FACS for GFP expression. Data were normalized with respect to rgHRSV infection as follows:  $100 \times \frac{[\text{MFI}_{(\text{infected cells in presence of each heparin dose})} - \text{MFI}_{(\text{uninfected cells})}]}{[\text{MFI}_{(\text{infected cells without heparin})} - \text{MFI}_{(\text{uninfected cells})}]}$

### **Cell staining and FACS analysis**

Fresh spleen cells, MACS-purified cells, and HEp-2 cells were monitored by flow cytometry. The antibodies used for staining were: Mouse SeroBlock FcR, FITC anti-CD3 (clone 7D169)(Serotec, Kidlington, OX, UK), FITC anti-HRSV (polyclonal), which recognizes HRSV F and G proteins (Chemicon International, Single Oak Drive Temecula, CA, USA), PE anti-CD11b (clone M1/70), allophycocyanin (AP) anti-B220 (clone RA3-GB2) (CD45R) (eBioscience, St Diego, CA, USA), FITC anti-CD86 (clone GL1), PE anti-CD4 (clone H12919), PE anti-CD8 (clone 53-6.7), PE anti-CD19 (clone 1D3), PE anti-MHC class I (H-2D<sup>b</sup>) (clone KH95), PE anti-MHC II (H-2 I-A<sup>b</sup>) (clone AF6-120.1), FITC goat IgG isotype control (polyclonal), FITC rat IgG isotype control (clone r35-95), PE rat IgG isotype control (clone R35-95), AP rat IgG isotype control (clone R35-95) (BD Pharmingen, San Diego, CA, USA). The 2F monoclonal Ab, which recognizes an epitope of the HRSV F protein <sup>46</sup>, was purified according to standard protocols, conjugated with the fluorochrome Cy5 using Cy5Ab

Labelling Kit according to the manufacturer's recommendations (Amersham Biosciences), and renamed 2F-Cy5.

Cells were first incubated with Mouse SeroBlock FcR (1  $\mu\text{g}/\text{mL}$  in FACS buffer) for 10 min at 4°C to block the Fc-receptor expressed by monocytes, macrophages, B lymphocytes and other spleen cells. Afterwards, cells were stained with the mAbs diluted in FACS buffer for 20 min at 4°C. Propidium iodide (BD Pharmingen) was added to the samples (1  $\mu\text{l}/\text{sample}$ ) for dead cell exclusion. Cells were then washed in cold FACS buffer three times to eliminate excess propidium iodide, and fixed with 1% para-formaldehyde in PBS. Samples were acquired on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using CellQuest Pro 2.0 software (BD Bioscience).

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The authors declare that they have no competing financial interests.

The abbreviations used are:

APC, Antigen presenting cells

CTL, Cytotoxic T lymphocytes

GAGs, glycosaminoglycans

GFP, green fluorescent protein

Heat-HRSV, Human respiratory syncytial virus inactivated at 56°C

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

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

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Table I

Summary of infection of different spleen subpopulations by HRSV

Subpopulation	0 hours <sup>a</sup>		24 hours		48 hours	
	No HRSV	+ HRSV	No HRSV	+ HRSV	No HRSV	+ HRSV
CD4 <sup>+</sup>	2±2% <sup>b</sup>	10±4%	2±1%	15±3%	4±1%	15±4%
CD8 <sup>+</sup>	1±1%	11±3%	3±2%	14±4%	4±4%	13±5%
B220 <sup>+</sup>	3±1%	19±10%	4±1%	56±18%	5±4%	80±11%

<sup>a</sup> Time post-infection.<sup>b</sup> Percentage of each subpopulation of splenocytes positive for HRSV antigens expressed as mean ± SD of five different experiments.

## FIGURE LEGENDS

### **Figure 1. Infection of splenocytes by HRSV.**

Splenocytes were infected with A2 strain of HRSV at a MOI of 10 PFU/cell and incubated for 2 h at 37°C. A mock infected control was included as negative control. Then, cells were either directly stained (t = 0 h) with the polyclonal FITC-labelled anti-HRSV Ab, which recognizes HRSV F and G proteins, or cultured for 48 h (t = 48 h), and then stained with the same Ab. Samples were analyzed by FACS. The percentage of fluorescent positive cells is marked in each panel. The results shown are representative of five different experiments.

### **Figure 2. Infection of different spleen subpopulations by HRSV.**

Spleen cells were incubated with HRSV and stained with the FITC anti-HRSV Ab as indicated in the legend to Figure 1. Furthermore, specific mAbs, indicated on both sides of the Figure, were used to distinguish among different spleen subpopulations: PE anti-CD4 or PE anti-CD8 for T cell subpopulations, PE anti-CD11b for macrophages/monocytes, and AP anti-B220 for B lymphocytes. The percentage of each subpopulation of splenocytes positive for HRSV antigens is indicated in the upper-right quadrant of the respective dot plot. The results shown are representative of five different experiments.



**Figure 3. Expression of GFP in rgHRSV-infected spleen subpopulations.**

Either total splenocytes, purified CD3<sup>+</sup> T lymphocytes or purified B220<sup>+</sup> B cells were incubated with rgHRSV as in Fig. 1. The T cells were stained with anti-CD4, and anti-CD8 antibodies and gated. GFP expression was analyzed by flow cytometry in each spleen subpopulation. Conditions: no virus (shaded histogram), 0 h post-infection (thin line) and 48 h post-infection (thick line). The results shown are representative of five different experiments.

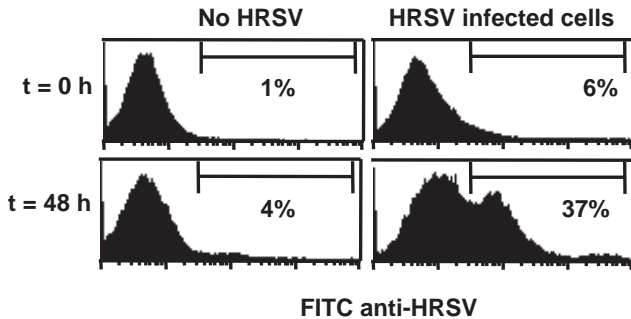
**Figure 4.- Effect of heparin on the efficiency of HRSV infection.**

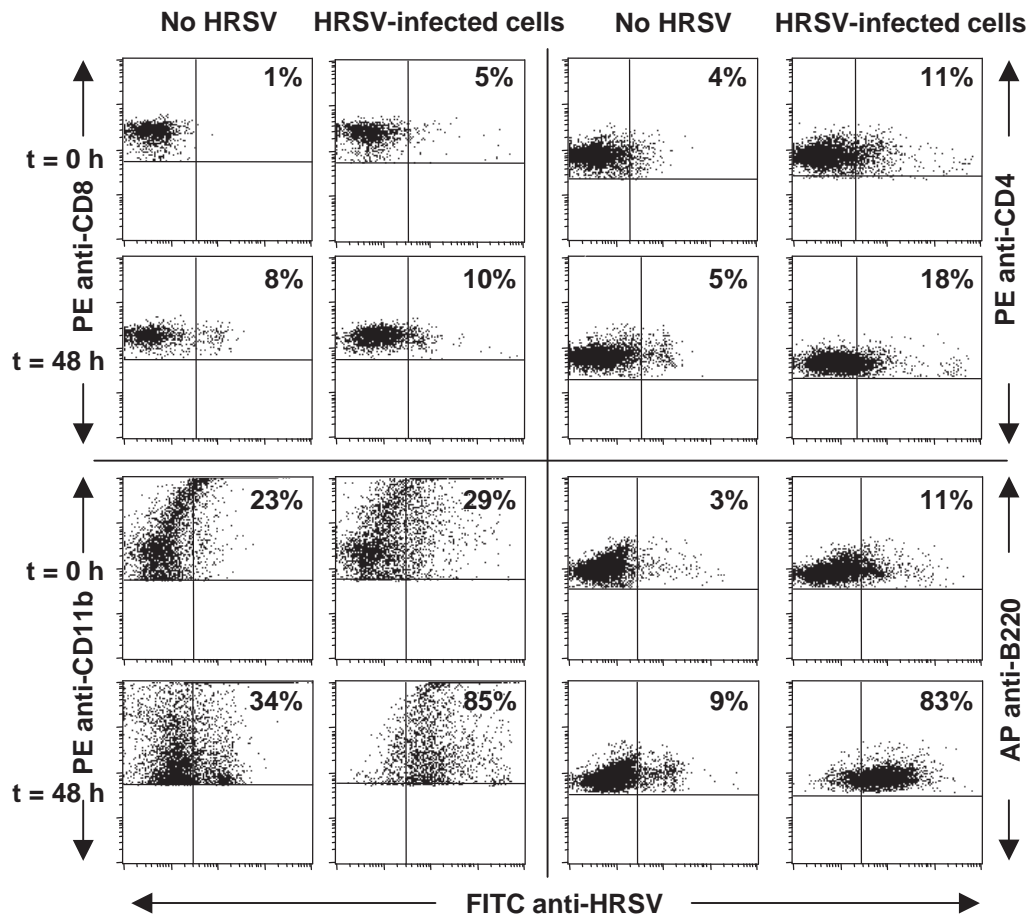
Serial heparin dilutions were incubated with rgHRSV for 30 min at 37°C. Subsequently, HEP-2 cells (left panel) or purified B cells (right panel) were infected with the preincubated virus using a MOI of 1 PFU/cell or 10 PFU/cell, respectively. After 24 h the percentage of infected cells was measured by monitoring GFP expression by flow cytometry. Data were normalized with respect to rgHRSV infection without heparin. The results shown are means of two different experiments.

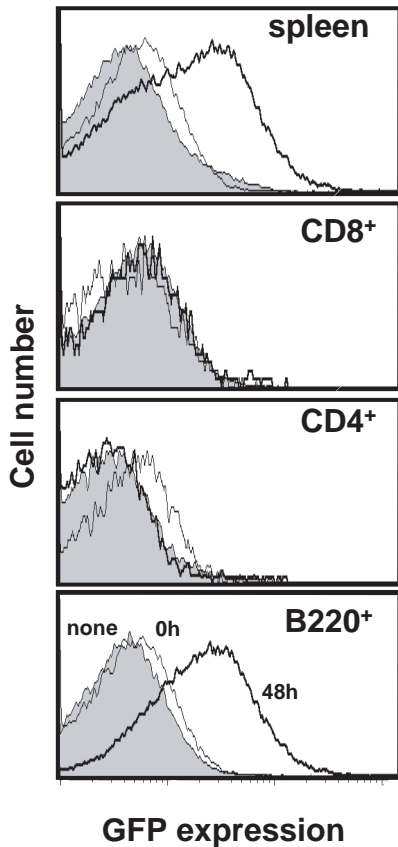
**Figure 5. Surface expression of MHC class I and class II and of CD86 in HRSV-infected B lymphocytes.**

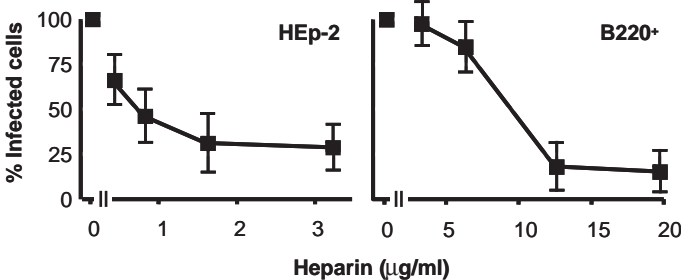
Purified B220<sup>+</sup> B cells were incubated with HRSV as in Figures 1 and 3, and then FACS analysis of HRSV F protein (panel A), MHC class I (panel B), MHC class II (panel C), and CD86 (panel D) expression were carried out. The code used was: isotypic control (dotted orange line), no HRSV (shaded histogram),

heat-HRSV (black line), UV-HRSV (blue line), HRSV (red line), and no HRSV with LPS (green line). The results shown are representative of three different experiments.









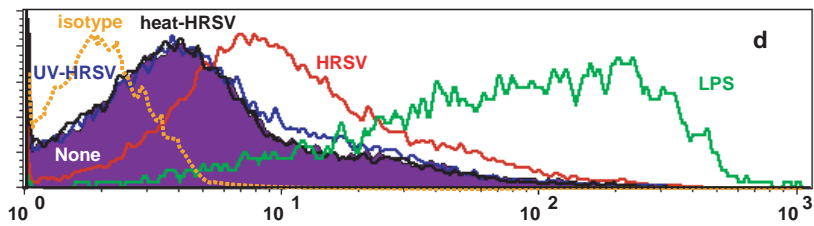
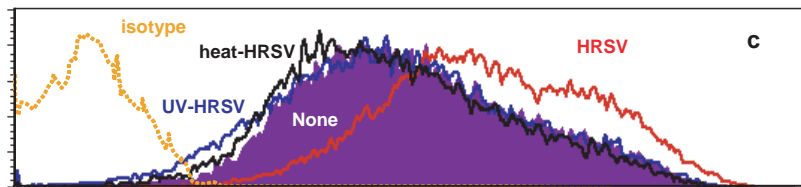
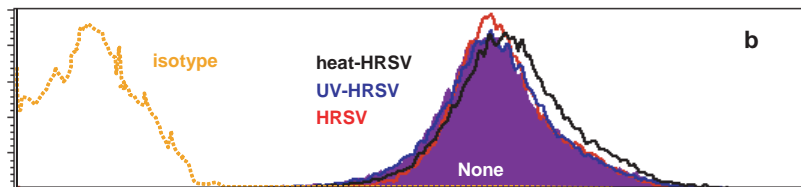
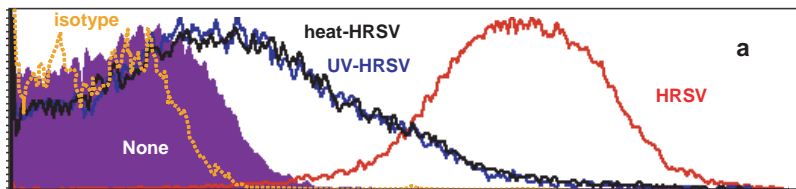


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