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RECEPTOR USAGE AND PATHOGENESIS IN ACUTE AND CHRONIC VIRAL INFECTION

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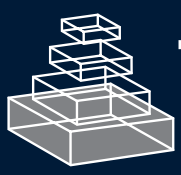
Yasuko Tsunetsugu-Yokota and
Kazutaka Terahara



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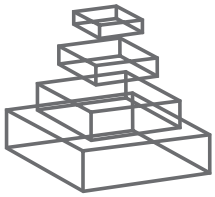

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RECEPTOR USAGE AND PATHOGENESIS IN ACUTE AND CHRONIC VIRAL INFECTION

Hosted By:
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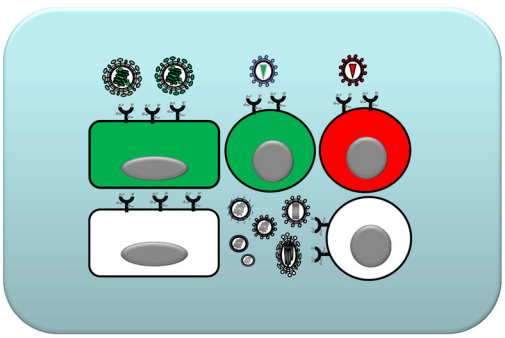

In the first phase of the viral life cycle, the virus enters cells using a specific cell surface receptor. Many viruses use multiple receptors: some of which are unique to a certain cell type, while others are ubiquitous in many cell types. Depending on the receptors used, the viral cell tropism is determined, which may result in the characteristic distribution of virus-infected cells/tissues. Obviously, virus entry is not the whole story. After the virus enters, various cellular proteins interact with it – some support virus replication, while others inhibit it. The intrinsic anti-viral cellular machinery differs among cell types; thus, viral cell tropism based on the receptor usage plays an important role in the pathogenesis. For example, HIV-1 utilizes one of two co-receptors, CXCR4 or CCR5, in addition to a major receptor, CD4. CCR5 expression is restricted to a certain subset of memory CD4+T cells, while CXCR4 expression is rather ubiquitous among CD4+T cells. Therefore, the latent infection of CCR5-utilizing HIV-1 in resting memory (CCR5+) T cells would be largely responsible for intermittent virus blimps accompanied by immune activation during chronic HIV infection. This may explain why CCR5-utilizing HIV-1 is preferentially isolated in the early phase of HIV-1 infection, even when the HIV-1 transmitter carries CXCR4-utilizing HIV-1 in abundance. In the case of the measles virus, CD150 (Signaling Lymphocyte Activation Molecule: SLAM) is a major receptor, while vaccine strains utilize CD46 when CD150 is not available. The third possible entry receptor would also be utilized for the infection of lung epithelial cells. The impact of these receptor usages in vivo on the disease outcome is now under investigation. We also know that the distinct use of host cell sugar moieties mediates species tropism in the influenza virus, and that a mutant virus breaking the species barrier is highly pathogenic in humans.

Thanks to the recent development of reverse genetics, it is now possible to visualize virus-infected cells, or even the virus itself, by using highly sensitive flow cytometery or microscopy. How viral cell tropism, determined by the receptor used, can affect the disease course of acute and chronic virus infection is an important question to address. In this Research Topic, we focus on studies connecting virus receptor usage and the pathogenesis of various viruses causing acute or chronic infection.

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EDITORIAL
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Receptor usage and the pathogenesis in acute and chronic virus infections

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In the first phase of the viral life cycle, the virus enters cells using a specific cell surface receptor. Many viruses use multiple receptors: some of which are unique to a certain cell type, whereas others are found in many cell types. After the virus enters into cells, various cellular proteins may interact with it; some support virus replication, while others inhibit it. Once virus succeeds to establish its life cycle in the target cell, the progeny viruses disseminate within the tissues or systemically via viremia. The intrinsic pro- or anti-viral cellular machinery differs among cell types. Thus, depending on the receptors used, the viral cell tropism is determined, resulting in the characteristic distribution of virus-infected cells/tissues and the disease outcome *in vivo*.

How viral cell tropism, determined by the receptor used, can affect the disease outcome in acute and chronic virus infection is a major subject under extensive investigation in Virology. To understand the mechanism which causes human diseases by viruses, that is, viral pathogenesis, we have studied various aspects of virus infection at a cell/tissue level or using animal models. In this context, the recent development of reverse genetics allows us to visualize virus-infected cells/tissues or even the virus itself. By applying such manipulated viruses to animal models, it is also possible to analyze the dynamics of virus infection *in vivo*.

In this Research Topic, we selected on studies connecting virus receptor usage and the pathogenesis of various viruses causing acute or chronic infection. Eventually, it could expand to cover the receptor-pathogenesis relationship in various acute and chronic virus infection. This research topic comprises an original research article on HIV-1, two opinions articles on the hepatitis C virus (HCV) and norovirus, while the remaining review articles on HTVL-1, measles virus (MV), mouse hepatitis virus (MHV), influenza virus, HCV, and enterovirus (EV) provide overviews on various aspects of viral pathogenesis. In all these review/opinion articles, at least one comprehensive table or figure is incorporated so that readers who are unfamiliar with these viruses can get a message at a glance.

With regards to the cell tropism of HIV-1, Terahara et al. (2012) presented his recent study using CCR5-tropic and CXCR4-tropic HIV-1 with distinct fluorescent reporter. These HIV-1s allowed us to detect HIV-infected cells at a different stage of infection and to evaluate the level of virus replication in $CD4^+$ T cells with distinct differentiation phenotype including $CCR5^+$ memory. In contrast, a receptor for HTLV-1 and related pathogenesis is still intriguing issue, which is described by Hoshino (2012) in his extensive review.

The two reviews on the MV (Kato et al., 2012; Takeda et al., 2012) were published at a very appropriate time as a third receptor for MV entry into epithelial cells, nectin 4, had just been discovered (Muhlebach et al., 2011; Noyce et al., 2011). Here, Kato et al. (2012) focused on the receptor usage of MV *in vivo* which may influences the disease outcome using monkey models, while Takeda et al. (2012) discussed about the dual-tropic nature of MV using SLAM and nectin 4 expressed in immune cells and epithelial cells, respectively.

Ito et al. (2012) addressed the importance of B cells as a reservoir for persistent HCV infection. In two reviews on HCV, Moriishi and Matsuura (2012) overviewed a current research focus on lipid components for the HCV pathogenesis, while Shoji et al. (2012) discussed about glucose metabolic disorders associated with HCV infection.

Nishimura and Shimizu (2012) and Yamayoshi et al. (2012), both of whom successfully identified two novel receptors for EV, overviewed the current knowledge about receptor usage and various diseases associated with EV infection. For the coronavirus, Taguchi and Hirai-Yuki (2012) overviewed studies on the receptor and related cellular factors for MHV, which may contribute to the mouse susceptibility to MHV infection.

As regards to the virus recognizing sugar moieties, Ramos and Fernandez-Sesma (2012) provided insights about the interaction of influenza A virus with sialic acid receptors on immune cells with special reference to the innate immune response. Shirato (2012) described about the norovirus with distinct genotypes which recognize a specific structure of sugar chain.

We will learn by these articles the fact that to identify a receptor is the first important step to know a virus, but many questions remain in order to fully understand human diseases caused by viruses. I would like to express my cordial thanks to all the contributors for this topic. I hope readers find the content interesting, but most importantly, that the information will prove very useful for future research.

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Fluorescent reporter signals, EGFP, and DsRed, encoded in HIV-1 facilitate the detection of productively infected cells and cell-associated viral replication levels

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Flow cytometric analysis is a reliable and convenient method for investigating molecules at the single cell level. Previously, recombinant human immunodeficiency virus type 1 (HIV-1) strains were constructed that express a fluorescent reporter, either enhanced green fluorescent protein, or DsRed, which allow the monitoring of HIV-1-infected cells by flow cytometry. The present study further investigated the potential of these recombinant viruses in terms of whether the HIV-1 fluorescent reporters would be helpful in evaluating viral replication based on fluorescence intensity. When primary $CD4^+$ T cells were infected with recombinant viruses, the fluorescent reporter intensity measured by flow cytometry was associated with the level of CD4 downmodulation and Gag p24 expression in infected cells. Interestingly, some HIV-1-infected cells, in which CD4 was only moderately downmodulated, were reporter-positive but Gag p24-negative. Furthermore, when the activation status of primary $CD4^+$ T cells was modulated by T cell receptor-mediated stimulation, we confirmed the preferential viral production upon strong stimulation and showed that the intensity of the fluorescent reporter within a proportion of HIV-1-infected cells was correlated with the viral replication level. These findings indicate that a fluorescent reporter encoded within HIV-1 is useful for the sensitive detection of productively infected cells at different stages of infection and for evaluating cell-associated viral replication at the single cell level.

Keywords: HIV-1, flow cytometry, EGFP, DsRed, Gag, productive infection

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) interacts with its primary receptor, CD4, and a co-receptor, usually CCR5 or CXCR4, to infect T cells, macrophages, and dendritic cells (Mcclure et al., 1987; Berger et al., 1999; Tsunetsugu-Yokota, 2008). Single cell analysis of HIV-1-infected cells is an essential approach to investigate the differential dynamics of HIV-1 infection and the cellular consequences for each of the HIV-1-targeted cell populations. To monitor HIV-1 infection, a recombinant HIV-1 encoding a reporter luciferase (Luc) gene, or indicator cells transduced with enzymatic reporters such as Luc, β-galactosidase, alkaline phosphatase, and chloramphenicol acetyl transferase, incorporated downstream of the HIV-1 long terminal repeats (LTR) have been widely used (Kar-Roy et al., 2000). However, these reporters require additional substrates or co-factors, and lysis or fixation of cells is required to show reporter activity, which makes the experimental process more complex. In addition, it is difficult to distinguish infected cells from uninfected cells using these reporter assays.

An alternative molecule, green fluorescent protein (GFP) and/or its derivatives, is a powerful reporter that does not require any substrates and co-factors to generate a reporter signal (Chalfie, 1995; Cubitt et al., 1995; Heim et al., 1995). Page et al. (1997) first used a GFP derivative, called enhanced green fluorescent protein (EGFP), as a fluorescent reporter molecule for HIV-1 and showed that infected cells were detectable and, more importantly, distinguishable from uninfected cells using flow cytometry. Furthermore, a red fluorescent protein, DsRed, has been used as an HIV-1 fluorescent reporter (Weber et al., 2006). The main benefit of such recombinant HIV-1 molecules is that the targeted cells do not require any modulation (e.g., transfection) of exogenous reporter genes and, therefore, they allow the characterization of intact HIV-1-infected cells. In most cases of previous recombinant HIV-1 strains, the *nef* gene was replaced with a reporter gene. Therefore, we previously constructed *nef*-intact, replication-competent, recombinant HIV-1 strains encoding either EGFP or DsRed, and showed that CXCR4-tropic X4 and CCR5-tropic R5 viruses replicate differently in $CD4^+$ T cells simultaneously infected with X4 HIV-1 encoding EGFP and R5 HIV-1 encoding

DsRed (Yamamoto et al., 2009). Such recombinant HIV-1 strains encoding a fluorescent reporter gene will be even more valuable because of recent advances in multicolor flow cytometry, which permit more detailed characterization of HIV-1-infected cells.

Flow cytometry is a reliable and convenient method for analysis at the single cell level. Because the transcriptional activity of HIV-1 can be quantitatively monitored in indicator cells according to the fluorescence intensity of an EGFP reporter driven by the HIV-1 LTR (Dorsky et al., 1996; Gervaix et al., 1997; Kar-Roy et al., 2000), we investigated whether the HIV-1-expressing fluorescent reporters EGFP and DsRed would allow the quantitative evaluation of viral replication using a flow cytometer. The results show that a fluorescent reporter signal generated by recombinant HIV-1 strains enables the detection of infected cells at various stages of the viral life cycle.

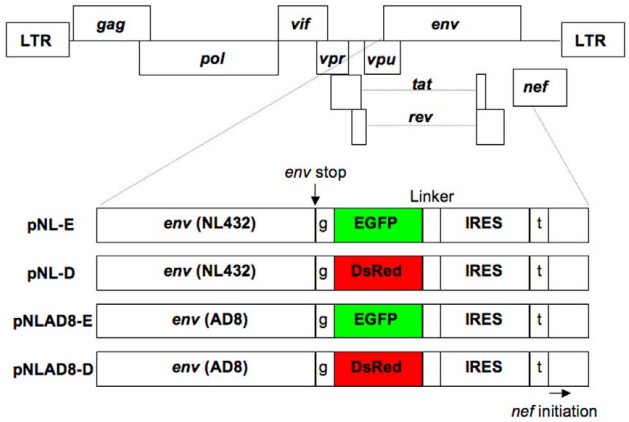

FIGURE 1 | Structure of the proviral DNA. The pNL432-based proviral clones encoded EGFP (pNL-E) or DsRed (pNL-D) for X4-tropic HIV-1_{NL-E} or HIV-1_{NL-D}, respectively, and the pNLAD8-based proviral clones encoded EGFP (pNLAD8-E) or DsRed (pNLAD8-D) for R5-tropic HIV-$1_{NLAD8-E}$ or HIV-$1_{NLAD8-D}$, respectively. EGFP or DsRed was not expressed as a fusion protein with Env due to the insertion of a single base after the Env stop codon. Nef was also independently expressed under the control of IRES. t (Thymine) and g (guanine) are additional DNA sequences.

MATERIALS AND METHODS

CELL PREPARATION

Human peripheral blood samples were collected from healthy donors after written informed consent. Sample collection was approved by the Institutional Ethical Committee of the National Institute of Infectious Diseases (NIID; Tokyo, Japan). Peripheral blood mononuclear cells (PBMCs) were separated on a Ficoll–Hypaque density gradient (Lymphosepal; IBL, Gunma, Japan) and $CD4^+$ T cells were negatively selected from the PBMCs using an EasySep Human $CD4^+$ T cell Enrichment Kit (StemCell Technologies, Vancouver, BC, Canada).

CEM cells stably expressing human CCR5 (CEM–CCR5) were established by transducing CEM cells with the human *ccr5* gene using a conventional mouse retrovirus system. CEM–CCR5 cells were maintained in complete RPMI medium (10% heat-inactivated fetal bovine serum, 100 μg/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine) supplemented with 1 μg/ml puromycin at 37°C.

PREPARATION OF HIV-1 VIRUS STOCKS

We previously constructed pNL432-based proviral clones encoding EGFP (pNL-E) or DsRed (pNL-D) for X4-tropic HIV-1_{NL-E} or HIV-1_{NL-D}, respectively, and pNLAD8-based proviral clones encoding EGFP (pNLAD8-E) or DsRed (pNLAD8-D) for R5-tropic HIV-$1_{NLAD8-E}$ or HIV-$1_{NLAD8-D}$, respectively (Yamamoto et al., 2009; **Figure 1**). To prepare the HIV-1 viral stocks, the human embryonic kidney cell line 293T was transfected with pNL-E, pNL-D, pNLAD8-E, or pNLAD8-D using the calcium phosphate precipitation method and then incubated for 48 h. Culture supernatants were filtered and frozen at −80°C. The amount of virus in each culture supernatant was measured using an in-house HIV-1 Gag p24 enzyme-linked immunosorbent assay (ELISA; Tsunetsugu-Yokota et al., 1995).

STIMULATION OF T CELL RECEPTORS

T cell receptors (TCR) were stimulated as described previously (Yamamoto et al., 2009) with some modifications. In brief, primary $CD4^+$ T cells were suspended in complete RPMI medium supplemented with 5% human plasma and stimulated with 5 μg/ml of immobilized anti-human CD3 monoclonal antibody (mAb; eBioscience, San Diego, CA) and 1 μg/ml of soluble anti-human CD28 mAb (eBioscience) in U-bottom, 96-well plates at 37°C for 4 (weak stimulation) or 24 h (strong stimulation).

HIV-1 INFECTION AND CELL CULTURE

Primary $CD4^+$ T cells (either unstimulated or pre-TCR-stimulated) or CEM–CCR5 cells were infected with 200 ng of p24-measured amounts of HIV-1_{NL-E}, HIV-1_{NL-D}, HIV-$1_{NLAD8-E}$, or HIV-$1_{NLAD8-D}$ per 1×10^6 cells by spinoculation at $1200 \times g$ for 2 h at 25 (conventional conditions) or 4°C (for CEM–CCR5 cells), as described previously (O'doherty et al., 2000; Dai et al., 2009). After spinoculation, cells were washed three times with PBS. Primary $CD4^+$ T cells were then suspended in complete RPMI medium supplemented with 5% human plasma. The cell suspensions derived from unstimulated or pre-TCR-stimulated $CD4^+$ T cells were settled onto U-bottom, 96-well plates with or without TCR-stimulation, respectively, at 37°C for 24 h. After the 24 h culture, cells were washed three times with PBS, suspended in complete RPMI medium supplemented with 5% human plasma and 50 U/ml recombinant interleukin-2, and cultured in U-bottom, 96-well plates at 37°C for up to 4 days.

FLOW CYTOMETRY

Cells were stained with fluorescence-conjugated mAbs as described previously (Yamamoto et al., 2009). The following mAbs were used for flow cytometry in various combinations: Pacific Blue-conjugated anti-human CD3 mAb (BioLegend, San Diego, CA, USA), phycoerythrin Cy7-conjugated anti-human CD4 mAb (BioLegend), and Alexa Fluor 700-conjugated anti-human CD8a mAb (BioLegend); and Nu24 mAb specific for HIV-1 Gag p24 (kindly provided by Dr. T. Sata, NIID, Tokyo, Japan) and conjugated to Alexa Fluor 647 using an Alexa Fluor 647 Protein Labeling Kit (Molecular Probes, Eugene, OR, USA). Dead cells were stained with propidium iodide or a LIVE/DEAD Fixable Dead Cell Stain

Kit (L34957; Invitrogen, Carlsbad, CA, USA). Intracellular staining (ICS) by Nu24 mAb was performed using a FIX and PERM Fixation and Permeabilization Kit (Invitrogen). Data collection was performed using a FACSCanto II (BD Bioscience, San Diego, CA, USA) and the data were analyzed using FACSDiva software (BD Bioscience) and FlowJo software (Tree Star, San Carlos, CA, USA).

QUANTIFICATION OF REPLICATED HIV-1 IN CELL CULTURE SUPERNATANTS

Human immunodeficiency virus type 1 replication was quantified in cell culture supernatants by ELISA and real-time RT-PCR. Gag p24 was measured using a RETRO-TEK HIV-1 p24 Antigen ELISA (ZeptoMetrix Corporation, Buffalo, NY, USA). For real-time RT-PCR, viral RNA was extracted using a QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) and subjected to real-time RT-PCR using a SuperScript III Platinum One-Step Quantitative RT-PCR System (Invitrogen), a set of HIV-1 *gag*-targeted primers, and a TaqMan probe as previously described (Saito et al., 2010). PCR was performed in an Mx3000P (Stratagene, La Jolla, CA, USA).

RESULTS

CD4 DOWNMODULATION IS ASSOCIATED WITH HIV-1 FLUORESCENT REPORTER INTENSITY

The cell surface CD4 molecule is downmodulated in HIV-1-infected cells in response to the HIV-1 components Env, Nef, and Vpu (Malim and Emerman, 2008). Therefore, to investigate the correlation between the level of CD4 downmodulation and the HIV-1 fluorescent reporter intensity, primary $CD4^+$ T cells infected with HIV-$1_{NL\text{-}E}$, HIV-$1_{NL\text{-}D}$, HIV-$1_{NLAD8\text{-}E}$, or HIV-$1_{NLAD8\text{-}D}$ followed by TCR-stimulation for 1 day and cultivation for a further 4 days were analyzed by flow cytometry. As shown in **Figure 2** (left panels), HIV-1-infected cells expressing a fluorescent reporter signal, EGFP, or DsRed, were detected, although the numbers varied between individual donors ($n = 3$–4): about 10–30% for X4-tropic HIV-$1_{NL\text{-}E}$-infected and HIV-$1_{NL\text{-}D}$-infected cells and 1–10% for R5-tropic HIV-$1_{NLAD8\text{-}E}$-infected and HIV-$1_{NLAD8\text{-}D}$-infected cells. However, the number of HIV-1^+ cells was comparable between HIV-$1_{NL\text{-}E}$ and HIV-$1_{NL\text{-}D}$ (X4-tropic), and between HIV-$1_{NLAD8\text{-}E}$ and HIV-$1_{NLAD8\text{-}D}$ (R5-tropic) within each donor, showing that the fluorescent reporter genes encoded within the HIV-1 proviral genome did not affect HIV-1 infectivity as described previously (Yamamoto et al., 2009). When we categorized $CD3^+CD8^-$ T cells into three fractions (HIV-1-negative, -dull, and -high) based on the fluorescence intensity of EGFP and DsRed, we found that CD4 was strongly downmodulated in the HIV-1 high fraction in all the HIV-1 strains (**Figure 2**, right panels). Interestingly, CD4 was also downmodulated in the HIV-1 dull fraction, but the level was modest compared with that in the HIV-1 high fraction (**Figure 2**, right panels). These results indicate that the level of CD4 downmodulation is associated with HIV-1 fluorescent reporter intensity.

FIXATION/PERMEABILIZATION WEAKENS THE HIV-1 FLUORESCENT REPORTER SIGNAL

To investigate the correlation between HIV-1 fluorescent reporter intensity and viral replication levels, we attempted to perform ICS for Gag p24 in HIV-1-infected cells prepared as described above. When we observed X4-tropic HIV-$1_{NL\text{-}E}$-infected and HIV-$1_{NL\text{-}D}$-infected cells from three donors by flow cytometry, we noticed that fixation/permeabilization, an essential step for ICS, weakened the fluorescent reporter signal for both EGFP and DsRed. **Figure 3** shows the flow cytometry profiles obtained for EGFP and DsRed at identical photomultiplier tube (PMT) voltages between intact (untreated) cells and fixed/permeabilized cells to visualize the differences in fluorescent reporter intensity. $DsRed^+$ cells were not properly separated from $DsRed^-$ cells within the population treated by fixation/permeabilization; the frequency of $DsRed^+$ cells was, therefore, markedly decreased. No adjustment of the flow cytometer settings, including PMT voltage and compensation, improved the blunted fluorescent reporter signal generated after fixation/permeabilization. Nevertheless, the number of $EGFP^+$ cells within the intact cell and fixed/permeabilized cell populations was comparable. Similar results were obtained for R5-tropic HIV-$1_{NLAD8\text{-}E}$ and HIV-$1_{NLAD8\text{-}D}$ (data not shown). Taken together, these results indicate that it is preferable to use an EGFP reporter when the fixation/permeabilization of cells is required.

HIV-1 FLUORESCENT REPORTER SIGNALS RELIABLY DETECT PRODUCTIVELY INFECTED CELLS SHOWING DIFFERENT VIRAL REPLICATION LEVELS

Following the results shown in **Figure 3**, we next assessed viral replication levels in the HIV-$1_{NL\text{-}E}$ infection group (5 days culture) from six donors using Gag p24 ICS (**Figure 4**). A representative flow cytometric analysis showed that not all $EGFP^+$ cells were Gag^+ and *vice versa*. When CD4 expression levels were compared in each of the four cell fractions based on the expression patterns of EGFP and Gag p24 ($EGFP^+Gag^+$, $EGFP^+Gag^-$, $EGFP^-Gag^+$, and $EGFP^-Gag^-$), the strongest downmodulation of CD4 was observed in $EGFP^+Gag^+$ cells (red fraction). CD4 downmodulation was moderate in $EGFP^+Gag^-$ cells (green fraction). However, CD4 was not downmodulated at all in $EGFP^-Gag^+$ cells (blue fraction) and the expression level of CD4 was the same as that in $EGFP^-Gag^-$ cells (black fraction). We further divided the $EGFP^+Gag^+$ cells (red fraction) into Gag^{hi} (brown fraction) and Gag^{lo} cells (pink fraction) and compared the expression levels of EGFP and CD4 with those of Gag p24. Gag^{hi} cells (brown fraction) showed the strongest expression of EGFP and the strongest downmodulation of CD4. Gag^{lo} cells (pink fraction) showed an intermediate level of EGFP expression [between that of Gag^{hi} cells (brown fraction) and that of $EGFP^+Gag^-$ cells (green fraction)] and CD4 expression [between that of Gag^{hi} cells (brown fraction) and $EGFP^-Gag^-$ cells (black fraction)]. These results indicate that the expression level of EGFP correlates with that of Gag p24 in HIV-1-infected cells in which CD4 is downmodulated.

HIV-1-BOUND OR -INTERNALIZED CELLS ARE ALSO DETECTED BY Gag p24 ICS

Because CD4 downmodulation was not observed in $EGFP^-Gag^+$ cells (**Figure 4**; blue fraction), it is possible that these cells may still be bound by or have internalized HIV-1 but have not produced virions. Therefore, we next investigated the kinetics of $EGFP^-Gag^+$ cells during 5 days post-infection. Primary $CD4^+$

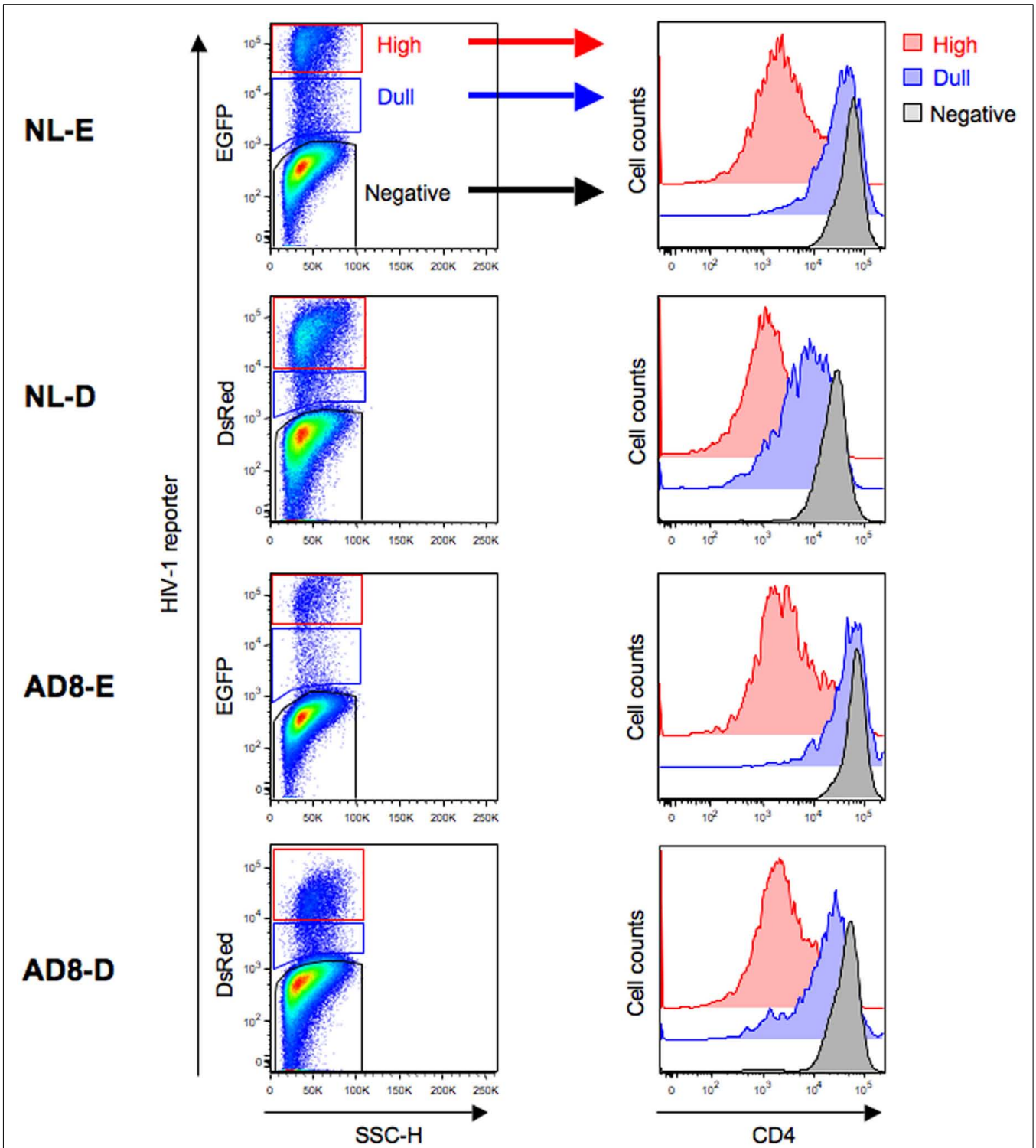

FIGURE 2 | Flow cytometry analysis of HIV-1-infected cells. Unstimulated CD4$^+$ T cells were infected with HIV-1_{NL-E}, HIV-1_{NL-D}, HIV-$1_{NLAD8-E}$, or HIV-$1_{NLAD8-D}$ and cultured for 5 days (including the initial 1 day culture with TCR-stimulation). (Left panels) representative pseudo-color plot profiles for the Dead$^-$/CD3$^+$/CD8$^-$-gated cell fractions from three donors. Cells were classified as high (red), dull (blue), and negative (black) based on the HIV-1 reporter intensity. (Right panels) histograms of CD4 expression by the high (red), dull (blue), and negative (black) fractions defined.

T cells from three donors were infected with HIV-1_{NL-E} followed by TCR-stimulation for 1 day and cultivation for a further 4 days. **Figure 5A** shows a representative flow cytometric analysis. At 1 day post-infection, 17.6% of Gag p24$^+$ cells were observed,

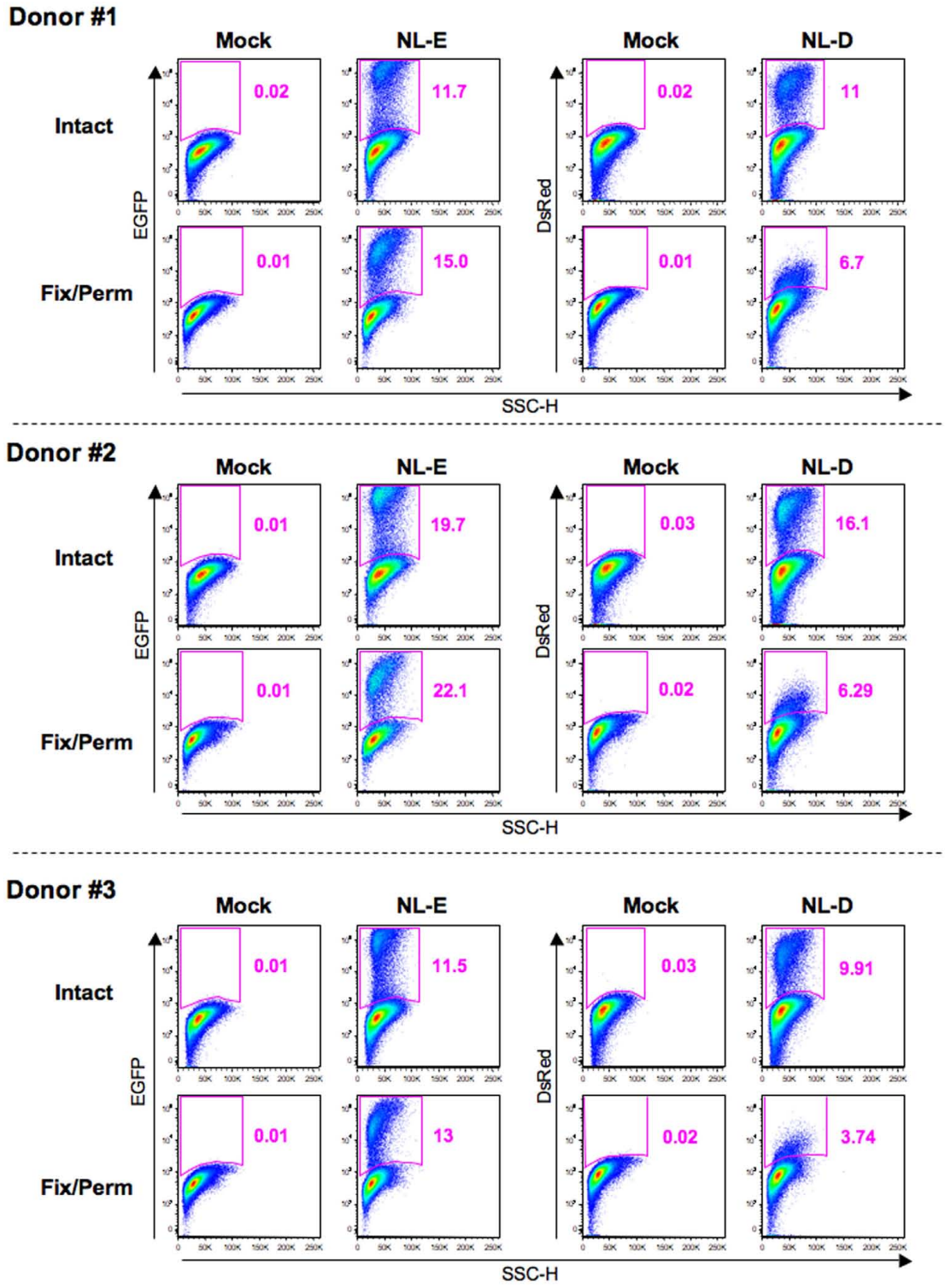

FIGURE 3 | Influence of fixation/permeabilization treatment on HIV-1 fluorescent reporter signals. Pseudo-color plot profiles for the Dead$^-$/CD3$^+$/CD8$^-$-gated cell fractions from the mock, HIV-1$_{NL-E}$ infection (NL-E), and HIV-1$_{NL-D}$ infection (NL-D) groups from all three donors tested. Analyzed cells were prepared as outlined in the legend to **Figure 2** and then either fixed/permeabilized (fix/perm) or not (intact).

despite the fact that no EGFP$^+$ cells were detected. At 2 days post-infection, the proportion of EGFP$^-$Gag$^+$ cells was decreased and EGFP$^+$ cells including Gag p24$^+$ and Gag p24$^-$ cells became to be observed, suggesting that initially infecting HIV-1 was

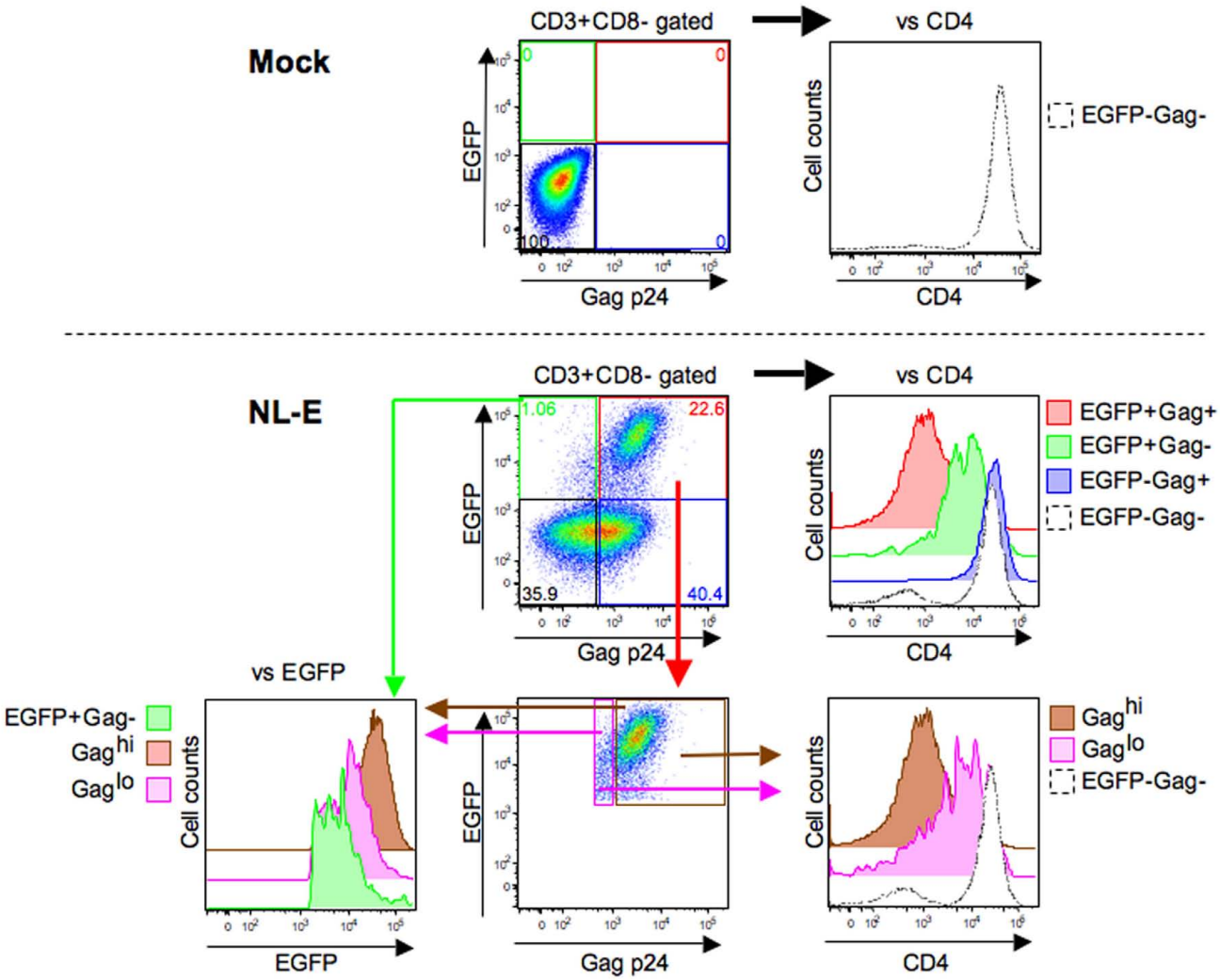

FIGURE 4 | Correlation between HIV-1 fluorescent reporter signal and Gag p24 expression. Representative flow cytometric analyses of the mock and HIV-1_{NL-E} infection groups from six donors. These infection groups were prepared as outlined in the legend to **Figure 2**. The $EGFP^+Gag^+$ (red), $EGFP^+Gag^-$ (green), $EGFP^-Gag^+$ (blue), $EGFP^-Gag^-$ (black), Gag^{hi} (brown), and Gag^{lo} (pink) cell fractions were categorized based on the expression patterns of EGFP and Gag p24. The expression levels of CD4 and EGFP in each cell fraction were compared according to their histogram profiles.

degraded and/or replaced with replication-competent proviruses. After 3 days post-infection, $EGFP^+$ cells were clearly visible and the proportion of $EGFP^-Gag^+$ cells turned to be increased, suggesting that progeny virus infection occurred. Because the CD4 expression levels were identical between $EGFP^-Gag^+$ cells and $EGFP^-Gag^-$ cells throughout the culture period, Gag p24 ICS must have detected cells that had bound or internalized HIV-1.

CEM–CCR5 cells, which are almost as susceptible to X4 and R5 HIV-1 fusion (data not shown), were used to confirm that Gag p24 ICS did indeed detect HIV-1-bound cells. Also, because it has been suggested that spinoculation at 25°C may induce HIV-1 fusion to the targeted cells (Dai et al., 2009), we tested Gag p24 ICS using CEM–CCR5 cells immediately after spinoculation with X4-tropic HIV-1_{NL-E} or R5-tropic HIV-1_{AD8-E} at 4°C. When cells were not fixed/permeabilized, no Gag $p24^+$ cells were detected by flow cytometry (**Figure 5B**, upper panels); however, when cells were fixed/permeabilized, a substantial proportion of Gag^+ cells was detectable in both the HIV-1_{NL-E} and HIV-1_{AD8-E} infection groups (**Figure 5B**, lower panels). Taken together, these results indicate that cells that have bound or internalized HIV-1 can be detected using flow cytometry for Gag p24 ICS.

THE INTENSITY OF THE HIV-1 FLUORESCENT REPORTER SIGNAL DEPENDS ON TCR-MEDIATED ACTIVATION LEVELS

T cell receptors-mediated activation of HIV-1-infected $CD4^+$ T cells increased productive viral replication, although the signaling pathway responsible may be different for X4 and R5 HIV-1 (Popik and Pitha, 2000). We investigated whether the intensity of the HIV-1 fluorescent reporter signal was affected by TCR-mediated

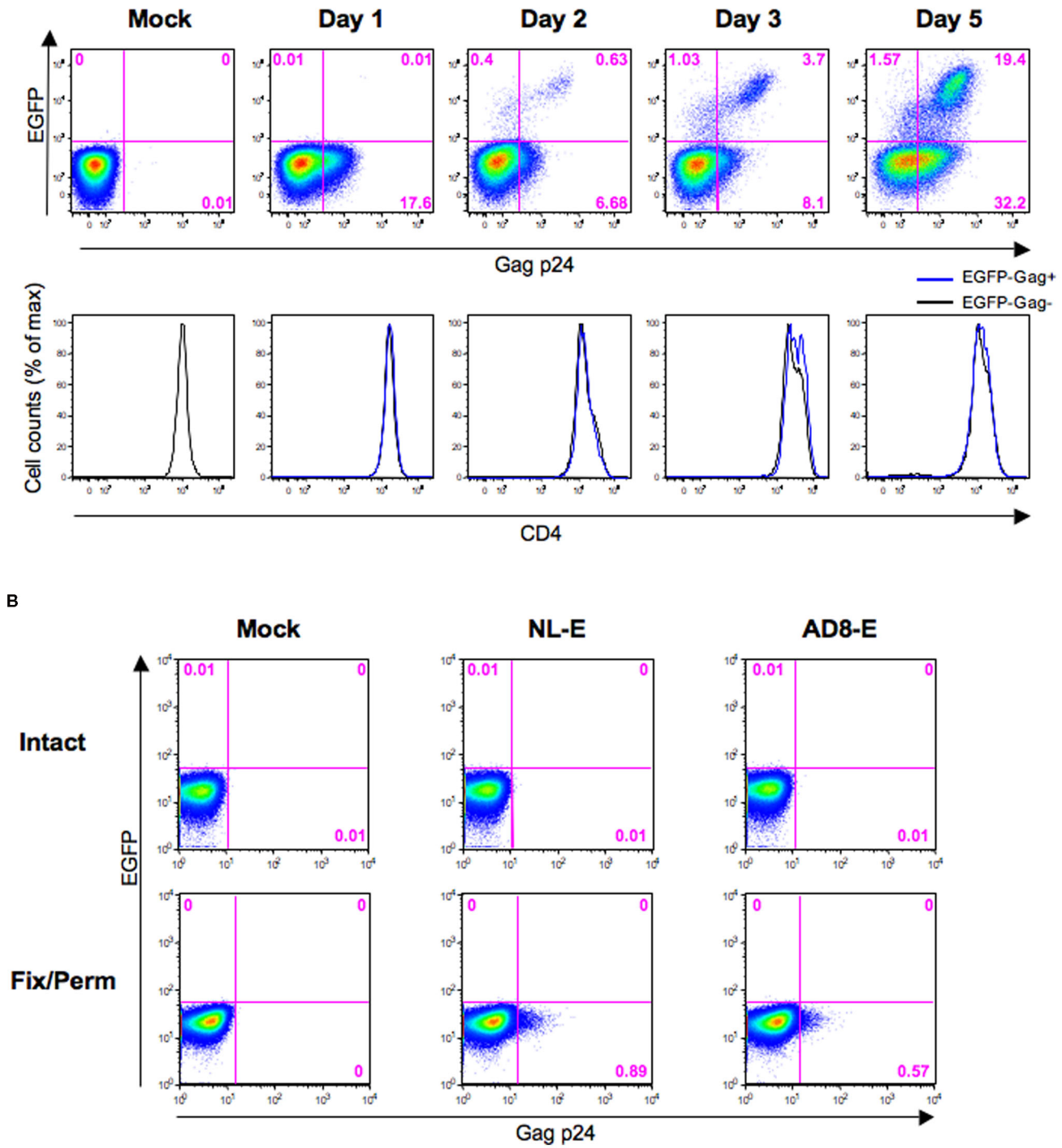

FIGURE 5 | Evaluation of Gag p24 ICS for HIV-1-internalized and -bound cells. (A) Representative flow cytometric analyses of the HIV-1_{NL-E} infection (NL-E) and mock (Mock) groups from three donors in which unstimulated primary $CD4^+$ T cells were infected with HIV-1_{NL-E} or not, respectively, and cultured for 5 days (including the initial 1 day culture with TCR-stimulation). The expression patterns of EGFP and Gag p24 are indicated in the pseudo-color plot profiles for the $Dead^-/CD3^+/CD8^-$-gated cell fractions (upper panels). The expression level of CD4 in $EGFP^-Gag^+$ and $EGFP^-Gag^-$ cells is indicated in the histograms by the blue and black lines, respectively (lower panels). **(B)** Representative flow cytometric analyses of Gag p24 staining. CEM–CCR5 cells were infected with HIV-1_{NL-E} (NL-E), HIV-1_{AD8-E} (AD8-E), or not (Mock). Immediately after spinoculation at 4˚C, the cells were washed and fixed/permeabilized (fix/perm) or not (intact) prior to Gag p24 staining.

activation levels. In this experiment, primary $CD4^+$ T cells from four donors were individually pre-stimulated via the TCR for 4 (weak stimulation) or 24 h (strong stimulation), infected with HIV-$1_{NL\text{-}E,}$ and then cultured for a further 3 days. First, we confirmed that this experimental protocol allowed the preferential production of HIV-$1_{NL\text{-}E}$ upon strong stimulation in all donors by examining the cell culture supernatants by ELISA (**Figure 6A**) and real-time RT-PCR (**Figure 6B**). Flow cytometric analysis of intact cells showed that HIV-$1_{NL\text{-}E}{}^+$ ($EGFP^+$) cells were more prevalent after strong stimulation than after weak stimulation, although the proportion of HIV-$1_{NL\text{-}E}{}^+$ cells varied among individuals (**Figure 6C**, upper and middle panels). The PMT voltage was optimized for EGFP to prevent excessive EGFP signaling (**Figures 2** and **3**). Of note, EGFP expression by HIV-$1_{NL\text{-}E}{}^+$ cells was lower in the weak stimulation group than in the strong stimulation group (as observed in donors #4 and #5), and EGFP expression in the weak stimulation group approached that in the strong stimulation group in parallel with the increase in the number of HIV-$1_{NL\text{-}E}{}^+$ cells (as observed in donors #6 and #7; **Figure 6C**, lower panels). Taken together, these results show that the intensity of the fluorescent reporter is highly correlated with the viral replication level.

DISCUSSION

Flow cytometric analysis is a reliable and convenient method for detecting HIV-1-infected cells at a single cell level. Here, we studied the potential usefulness of several HIV-1 fluorescent reporters that have been published previously (Yamamoto et al., 2009). We examined whether they would be helpful for evaluating viral replication levels based on their fluorescence intensity. In this study, we used recombinant HIV-1 encoding either EGFP or DsRed to show that the fluorescence intensity of the EGFP and DsRed reporters was associated with the level of CD4 downmodulation (**Figure 2**). Furthermore, we showed that EGFP intensity was associated with the expression level of Gag p24 (**Figure 4**). These findings clearly indicate that fluorescent reporter intensity is useful for evaluating viral replication levels. To confirm this argument, we further compared the fluorescent reporter intensity of HIV-1-infected cells that were strongly or weakly stimulated via the TCR. As expected, higher levels of HIV-1 replication/production occurred in strongly stimulated cells from all the donors tested (**Figure 6A,B**). Although the proportion and EGFP intensity of the HIV-1-infected cells varied among individuals, this might be due to differing susceptibility to HIV-1 and/or TCR-stimulation. Thus, the variability in EGFP expression is rather favorable to our argument, as increased EGFP intensity was associated with an increase in the number of HIV-1-infected cells after weak stimulation (**Figure 6C**).

Although Gag p24 ICS is usually used for flow cytometric analysis of other markers, we showed that it can also be used to detect cells that have internalized or bound HIV-1 (**Figure 5A,B**). However, Gag p24 ICS did not appear sensitive enough to detect HIV-1-infected cells because some HIV-1-infected cells in which CD4 was moderately downmodulated were identified as positive for EGFP but negative for Gag p24 (**Figure 4**). Bosque and Planelles (2009) also identified a small population of such reporter-positive but Gag p24-negative cells by flow cytometry when $CD4^+$ T cells were infected with EGFP-encoded DHIV incorporating a small out-of-frame deletion in the gp120-encoding area and pseudotyped with X4-tropic HIV-1_{LAI}, and assumed that these cells were at an early stage of the infection process and did not display late viral proteins. Therefore, our own findings indicate that it is the HIV-1 fluorescent reporter, rather than Gag p24 staining, that reliably detects HIV-1-infected cells at different stages of infection in flow cytometry experiments.

It is known that maturation of DsRed for coloration is usually slower compared with EGFP (Bevis and Glick, 2002; Maruyama et al., 2004). When we focused on the HIV-1 dull fraction in **Figure 2**, we found that CD4 downmodulation was stronger in $DsRed^+$ cells than in $EGFP^+$ cells. These results suggest that the EGFP reporter is preferable to the DsRed reporter for detection of earlier stage of infection. Furthermore, the detrimental effect of fixation/permeabilization on fluorescent reporter intensity, particularly when using the DsRed reporter, should be noted (**Figure 3**). Although the detailed mechanism remains obscure, this may result from the lower fluorescence intensity of DsRed compared with EGFP. A similar phenomenon was described regarding fixation with 3% paraformaldehyde, which significantly decreases the fluorescence intensity of DsRed, although specific data were not provided (Weber et al., 2006). Regardless of the weakened signal, the EGFP reporter is still compatible with fixation/permeabilization because the proportion of $EGFP^+$ cells was comparable between intact cells and fixated/permeabilized cells (**Figure 3**). Therefore, the EGFP reporter still maintains an advantage for analyses of cytokine/chemokine production and proliferation assays based on Ki-67 expression, for which ICS is necessary.

The HIV-1 fluorescent reporter has a potential application in molecular biology. In general, ICS-treated cells are not suitable for analysis using molecular biology techniques, since formaldehyde-based fixation (required for ICS) makes RNA extraction and reverse transcription and quantification problematic (Farragher et al., 2008) because of chemical cross-linking of proteins and nucleic acids (Kuykendall and Bogdanffy, 1992; Finke et al., 1993; Park et al., 1996), degradation of RNA (Bresters et al., 1994), and covalent modification of RNA via the addition of monomethylol groups to the bases (Masuda et al., 1999); therefore, by using the HIV-1 fluorescent reporter, HIV-1-infected cells can be sorted/purified without the need for fixation, allowing further characterization at a molecular level.

Given the usefulness of the HIV-1 fluorescent reporter shown here, it would also be very useful for investigating the mechanisms involved in the selective replication of R5 HIV-1 over X4 HIV-1 during the acute phase *in vivo* (Wolinsky et al., 1992; Zhu et al., 1993; van't Wout et al., 1994) and in cell culture systems *in vitro* (Schweighardt et al., 2004; Roy et al., 2005). We previously developed an *in vitro* dual infection model using EGFP-encoded X4 HIV-1 (HIV-$1_{NL\text{-}E}$) and DsRed-encoded R5 HIV-1 (HIV-$1_{AD8\text{-}D}$) and showed that the increase in the proportion of X4 HIV-1-infected cells is dependent upon their activation level (Yamamoto et al., 2009). Furthermore, the results of the present study show that the fluorescence intensity of the reporter molecule

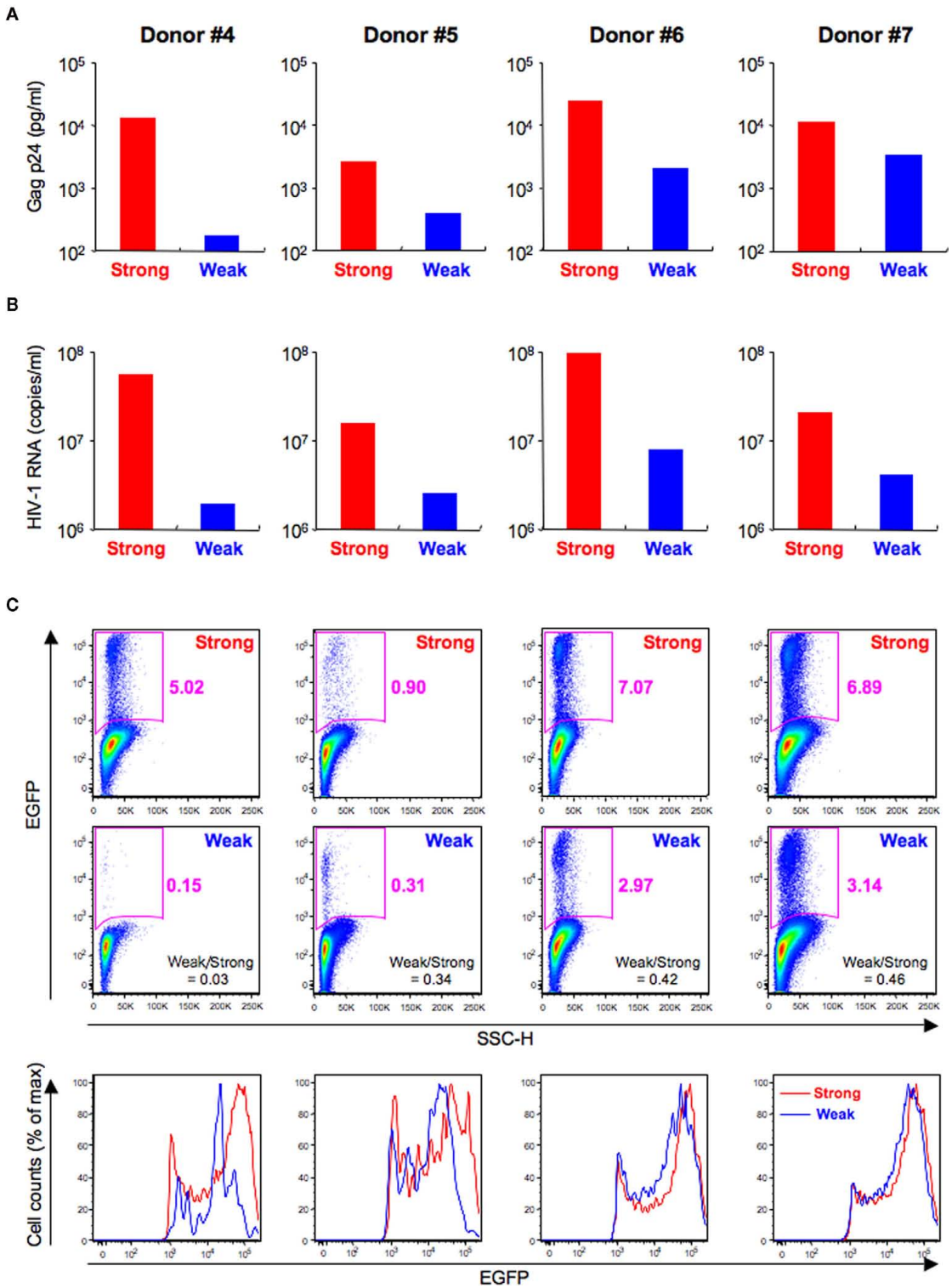

FIGURE 6 | Differences of HIV-1 replication according to TCR-mediated activation levels. Primary $CD4^+$ T cells from four donors (Donor #4–7) were pre-stimulated via TCR for 4 (Weak; blue) or 24 h (Strong; red) and then infected with $HIV\text{-}1_{NL\text{-}E}$ and cultured for 3 days. **(A)** ELISA for HIV-1 Gag p24 in the culture supernatants. **(B)** Quantitative real-time RT-PCR for HIV-1 RNA in the culture supernatants. **(C)** Flow cytometric analysis of intact cells showing pseudo-color plot profiles ($Dead^-/CD3^+/CD8^-$-gated cell fraction; upper and middle panels) and histogram profiles ($Dead^-/CD3^+/CD8^-/EGFP^+$-gated cell fraction; lower panels).

can be used to assess the level of viral replication in infected cells; therefore, by focusing on the HIV-1-infected cells and the fluorescent reporter intensity in the dual infection model, the detailed mechanism(s) of HIV-1 infection/pathogenesis can be clarified. In this regard, we have been investigating the dynamics of HIV-1 infection *in vivo* using humanized mice infected simultaneously with EGFP-encoded X4 HIV-1 (HIV-1_{NL-E}) and DsRed-encoded R5 HIV-1 (HIV-1_{AD8-D}; Ishige et al., in preparation). We believe that the advantages of the recombinant HIV-1 fluorescent reporter will contribute to the further understanding of HIV-1 infection/pathogenesis.

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Cellular factors involved in HTLV-1 entry and pathogenicity

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Human T cell leukemia virus type 1 (HTLV-1) is the causative agent of adult T cell leukemia (ATL) and HTLV-1 – associated myelopathy and tropical spastic paraparesis (HAM/TSP). HTLV-1 has a preferential tropism for CD4 T cells in healthy carriers and ATL patients, while both CD4 and CD8 T cells serve as viral reservoirs in HAM/TSP patients. HTLV-1 has also been detected other cell types, including monocytes, endothelial cells, and dendritic cells. In contrast to the limited cell tropism of HTLV-1 *in vivo*, the HTLV receptor appears to be expressed in almost all human or animal cell lines. It remains to be examined whether this cell tropism is determined by host factors or by HTLV-1 heterogeneity. Unlike most retroviruses, cell-free virions of HTLV-1 are very poorly infectious. The lack of completely HTLV-1-resistant cells and the low infectivity of HTLV-1 have hampered research on the HTLV entry receptor. Entry of HTLV-1 into target cells is thought to involve interactions between the *env* (Env) glycoproteins, a surface glycoprotein (surface unit), and a transmembrane glycoprotein. Recent studies have shown that glucose transporter GLUT1, heparan sulfate proteoglycans (HSPGs), and neuropilin-1 (NRP-1) are the three proteins important for the entry of HTLV-1. Studies using adherent cell lines have shown that GLUT1 can function as a receptor for HTLV. HSPGs are required for efficient entry of HTLV-1 into primary CD4 T cells. NRP-1 is expressed in most established cell lines. Further studies have shown that these three molecules work together to promote HTLV-1 binding to cells and fusion of viral and cell membranes. The virus could first contact with HSPGs and then form complexes with NRP-1, followed by association with GLUT1. It remains to be determined whether these three molecules can explain HTLV-1 cell tropism. It also remains to be more definitively proven that these molecules are sufficient to permit HTLV-1 entry into completely HTLV-1-resistant cells.

Keywords: epidemiology, cell tropism, heparan sulfate, glucose transporter GLUT1, neuropilin-1, syncytia formation, Env proteins, Env isomerization

INTRODUCTION

Human T cell leukemia virus type 1 (HTLV-1) or T-lymphotrophic virus type 1 is a member of the deltaretrovirus family, which includes the simian T-lymphotrophic virus type 1 (STLV-1) and bovine leukemia virus (BLV). There are four known strains of HTLV, i.e., HTLV-1, HTLV-2, HTLV-3, and HTLV-4. HTLV-1 and HTLV-2 are prevalent worldwide (Edlich et al., 2000; Proietti et al., 2005), while HTLV-3 and HTLV-4 have been identified only in Central Africa (Mahieux and Gessain, 2009). HTLV-1 and STLV-1 are highly related; HTLV-2 and HTLV-3 are closely related to STLV-2 and STLV-3, respectively. Thus, the HTLVs are thought to derive from interspecies transmission between monkeys and humans. The genetic variation among HTLV-1 strains is less than 8%, and HTLV-1 and HTLV-2 show 70% nucleotide homology (Gessain et al., 1996; Feuer and Green, 2005).

Human T cell leukemia virus type 1 was first isolated in 1980 in a T cell line derived from a patient with cutaneous T cell lymphoma (Poiesz et al., 1980) and was shown to be the etiological agent of adult T cell leukemia (ATL; Hinuma et al., 1981; Yoshida et al., 1982; Takatsuki, 2005). In 1985, HTLV-1-seropositive patients in French Martinique were diagnosed with a neurodegenerative disorder, termed tropical spastic paraparesis (TSP; Gessain et al., 1985). A similar clinical disorder was reported in Japanese patients and was named HTLV-1 associated myelopathy (HAM; Osame et al., 1986; Osame and Igata, 1989). HTLV-2 is rarely pathogenic and is sporadically associated with neurological disorders (Hjelle et al., 1992; Araujo and Hall, 2004). There have been no known diseases associated with HTLV-3 or HTLV-4.

Here, the results from *in vivo* and *in vitro* studies concerning HTLV-1 infection, focusing on the mechanisms of HTLV-1 entry, are reviewed.

EPIDEMIOLOGY OF HTLV-1 INFECTION

An estimated 15–20 million persons are infected with HTLV-1 throughout the world. The virus is endemic in southwestern Japan, Central Africa, the Caribbean Islands, and Australia (aborigines) as well as in some regions of South America, Melanesia, Middle East, and India. Thus, HTLV-1 prevalence shows a quite strange ethnic distribution. In these endemic areas, the seroprevalence rates range from 0.1 to 30% (Blattner et al., 1982; Saxinger et al., 1984; Yanagihara et al., 1990; Gotuzzo et al., 2000; Sonoda et al., 2011). After prolonged latency periods of 40–60 years, approximately 5% of HTLV-1-infected individuals (6.6% of males and 2.1% of females) will develop ATL (Kajiyama et al., 1986; Murphy et al., 1989; Edlich

et al., 2000; Proietti et al., 2005; Sonoda et al., 2011). HTLV-2 is prevalent among intravenous drug users (IDUs) and is endemic among IDUs in the USA, Europe, South America, and Southeast Asia (Lee et al., 1989; Khabbaz et al., 1992; Fukushima et al., 1995).

Human T cell leukemia virus type 1 is transmitted through three major routes: (1) breastfeeding from mother-to-child, (2) sexual contact from male to female, and (3) needle sharing which mediates exposure to contaminated blood. Mother-to-child transmission through breastfeeding is the predominant mode. Transmission rates are approximately 20% for children born to infected mothers. Lifetime sexual transmission rates between spouses are 60% from infected males to females and only 0.4% from infected females to males (Kajiyama et al., 1986; Kusuhara et al., 1987; Murphy et al., 1989; Hino et al., 1994; Ureta-Vidal et al., 1999; Sonoda et al., 2011). These epidemiological characteristics of HTLV-1 infection are, or shall be, associated with virological properties of HTLV-1 that have been, or will be, clarified *in vitro*.

STRUCTURE AND GENE PRODUCTS OF HTLV-1 GENOME

Human T cell leukemia virus type 1 is an enveloped virus of approximately 100 nm in diameter. Newly synthesized viral particles attach to target cell receptors on the viral envelope (Env) and enter the target cell through membrane fusion. Entry is followed by capsid uncoating and content release into the cell cytoplasm. The viral RNA is reverse transcribed into double-stranded DNA by reverse transcriptase (RT), which is then transported to the nucleus and integrated into the host chromosome, forming the provirus.

Human T cell leukemia virus type 1 is a single-stranded diploid RNA virus, and the proviral genome of HTLV-1 is 9,030–9,040 nucleotides long containing two flanking long terminal repeat (LTR) sequences (**Figure 1**; Seiki et al., 1983). The LTRs of HTLV-1 comprise three components: a unique 3′ (U3) region, a repeated (R) region, and a unique 5′ (U5) region. The HTLV-1 genome is packaged in the viral core with the viral nucleocapsid protein (p15 NC), which is surrounded by capsid (p24 CA) and matrix (p19 MA) proteins.

The HTLV-1 genome encodes the structural proteins Gag (NC, CA, and MA) and Env and the enzymes RT, RNase H (RH), integrase (IN), and protease (Pro; **Figure 1**). The *env* gene encodes the surface unit (SU) gp46 and transmembrane unit (TM) gp21 proteins (Hattori et al., 1984; **Figure 2**).

The envelope is made of a lipid bilayer of cellular origin, which contains the virally encoded Env glycoproteins. The Env proteins are synthesized as a precursor protein, gp62, and cleaved into the gp46 and gp21 glycoprotein subunits. The SU protein gp46 is associated with the TM gp21, and the SU-TM complex is anchored to the viral or infected cell membrane through the membrane-spanning region (MSR) within TM (**Figures 3** and **4**). HTLV-1 Env is a trimer of the gp46-gp21 subunit pair. Cell receptor-binding activity is conferred by gp46 and the fusion activity is a function of gp21. The HTLV-1 Env proteins are genetically highly conserved among isolates, and the variability within their amino acid sequences ranges from 1 to 8% (Gessain et al., 1996; Feuer and Green, 2005).

The pX region encodes the accessory proteins as well as the regulatory proteins Tax and Rex (Sodroski et al., 1984; Cann et al., 1985; Felber et al., 1985; Fujisawa et al., 1985; **Figure 1**). The accessory genes are alternatively spliced and translated from different initiation sites. These genes encode novel proteins, such as p12/p8, p30, and p13 (Albrecht et al., 2000; Silverman et al., 2004; Nicot et al., 2005). The anti-sense region corresponding to the pX region and the *env* gene region encodes HBZ (Gaudray et al., 2002; Arnold et al., 2006; Matsuoka and Jeang, 2011; **Figure 1**). The expression of the HBZ gene has been detected in primary leukemic cells isolated from all ATL patients tested while the *tax* transcript was hardly detectable in them. The HBZ gene has growth-promoting activity *in vivo* and *in vitro*. These findings suggest that HBZ gene transcription is indispensable for the development of ATL.

CELLS INFECTED WITH HTLV-1 *IN VIVO*

Approximately up to 5% of HTLV-1-infected patients will develop ATL after a prolonged latency period (Uchiyama, 1997; Takatsuki, 2005; Sonoda et al., 2011). *In vivo*, the targets of virus-induced transformation are CD4+/CD45RO+ memory T cells. A monoclonal population of T cells showing CD3+/CD4+/CD8-/CD25+/HLA-DR+/CCR4+ cell surface markers also characterizes malignant cells of ATL (Uchiyama, 1997; Suzuki et al., 1998; Takatsuki, 2005; Hieshima et al., 2008). Regulatory T cells (Tregs)

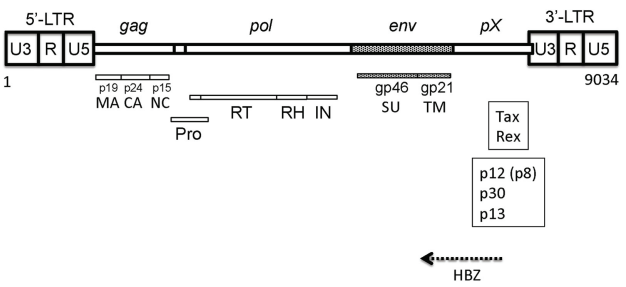

FIGURE 1 | Structure of HTLV-1 genome. The *gag* gene encodes the matrix (MA), capsid (CA), and nucleocapsid (NC) proteins. The *pol* gene encodes reverse transcriptase (RT), RNase H (RH), and integrase (IN). The pX region encodes p13, p12, p30, etc. HBZ is encodes by the anti-sense frame of the provirus.

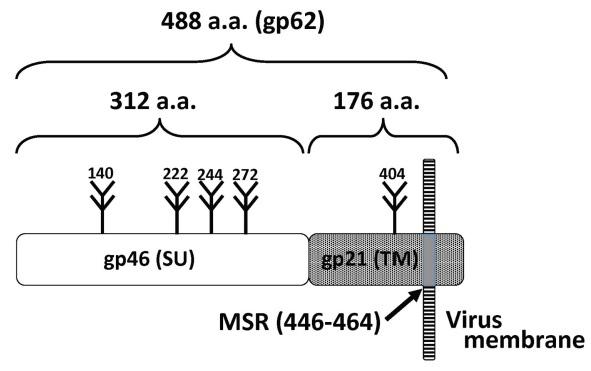

FIGURE 2 | Organization of Env proteins. The surface unit (SU) gp46 and transmembrane unit (TM) gp21 and are shown. Five potential N-glycosylation sites and the membrane-spanning region (MSR) are shown.

might also be infected in some patients (Chen et al., 2006; Yano et al., 2007; Abe et al., 2008; Toulza et al., 2009).

Human T cell leukemia virus type 1 – associated myelopathy and tropical spastic paraparesis primarily affects the thoracic spinal cord, and patients often show limb paraparesis (Osame et al., 1986; Osame and Igata, 1989). In the brains of some HAM/TSP patients, astrocytes are productively infected with HTLV-1. Several lines of evidence, such as IgG deposits in CNS parenchyma, indicate that the blood-brain barrier (BBB) is disrupted in HAM/TSP patients (Walter et al., 1994; Lehky et al., 1995). In addition, the infection of adherent cells *in vivo* has been reported: sweat gland epithelia, salivary glands, vascular endothelial cells, and mammary glands (Setoyama et al., 1998).

In infected individuals, HTLV-1 is primarily found in CD4+ T cells, while HTLV-2 has frequently been found in CD8+ T cells (Richardson et al., 1990; Ijichi et al., 1992). Although CD4+ T cells are the major reservoir, other hematopoietic cells [CD8+ T cells, B lymphocytes, monocytes, macrophages, dendritic cells (DCs), and megakaryocytes], synovial cells, and glial cells (astrocytes and microglial cells) have been infected with HTLV-1 (Kitajima et al., 1991; Koyanagi et al., 1993; Walter et al., 1994; Lehky et al., 1995; Hanon et al., 2000a; Nagai et al., 2001). It is intriguing that HTLV-I-specific, but not Epstein–Barr virus-specific, CD8+ T lymphocytes were shown to be preferentially infected with HTLV-1 (Wucherpfennig et al., 1992; Goon et al., 2002). The ratio of HTLV-1 genome-positive cells in some HAM/TSP patients was reported to be as high as 40–65% in the CD4+ and CD8+ subset. The proportion of cells expressing HTLV-1 RNA is 0.02–0.1% in both subsets. Other reports also show the expression of the HTLV-1 genome *in vivo* (Cho et al., 1995; Hanon et al., 2000b), although HTLV-1 RNA expression *in vivo* is known to be highly suppressed in HTLV-1 carriers and ATL patients.

Experiments using deuterated glucose labeling to quantify *in vivo* lymphocyte dynamics in HTLV-1-infected individuals revealed that CD4+/CD45RO+ and CD8+/CD45RO+ T lymphocyte proliferation was elevated in HTLV-1-infected subjects; about 10^{12} more lymphocytes were estimated to be produced per year in HTLV-1-infected subjects compared with HTLV-I-uninfected subjects (Asquith et al., 2007). This high cell turnover was correlated with Tax expression measured *ex vivo*. These findings suggest that HTLV-1 genes may be expressed somewhere *in vivo*. These virological, pathological, and clinical features of HTLV-1 infection can or will be explained by the cell tropism and pathogenicity of HTLV-1.

ANIMAL MODELS OF HTLV-1 INFECTION

Animal models of HTLV-1 infection and transmission have clarified the effects of neutralizing antibodies and viral and host factors on persistent HTLV-1 infections. Some non-human primates, rabbits, rats, and mice, can be infected with HTLV-1 (Lairmore et al., 2005). In these models, HTLV-1 is also detected in various types of hematopoietic cells and non-hematopoietic tissues, including the brain, lung, kidney, heart, liver, and thyroid.

Milk transmission of HTLV-1 in rabbits can be inhibited through passive immunizations using neutralizing antibodies or HTLV-1 Env vaccines (Akagi et al., 1985; Nakamura et al., 1987; Uemura et al., 1987; Kataoka et al., 1990; Sawada et al., 1991). Although there is a strong immune response to HTLV proteins, such Env or Tax, through the humoral or cellular immune system in humans, HTLV-1 can sustain life-long persistence in subjects and HTLV-1-infected lymphocytes can robustly proliferate as described above.

The HTLV-1 accessory proteins, p12/p8, p30, and p13, have been implicated in the transmission and spread of HTLV-1 *in vivo* or in HTLV-1 persistence in animal models, although these proteins were shown to have little effect on HTLV-1 infection *in vitro* (Albrecht et al., 2000; Silverman et al., 2004; Nicot et al., 2005; Lairmore et al., 2011). The viral protein p12 localizes in the endoplasmic reticulum (ER), binds major histocompatibility complex (MHC) class I in the ER, decreases surface expression of MHC-I, ICAM-1, and intercellular adhesion molecule-2 (ICAM-2), and inhibits the killing of HTLV-1-infected cells by natural killer cells. p8, Proteolytically cleaved from p12, increases T cell contact through LFA-1 clustering and augments the number and length of cellular conduits among T cells. p8 or even virions are transferred to neighboring T cells through these conduits (van Prooyen et al., 2010). The p30 protein will localize in the nucleus and retain the Tax/Rex messenger inside the nucleus and enhances cell survival by altering cell cycle regulation. p13 Localizes in the nucleus and mitochondria. p13 can modulate cell survival and have an pivotal role in early virus transmission and virus persistence *in vivo*.

INFECTION OF CELLS WITH HTLV-1 *IN VITRO*

Unlike most other viruses, cell-free HTLV-1 is poorly infectious, and efficient infection requires cell-to-cell contact (Clapham et al., 1983; Hoshino et al., 1983; Nagy et al., 1983; Fan et al., 1992; Mazurov et al., 2010). HTLV-1-infected cells produce small amounts and low titers of the virus. However, HTLV-1 has passed on from generation to generation among humans for a long time.

Adherent cells persistently infected with HTLV-1 have rarely been isolated (Hoshino et al., 1983; Ho et al., 1984; Hoxie et al., 1984; Yoshikura et al., 1984; Fan et al., 1992; Mazurov et al., 2010). However, the HTLV receptor has been detected in almost all vertebrate cells (Sutton and Littman, 1996; Trejo and Ratner, 2000; Manel et al., 2005a) in contrast to the limited cell tropism *in vivo*. Some cell lines will form numerous syncytia upon co-cultivation with HTLV-1-producing cells, while others are resistant to syncytia formation (Jassal et al., 2001).

Surface unit-Fc fusion proteins consisting of the Fc portion of immunoglobulin and HTLV-1 SU [either full-length gp46 or the N-terminal Receptor-Binding Domain (RBD) of SU; **Figure 3**] have been used to detect and analyze HTLV-1 receptors. The SU-Fc binds to almost all vertebrate cell lines and can often bind to cell lines considered to be negative for the receptor due to their resistance to Env-mediated syncytia formation (Sutton and Littman, 1996; Trejo and Ratner, 2000; Jassal et al., 2001; Manel et al., 2005a). The use of SU-Fc has, however, clarified that HTLV-1 receptors are lacking on the surface of resting or naive CD4+ and CD8+ T cells but HTLV-1 receptors can be rapidly upregulated by treating them with PHA/IL-2, TGF-β, or interleukin-7 (Moriuchi and Moriuchi, 2002; Manel et al., 2003a; Nath et al., 2003; Jones et al., 2005a). These properties have been used to characterize an HTLV-1 receptor candidate as described below.

VIRAL SYNAPSE AND BIOFILM-LIKE STRUCTURE IN HTLV-1 INFECTION

Special structures permitting efficient transmission of HTLV-1 have been identified. Namely, viral or virological synapses (VS), similar to immune or immunological synapses, can be made after cell-to-cell contacts between HTLV-1-infected and uninfected T cells (Igakura et al., 2003; Jenkins et al., 2009). Lymphocyte contact will rapidly induce polarization of the cytoskeleton, especially the microtubule-organizing center (MTOC), of HTLV-1-infected cells at the cell–cell junction: HTLV-I genome and Gag proteins accumulate at the junction. Subsequently, polarization is promoted by Tax, and the formation of VS leads to the entry of viral particles, viral proteins, and genomic RNA into uninfected target cells (Igakura et al., 2003; Nejmeddine et al., 2005; Jenkins et al., 2009). VS also lead to the efficient transmission of human immunodeficiency virus type 1 (HIV-1): its transmission takes place even in the presence of neutralizing antibodies. The viral envelope protein is required for the transmission of HTLV-1, suggesting that complete HTLV-1 virions are transferred across the synapse (Nejmeddine et al., 2009). Electron tomography combined with the immunostaining of viral proteins has demonstrated the presence of enveloped HTLV-1 particles in the VS formed between naturally infected lymphocytes and uninfected cells (Majorovits et al., 2008).

More recently it was shown that HTLV-1 virions are stored outside the cells within a protective microenvironment: specific components of the extracellular matrix, including collagen, galectin-3, and tetherin, form a biofilm-like structure on the outer surface of infected cells (Pais-Correia et al., 2009). Following contact with T cells, these structures are rapidly transferred from infected lymphocytes to uninfected cells, resulting in the infection of target cells. HTLV-1 p8 promotes the formation of cellular conduits between T cells as described above (van Prooyen et al., 2010). These three structures will promote highly efficient transmission of the virus from cells to cells *in vivo*, as compared to the transmission of cell-free virus produced by infected cells to uninfected cells.

INFECTION OF DENDRITIC CELLS

Dendritic cells can also be infected with HTLV-1 (Macatonia et al., 1992). Recently, it was demonstrated that DCs could play a central role in HTLV-1 infection *in vivo*. DCs participate in the cell-to-cell transmission of HTLV-1 in two ways: (1) DCs can capture and transfer HTLV-1 virions to fresh T cells and (2) infected DCs can transmit newly made virions to fresh T cells (Macatonia et al., 1992; Knight et al., 1993; Ceccaldi et al., 2006). Monocyte-derived DCs form syncytia and can be infected with HTLV-1 upon co-culture with HTLV-1-producing lymphocytes. Monoclonal antibodies (MAbs) against DC-specific ICAM-3-grabbing non-integrin (DC-SIGN) can inhibit the formation of HTLV-1-induced syncytia. HTLV-1, however, does not bind directly to DC-SIGN, and the enhanced syncytium formation is mediated through the interaction of DC-SIGN with ICAM-2 and ICAM-3.

More recently, DCs have been shown to be readily infected through cell-free HTLV-1 (Jones et al., 2008). Cell-free HTLV-1 can efficiently infect myeloid and plasmacytoid DCs (pDC). DCs exposed to HTLV-1 efficiently transfer virus to autologous primary CD4+ T cells. This DC-mediated transfer of HTLV-1 involves heparan sulfate proteoglycans (HSPGs), neuropilin-1 (NRP-1), and DC-SIGN (Jain et al., 2009). Thus, DCs can facilitate HTLV-1 transmission, dissemination, and persistence through different mechanisms *in vivo*.

OTHER CELLULAR FACTORS AFFECTING HTLV-1 INFECTION *IN VITRO*

Many cellular factors that affect HTLV-1-induced syncytium formation have been identified. HTLV-1 Tax affects the expression of numerous molecules, such as vascular cell adhesion molecule 1 (VCAM-1) or ICAM-1 (Tanaka et al., 1995; Hildreth et al., 1997; Daenke et al., 1999). VCAM-1 promotes HTLV-1-induced syncytia formation. ICAM-1, ICAM-3, or VCAM-1 is required for syncytia formation in K562 induced by HTLV-1-producing MT-2 cells. Antibodies against β-integrins can inhibit this syncytium formation (Daenke et al., 1999). MAbs that inhibit HTLV-1-induced syncytium formation were produced in mice and shown to recognize class II MHC molecules (Hildreth, 1998). The heat shock cognate protein 70 (HSC70) directly binds to gp46. The transduction of human HSC70 cDNA into mouse cells, however, did not support HTLV-I entry: HSC70 markedly promoted syncytium formation upon co-cultivation with HTLV-I-producing cells but did not facilitate HTLV-I entry (Fang et al., 1999). Tetraspanin (CD82) was also shown to bind HTLV-1 Env proteins (Pique et al., 2000; Mazurov et al., 2006). Thus, HTLV-1-induced syncytium formation is affected by HSC70, CD82, ICAM-1, ICAM-3, LFA-1, VCAM-1, integrin β2, integrin β7, or Dlg protein (Blot et al., 2004). These factors might also affect the spread and pathogenicity of HTLV-1 *in vivo*.

Plasma membrane microdomains rich in cholesterol are important for the entry of many viruses, including retroviruses. The depletion of cholesterol using cyclodextrin inhibits the entry of HTLV-1 and HTLV-I pseudotypes, whereas it did not affect the receptor-binding activity of HTLV-1. Using SU-Fc, the HTLV-I receptor was shown to co-localize with a raft-associated marker. Some MAbs that inhibited HTLV-1-induced syncytium formation recognized proteins in lipid rafts. The results of these studies suggest that lipid rafts might play a role in HTLV-1-induced syncytium formation and that cholesterol is required in a post-binding step (Wielgosz et al., 2005). Intact lipid rafts are also necessary for the formation of the VS as described above (Igakura et al., 2003; Jolly and Sattentau, 2005).

ENHANCEMENT OF HTLV-1 INFECTION BY HSPG

The HSPG family is composed of core proteins, such as syndecans or glypicans, associated with one or more of the sulfated polysaccharide side chains heparan sulfate (HS) glycosaminoglycans. HSPGs have been shown to bind to many viruses, such as herpes simplex viruses, flaviviruses, picornaviruses, and HIV-1 (Ibrahim et al., 1999; Shukla et al., 1999) and promote their receptor-mediated entry.

The presence of HSPGs on the cell surface has been shown to affect HTLV-1 binding and viral entry (Okuma et al., 2003; Pinon et al., 2003a). Osteoprotegerin (OPG) inhibited the HTLV-1 infection of cell lines, indicating that this inhibition is due to the binding of OPG to cellular HS (Okuma et al., 2003). HTLV-1 SU-Fc binds to HSPGs on mammalian cells, and the enzymatic removal of

HSPGs from HeLa and CHOK1 cells also reduced SU-Fc binding and plating of the HTLV-1 pseudotype (Pinon et al., 2003a). As compared with SU-Fc binding in HSPG-positive CHOK1 hamster cells, the plating efficiency of HTLV-1 in a CHOK1 subline completely lacking HSPG was markedly reduced but apparently detectable. Thus, HSPGs might not be absolutely required for HTLV-1 entry.

More recently, HSPGs were also found to promote the HTLV-1 infection of primary CD4+ T cells and DCs (Jones et al., 2005b, 2006). Resting CD4+ T cells are negative for detectable levels of HSPGs and HTLV-1 receptor(s). HSPGs are expressed following immune activation, and the cells become susceptible to HTLV-1 (Nath et al., 2003; Jones et al., 2005a). The enzymatic removal of HSPGs on the surface of primary CD4+ T cells also reduced the binding of HTLV-1 SU and HTLV-1 virions. HTLV-1 is more dependent on HSPGs than HTLV-2 (Jones et al., 2006). Using HTLV-1/HTLV-2 recombinants, it was shown that HTLV-1 binding to HSPGs requires the C-terminal domain (CTD) of HTLV-1 SU (amino acids 215–313; **Figure 3**). Thus, HSPGs play an important role in the binding and entry of HTLV-1 into primary CD4+ T cells. Recently, we observed that the susceptibilities of established cell lines to HTLV-1 are well correlated with the number of HS chains, especially heparin-like regions, on the cell surface and are inversely correlated with the length of the HS chains (Tanaka et al., 2012).

EFFECTS OF GLUT1 ON HTLV-1 INFECTION

The observation that the transduction of HTLV-1 SU into cells delays medium acidification led to an investigation of glucose transporter 1 (GLUT1) as a candidate HTLV-1 receptor. Subsequently, GLUT1 was found to bind to HTLV-1 SU and promote HTLV-1 entry into adherent cell lines (Manel et al., 2003b). GLUT1 is a 12-membrane-spanning receptor for glucose transport across the cell membrane. GLUT1 is expressed in all of mammalian cell lines that have been tested. The transduction of GLUT1 increases the susceptibility of markedly HTLV-1-resistant cell lines, such as MDBK, to HTLV-1 Env-mediated cell fusion and infection (Coskun and Sutton, 2005). Antibodies against GLUT1, especially against extracellular loop 1 (ECL1), inhibit syncytia formation, and the infection of primary CD4+ T lymphocytes (Manel et al., 2005b; Jin et al., 2006a). Similar to HSPGs, GLUT1 is not detected in resting lymphocytes. The treatment of resting T lymphocytes with TGF-β or IL-7 rapidly induces GLUT1 expression on the cell surface (Jones et al., 2005a).

Quantitative analyses have shown that CD4+ T cells, which are the primary target of HTLV-1, express higher levels of HSPGs than CD8+ T cells. In contrast, CD8+ T cells, which are the primary targets of HTLV-2, express GLUT1 at much higher levels than CD4+ T cells. These studies suggest that the difference in the *in vitro* cellular tropism between HTLV-1 and HTLV-2 (Jones et al., 2006) and the *in vivo* tropisms of these viruses can be explained through different interactions between HTLV-1 and HTLV-2 Env proteins and CD4+ or CD8+ T cells.

The glioblastoma U87 cell line expresses low levels of GLUT1 due to a deletion in the *GLUT1* gene but is easily infected by the HTLV-1 pseudotype (Jin et al., 2006b). Among U87 cell clones, the levels of cell surface GLUT1, however, correlate well with the plating efficiency of HTLV-1 pseudotype viruses, but not their interactions with SU-Fc. These findings together with other observations suggest that molecules other than GLUT1 are also involved in HTLV-1 entry steps, i.e., attachment and binding, and GLUT1 affects the post-binding or fusion step of HTLV-1 infection.

EFFECTS OF NEUROPILIN-1 ON HTLV-1 INFECTION

In 2006, neuropilin-1 (NRP-1) was reported to display the expected properties of a HTLV-1 receptor. NRP-1 is a 130-kDa single membrane-spanning glycoprotein, primarily modified by heparan sulfate (HS), or chondroitin sulfate (Soker et al., 1998; Shintani et al., 2006). NRP-1 is highly conserved among vertebrate species. NRP-1 is a receptor for the chemo-repellent semaphorin III (Sema III), which is a secreted protein known to be necessary for neuronal growth and development. NRP-1 can also bind to VEGF165. VEGF is a polypeptide growth factor with five alternatively spliced isoforms; VEGF165 is the most abundant isoform. NPR-1 is one of the immune synapse factors that is primarily expressed in T cells; its expression is not detectable in resting T cells but is rapidly upregulated after activation (Romeo et al., 2002; Tordjman et al., 2002). pDC and endothelial cells also express NRP-1. Although the *in vivo* expression of NRP-1 is relatively limited, NRP-1 is expressed in many tumor cells and in almost all established cell lines (Soker et al., 1998).

Human T cell leukemia virus type 1 SU inhibits T cell proliferation in a mixed lymphocyte reaction, while anti-NRP-1 antibodies also inhibit T cell proliferation (Ghez et al., 2006). The possibility of the involvement of NRP-1 in HTLV entry has also been examined (Shintani et al., 2006). NRP-1 over expression increases HTLV-1 Env-mediated syncytium formation and promotes HTLV-1 and HTLV-2 infection, whereas the down-regulation of endogenous NRP-1 inhibits HTLV infection (Ghez et al., 2006). Therefore, NRP-1 expression levels *in vivo* and *in vitro* might explain HTLV-1 infection and tropism.

VEGF165 and HTLV-1 SU competitively and HS-dependently bind to NRP-1. The KPXR motif is critical for the direct binding of VEGF165 to NRP-1 and this motif is also found in HTLV-1 SU. The pentapeptide sequence of SU corresponding to this motif (a.a. 90–94, KKPNR) is highly conserved among HTLV-1 strains, and this peptide blocks HTLV-1 entry into primary T cells or DCs (Lambert et al., 2009). These findings indicate that NRP-1 plays a pivotal role in HTLV-1 binding. Because NRP-1 is modified by HS (Shintani et al., 2006), HTLV-1 SU might interact directly with HS conjugated to NRP-1. Thus, HTLV-1 SU will bind to NRP-1 in two ways: an HSPG-mediated indirect interaction and a KKPNR sequence-mediated direct interaction. This motif is also the target of neutralizing antibodies (Palker et al., 1992; **Figure 3**).

Important roles for NPR-1 and HSPG in HTLV-1 infection of DCs and CD4+ T cells have also been shown (Ghez et al., 2006; Jones et al., 2009; Lambert et al., 2009). GLUT1 and NRP-1 are concentrated in viral synapses (Romeo et al., 2002). In contrast, the effects of VEGF165 and anti-GLUT1 on the infection of U87 cells, primary astrocytes or HeLa cells appear to be more complicated (Jin et al., 2006b, 2010).

A RECEPTOR COMPLEX MODEL OF HTLV-1 ENTRY

Recent studies have revealed that HTLV-1 infection *in vitro* involves interactions with three different molecules, HSPG, NRP-1, and GLUT1. HTLV-1 SU can bind to NRP-1 in two ways: an HSPG-mediated indirect interaction and/or a KKPNR sequence-mediated direct interaction as described above. NRP-1 and GLUT1 can also form a complex in the presence of HTLV-1 Env (Ghez et al., 2006; Jones et al., 2009; Lambert et al., 2009). NRP-1 and GLUT1 as well as HS and NRP-1 will work together to promote HTLV-1 entry. The level of GLUT1 expression on target cells correlates well with the titer of HTLV-1 pseudotypes, but not with the level of HTLV-1 binding (Delamarre et al., 1994; Manel et al., 2005b; Jones et al., 2009), indicating a role for GLUT1 in the fusion step that follows the initial binding.

The findings described above lead to a multi-receptor model for HTLV-1 entry (Ghez et al., 2010; Jones et al., 2011). Namely, during the initial binding phase, the CTD of HTLV-1 SU interacts with HSPGs. This interaction would enhance the probability and stability of the HTLV-1 SU interaction with NRP-1. This binding to NRP-1 triggers a conformational change to expose the GLUT1-binding domain. Finally, the interaction of SU with GLUT1 triggers the translocation of TM gp21. In this model, NRP-1 is the primary binding receptor, while GLUT1 is the fusion receptor.

ROLES OF SU PROTEIN gp46 IN HTLV-1 INFECTION

The HTLV-1 *env* gene encodes a 488 amino acid precursor protein, and a 62-kDa protein (gp62) is produced after the addition of five N-glycan chains, four in SU, and one in TM. gp62 is cleaved into the SU gp46 and TM gp21 subunits (**Figures 2–4**). SU-TM complexes are organized as trimers and transported to the surface of infected cells (Delamarre et al., 1996). Site-directed mutagenesis demonstrates that the glycosylation of each site is required for syncytium formation (Pique et al., 1992).

Human T cell leukemia virus type 1 gp46 SU can be organized into four structural domains (**Figure 3**): a signal peptide (residues 1–25), an N-terminal region (26–180), a proline-rich region (PRR; 181–215), and a CTD (216–312). The first 155 amino acid residues after the signal peptide are identified as the RBD (Delamarre et al., 1994; Kim et al., 2004). This organization of SU is similar to other retroviruses (Lavillette et al., 1998). The binding of RBD to viral receptors transmits a PRR-controlled signal to CTD, and this interaction leads to the activation of the fusion function of gp21 TM. The PRR domain is shown to be a hinge region that is subject to the conformational changes induced by receptor-binding.

The functional domains of the HTLV-1 Env have also been identified through analyses of the effects of neutralizing antibodies and specific mutations in Env on HTLV-1 infectivity (Kuroki et al., 1992; Palker et al., 1992; Baba et al., 1993; Desgranges et al., 1994; Tanaka et al., 1994; Londos-Gagliardi et al., 1996). The residues between 100 and 200 amino acids of the SU have been shown to be the targets of neutralizing antibodies. The mutations introduced in these regions reduce the ability of HTLV-1 Env to induce syncytium formation and virus infection. Antibodies in 78% of sera of HTLV-I seropositive subjects reacted with a synthetic peptide corresponding to amino acids 190–209 (Londos-Gagliardi et al., 1996). A synthetic peptide, P-197, consisting of amino acids 197–216 also strongly inhibits HTLV-1-induced cell fusion of various cells, such as Molt-4 and HeLa (Brighty and Jassal, 2001). The rat monoclonal antibody LAT-27 recognizes a peptide epitope (amino acids 191–196) and potently neutralizes cell-free HTLV-1 infection and inhibits HTLV-1-induced syncytia formation (Tanaka et al., 1991). Antibodies binding to the PRR domain will prevent SU structural rearrangements leading to membrane fusion, rather than blocking interactions between SU and the cellular receptors. Some neutralizing antibodies may recognize secondary or tertiary structures of HTLV-1 Env proteins, and so the regions recognized by these antibodies are not necessarily located in the functional domains for HTLV-1 entry.

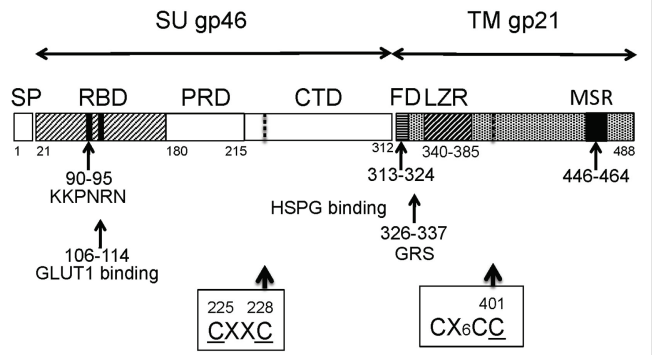

FIGURE 3 | The structure of Env proteins. The CXXC and CX6CC motifs are shown by dotted lines. Abbreviations: SP, signal peptide; RBD, receptor-binding domain; PRD, proline-rich domain; CTD, C-terminal domain; FD, fusion domain; GRS, glycine-rich segment; LZR, leucine zipper-like region; MSR, membrane-spanning region.

Furthermore, the adsorption of the neutralizing activities using a set of shorter peptides has shown that the six residues (KKPNRN; position 90–95) comprise the minimal neutralizing epitope, while residues 86–107 are involved in interactions with NRP-1 and GLUT1 (**Figure 3**). Specifically, a pentapeptide (residues 90–94) blocks the binding of HTLV-1 to cells and NRP-1 (Lambert et al., 2009). Similarly, site-directed mutagenesis experiments showed that D106 and Y114 of SU are important for GLUT1-binding (Manel et al., 2003b). These studies have suggested regions important for the interaction of HTLV-1 SU with the cellular factors (**Figure 3**).

ROLES OF TM PROTEIN gp21 IN HTLV-1 INFECTION

Retrovirus entry into cells occurs through the fusion of the viral envelope either with the cell membrane or with the endosomal membrane after internalization through an endocytic pathway (Mothes et al., 2000; Marsh and Helenius, 2006). The entry process starts by a binding step mediated by SU as described above and a subsequent TM-mediated fusion step. During virus production, TM is maintained in an inactive conformation through an association with SU, and the fusion domain (FD) in TM is not exposed. This conformation prevents premature Env activation that will lead to virus inactivation. The hydrophobic FD is located at its N-terminal region (**Figure 3**). The dissociation of TM from the SU-TM complex results in the projection of FD into the cell membrane. This dissociation involves the formation of a six-helix

coiled-coil bundle that induces membrane fusion (Maerz et al., 2000; Marsh and Helenius, 2006; Mirsaliotis et al., 2007; Lamb et al., 2009).

Four domains conserved in retroviral TM glycoproteins are also present in HTLV-1 TM (Kobe et al., 1999): (1) an amino-terminal hydrophobic domain with characteristics of a FD (Kim et al., 2000); (2) a leucine zipper-like region (LZR) with an amphipathic α-helical structure capable of self-association as a coiled-coil (Maerz et al., 2000; Pinon et al., 2003b; Mirsaliotis et al., 2007; Lamb et al., 2009); (3) a domain containing the conserved motif, which forms an intramolecular disulfide link; and (4) a FD linked to the coiled-coil core through a conserved sequence rich in glycine [glycine-rich segment (GRS), M(326) to S(337); Wilson et al., 2005; **Figure 3**].

Synthetic peptides based on the leash and α-helical region (LHR) of gp21 are potent inhibitors of virus entry into cells. Inhibitory peptides target the triple-stranded coiled-coil motif of the fusion-active gp21 and block its conformational change (Mirsaliotis et al., 2008): These peptides are expected to function in a similar way to clinically available enfuvirtide (T-20) type inhibitors of HIV-1.

ISOMERIZATION IN HTLV-1 Env PROTEINS

The SU and TM subunits are associated through an inter-subunit disulfide link. Receptor-binding or low pH in the endosome decreases this association, leading to dissociation of the SU subunit from the virus and translocation of the TM subunit for membrane fusion (Pinter et al., 1997; Barnard et al., 2004; Wallin et al., 2004; Li et al., 2008). In the case of murine leukemia virus (MuLV) Env, the inter-subunit disulfide has been shown to link the CXXC motif in SU to the conserved CX_6CC motif in the TM, and these motifs are also conserved in deltaretroviruses, such as HTLV-1. The inter-subunit disulfide is then rearranged into a disulfide isomer within the motif (**Figures 3** and **4**).

Human T cell leukemia virus type 1 Env proteins contain the disulfide isomerization motifs C(225)XXC(228; e.g., CIVC in gp46 SU) and CX_6CC(401; e.g., CKALQERCC in gp21 TM; **Figures 3** and **4**). Thus, it has been proposed that C(228) is linked to C(401; Li et al., 2008). This disulfide link will be rearranged through isomerization into the intra-subunit disulfide link between C(225) and C(228). This rearrangement results in SU dissociation and TM translocation for membrane fusion. Thus, similar to MuLV Env, the membrane fusion of HTLV-1 Env was shown to occur under neutral conditions at the cell surface (Li et al., 2008; **Figure 4**). A set of these motifs is lacking in Rous sarcoma virus or HIV-1 but BLV (Barnard et al., 2004; Li et al., 2008).

Murine leukemia virus Env shows low spontaneous inter-subunit disulfide isomerization activity (Li et al., 2008), and MuLV and BLV are relatively stable in culture medium (Shinagawa et al., 2012). In contrast, cell-free HTLV-1 shows a low infectivity. The low infectivity might be related to a high spontaneous inter-subunit disulfide isomerization activity. We observed that the spontaneous dissociation of SU from SU-TM complexes in HTLV-1 virions occurs readily over a wide range of temperatures: the half-life of HTLV-1 infectivity at various temperatures (0–37°C) is as short as 30–60 min (Shinagawa et al., 2012). Derse et al. (2001) have, however, reported that the half-life of HTLV-1 is about 3.5 h at 37°C: in their system, HTLV-1 pseudotype was used and its infectivity was measured by luciferase assays. We have assumed that the isomerization of HTLV-1 Env will occur spontaneously and rapidly in culture medium and HTLV-1 will easily lose the infectivity. HTLV-1 can afford to have this property since most HTLV-1 infections *in vivo* occur from cell-to-cell.

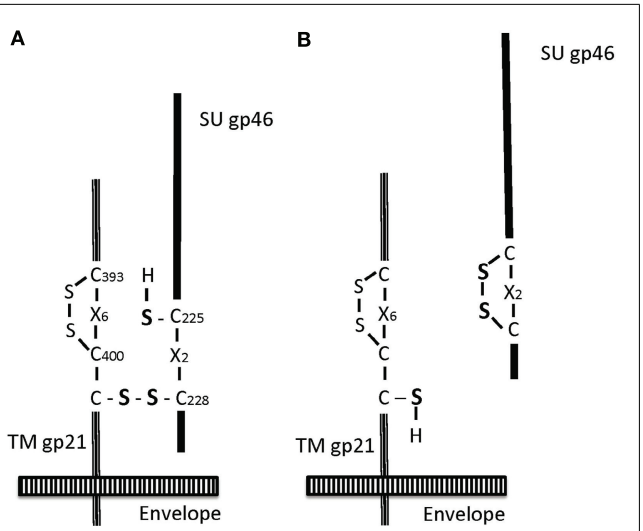

FIGURE 4 | Isomerization of Env proteins. The scheme is shown according to the model by Li et al. (2008), Pinter et al. (1997). The disulfide bond is present between C(228) of SU gp46 and C(401) of TM gp21 **(A)**. Upon the isomerization, this bond is cleaved and the new disulfide bond is made between C(225) and C(228) of gp46, leading to SU dissociation and TM translocation **(B)**.

CONCLUSION

Cellular factors important for HTLV-1 infection and persistence *in vivo* and *in vitro* have been investigated for 30 years. Recently, three factors have been found to play crucial roles in HTLV-1 entry: HSPGs, glucose transporter GLUT1, and neuropilin-1 (NRP-1). These molecules have been shown to form complexes with HTLV-1 SU to promote HTLV-1 entry into primary cells and established cell lines. The functional domains of the HLTV-1 Env proteins and their interactions with these molecules have been elucidated. However, mutant cells lacking an enzyme synthesizing HS, xylosyl transferase, or mutant cells expressing little GLUT1 are apparently susceptible to HTLV-1. It has not been proven whether cells that are completely resistant to HTLV-1 will become susceptible to HTLV-1 when all or a combination of these three molecules are transduced into the cells.

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Cell tropism and pathogenesis of measles virus in monkeys

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Measles virus (MV) is an enveloped negative strand RNA virus belonging to the family of Paramyxoviridae, genus *Morbillivirus*, and causes one of the most contagious diseases in humans. Experimentally infected non-human primates are used as animal models for studies of the pathogenesis of human measles. We established a reverse genetics system based on a highly pathogenic wild-type MV. Infection of monkeys with recombinant MV strains generated by reverse genetics enabled analysis of the molecular basis of MV pathogenesis. The essential *in vivo* function of accessory genes was indicated by infecting monkeys with recombinant MV strains deficient in the expression of accessory genes. Furthermore, recombinant wild-type MV strains expressing enhanced green fluorescent protein enabled visual tracking of MV-infected cells *in vitro* and *in vivo*. To date, three different molecules have been identified as receptors for MV. Signaling lymphocyte activation molecule (SLAM, also called CD150), expressed on immune cells, is a major receptor for MV. CD46, ubiquitously expressed in all nucleated cells in humans and monkeys, is a receptor for vaccine and laboratory-adapted strains of MV. The newly identified nectin-4 (also called poliovirus-receptor-like-4) is an epithelial cell receptor for MV. However, recent findings have indicated that CD46 acts as an MV receptor *in vitro* but not *in vivo*. The impact of the receptor usage of MV *in vivo* on the disease outcome is now under investigation.

Keywords: measles virus, monkey, pathogenesis, tropism, reverse genetics, receptor, EGFP

INTRODUCTION

Measles is a febrile disease that typically occurs in small children; the incubation period is 10–14 days, after which clinical symptoms such as fever, coughing, and a characteristic rash appears. Since measles is accompanied by immunosuppression, it has a high frequency of complication with secondary bacterial infections, such as otitis media or pneumonia. Although developed countries are eradicating measles by promoting effective vaccination, measles remains an important issue, especially in developing countries (Griffin, 2007).

Measles virus (MV), belonging to the genus *Morbillivirus* of the family Paramyxoviridae, is an enveloped virus with a non-segmented negative strand RNA genome. The MV genome has six genes that encode the nucleocapsid (N), phospho (P), matrix (M), fusion (F), hemagglutinin (H), and large (L) proteins (**Figure 1A**). MV contains two envelope glycoproteins: the H protein, which is responsible for receptor binding and is important for determining cell tropism of MV; and the F protein, which mediates membrane fusion (Navaratnarajah et al., 2009). The P gene encodes the P protein and the non-structural V and C proteins. The V and C proteins are important for antagonizing the host interferon (IFN) response (Gerlier and Valentin, 2009).

To date, three different molecules have been identified as receptors for MV. Signaling lymphocyte activation molecule (SLAM, also called CD150), expressed in certain immune system cells including activated B and T lymphocytes, mature dendritic cells, and macrophages, is a receptor for wild-type MV and vaccine and laboratory-adapted strains of MV (Tatsuo et al., 2000). CD46 (also called membrane cofactor protein), expressed in all human and monkey nucleated cells, is a receptor for vaccine and laboratory-adapted strains of MV (Dorig et al., 1993; Naniche et al., 1993). Recently, nectin-4 [also called poliovirus-receptor-like-4 (PVRL4)] has been identified as the epithelial receptor for wild-type MV (Muhlebach et al., 2011; Noyce et al., 2011).

Several animal models have been used for studying the pathogenesis of MV (Griffin, 2007). Cotton rats, rats, hamsters, mice, and ferrets can be infected with MV and are commonly used as small animal models for MV pathogenesis. After identification of CD46 and SLAM as MV receptors, numerous transgenic and knock-in mice expressing human CD46 and/or SLAM were established and intensively used to study different aspects of MV infection (Sellin and Horvat, 2009). However, non-human primates are the only animals that exhibit acute disease similar to that seen in humans. In this review, we discuss recent findings regarding tropism and pathogenesis of MV; these findings were obtained by infecting monkeys with recombinant wild-type MV.

HISTORICAL BACKGROUND OF MONKEY MODELS

When infected with measles, monkeys exhibit similar symptoms as seen in humans. This was reported as early as 1911, after inoculating monkeys with blood from measles patients (Anderson and Goldberger, 1911). In 1921, it was reported that measles could be transmitted from humans to monkeys by placing a filtered throat swab from a measles patient into the tracheae of monkeys (Blake and Trask, 1921a). These authors also performed histological analysis of infected monkeys (Blake and Trask, 1921b). However,

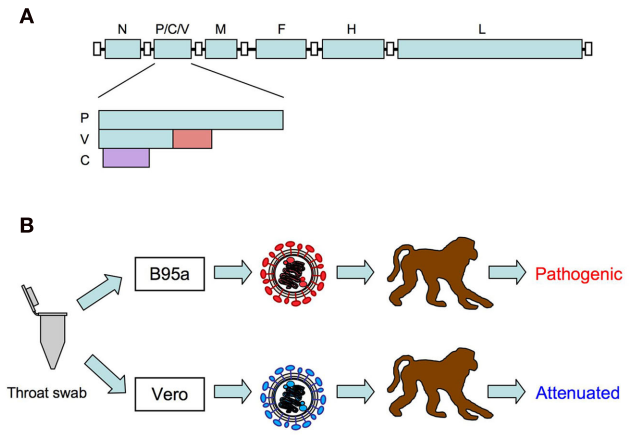

FIGURE 1 | (A) Schematic diagram of measles virus genome. The P gene encodes the P protein and the non-structural V and C proteins. The V protein is translated from an edited RNA with a non-templated G residue. The V protein-specific region is indicated in red. The C protein is translated from an alternative open reading frame (purple) initiated by the second AUG. **(B)** Isolation of wild-type MV using B95a cells. Pathogenic wild-type MV strains can be isolated by inoculating throat swabs from measles patients in B95a cells. MV strains isolated in Vero cells lose their pathogenicity.

at this time, "MV" had not yet been discovered. MV was first isolated in 1954 from a specimen obtained from a measles patient (Enders and Peebles, 1954). Enders and Peebles (1954) inoculated human and monkey cell cultures with a throat swab taken from a young boy named David Edmonston and isolated MV from these cultures. After this, it was discovered that MV isolated from normal human renal cells caused clinical signs similar to those of human measles in monkeys (Peebles et al., 1957). Since then, numerous studies have been carried out by infecting monkeys with MV, measles vaccines, or specimens from measles patients (Griffin, 2007). In such experiments, two species of monkeys, cynomolgus monkey (*Macaca fascicularis*) and rhesus monkey (*Macaca mulatta*), serve as good animal models. New World monkeys are more susceptible to MV than Old World monkeys, and infection of marmoset (*Saguinus mystax*) with MV results in a fulminant disease (Levy and Mirkovic, 1971; Albrecht et al., 1980).

In the past, it was well known that infection of monkeys with materials from measles patients induced clinical signs similar to those of human measles (Nii et al., 1964; Yamanouchi et al., 1970; Sakaguchi et al., 1986). However, curiously enough, infection of monkeys with MV isolated and propagated in cultured cells did not always induce these clinical signs (Enders et al., 1960; Yamanouchi et al., 1970; van Binnendijk et al., 1994). This riddle was solved by the introduction of B95a cells (a marmoset B-lymphoid cell line) for isolation and propagation of MV (Kobune et al., 1990). Kobune et al. (1990, 1996) found that MV strains could be efficiently isolated in B95a cells using materials from measles patients. More importantly, MV strains isolated from B95a cells retained their original pathogenicity in monkeys. These studies indicated that vaccine and laboratory-adapted strains of MV previously isolated from non-lymphoid cells such as Vero cells were not true MV (**Figure 1B**). A decade later, it was found that the MV receptor SLAM is highly expressed on B95a cells (Tatsuo et al., 2000), which accounts for the efficient isolation of pathogenic MV from patient samples. Similar to MV strains isolated from B95a cells, MV strains isolated and propagated in monkey mononuclear cells, human cord blood cells, human B lymphoblastoid cell lines, and Vero cells expressing SLAM replicated well in monkeys and induced clinical signs of measles (van Binnendijk et al., 1994; McChesney et al., 1997; Zhu et al., 1997; Auwaerter et al., 1999; El Mubarak et al., 2007; Bankamp et al., 2008). These results suggest that expression of SLAM on cells used for isolation is important for isolation of pathogenic MV.

REVERSE GENETICS OF MV

Reverse genetics refers to the methods used for recovering infectious viruses from the cDNA that encodes the viral genome. By using this method, mutations or extra transcription units can be introduced into viral genomes by the modification of cDNA plasmids. Reverse genetics of MV was first established based on the Edmonston vaccine strain (Radecke et al., 1995). However, as previously mentioned, viruses derived from the Edmonston vaccine strain do not induce clinical symptoms of measles in monkeys. Therefore, reverse genetics of pathogenic wild-type MV was needed for the study of MV pathogenesis in monkeys. To this end, we first determined the complete nucleotide sequence of the genome of the pathogenic wild-type IC-B strain (NC_001498/AB016162; Takeuchi et al., 2000), which was isolated in Tokyo in 1984 by using B95a cells (Kobune et al., 1990). Then, we constructed a complete cDNA plasmid of the IC-B strain named p(+)MV323, and successfully recovered infectious MV (IC323 strain) from the p(+)MV323 plasmid (Takeda et al., 2000). Importantly, the IC323 strain induced clinical signs such as rash, Koplik's spots, and lymphopenia similar to human measles in infected monkeys, indicating that the IC323 strain retains the original pathogenicity of the IC-B strain. Now, infectious MV strains can be easily recovered from cDNA plasmids by using an improved protocol (Takeda et al., 2005).

Reverse genetics of other wild-type MV strains has been reported for the HL strain isolated in Japan (Terao-Muro et al., 2008) and the KS strain isolated in Sudan (Lemon et al., 2011). For vaccine strains of MV, reverse genetics for the Schwarz/Moraten vaccine strain (Combredet et al., 2003; del Valle et al., 2007) and the AIK-C vaccine strain (Nakayama et al., 2001) have also been reported. Reverse genetics for vaccine strains are being used as a platform to generate new multivalent vaccines expressing antigens of other pathogens (Billeter et al., 2009) and oncolytic viruses for cancer therapy (Russell and Peng, 2009).

FUNCTION OF MV ACCESSORY PROTEINS *IN VIVO*

The P gene of MV encodes two non-structural proteins, namely the C and V proteins. However, the function of the C and V proteins *in vivo* was not well understood. The C protein is a small (186 amino acid), highly positively charged protein. To study the function of the C protein in the context of the natural course of MV pathogenesis, we generated an IC323 strain deficient in the expression of the C protein, wtMV(C−), by using the reverse genetics of wild-type MV (Takeuchi et al., 2005). Notably, the growth of wtMV(C−) in cynomolgus monkeys was dramatically reduced

when compared to the IC323 strain. A similar growth defect of the IC323 strain deficient in the expression of the C protein, C^{ko}, *in vivo* was observed in rhesus monkeys (Devaux et al., 2008). Interestingly, C^{ko} induced more inflammatory cytokines such as tumor necrosis factor alpha (TNF)-α and interleukin (IL)-6 and interferon (IFN)-α and -β in infected monkeys.

The V protein is translated from an edited mRNA of the P gene (Griffin, 2007). Thus, the amino-terminal domain of the V protein has the same amino acid sequence as the P protein, and the carboxyl-terminal domain of the V protein has a highly conserved amino acid sequence forming a zinc-binding domain, which is important for function as an interferon antagonist (Gerlier and Valentin, 2009). An IC323 strain deficient in the expression of the V protein, V^{ko}, was generated by introducing nucleotide mutations in the RNA-editing signal in the P gene (Devaux et al., 2008). The growth of V^{ko} in infected rhesus monkeys was lower than that of the parental IC323 strain. V^{ko} induced more inflammatory cytokines (TNF-α and IL-6) and IFN-α and -β. An IC323 strain unable to antagonize STAT1 function, STAT1-blind virus, was generated by introducing three amino acid substitutions in the shared domain of the P and V proteins (Devaux et al., 2011). The STAT1-blind virus induced short-lived viremia and no clinical signs in infected rhesus monkeys. This virus induced more inflammatory cytokines (TNF-α and IL-6) and a Th1/Th2 balance cytokine (IL-12) in infected monkeys. Taken together, these findings indicate that the C and V proteins are not non-essential gene products as previously thought, but are stringently required for antagonizing host innate immune and inflammatory responses *in vivo*.

In contrast, *in vitro* studies indicated that the V protein blocks IFN-α/β signal transductions in infected cells, inhibits TLR7-mediated IFN-α production in human plasmacytoid dendritic cells, and inhibits IFN induction in infected cells by interacting with MDA5 (Gerlier and Valentin, 2009). Furthermore, the C protein appears to inhibit IFN induction in infected cells by regulating viral RNA synthesis (Nakatsu et al., 2008). Thus, it is necessary to elucidate whether the *in vivo* phenotypes of C- and V-deficient viruses are similar to the *in vitro* phenotypes.

MV TROPISM *IN VIVO*

Recent advances in the study of virus tropism have included the introduction of enhanced green fluorescent protein (EGFP)-expressing viruses. *In vivo* tropism of *Morbillivirus* can be visualized with high sensitivity in living animals as well as tissue samples by using EGFP-expressing recombinant canine distemper viruses (von Messling et al., 2004). Similarly, MV target tissues or organs can be visualized with high sensitivity by infecting cynomolgus monkeys with an EGFP-expressing IC323 strain (**Figure 2**). de Swart et al. (2007) infected rhesus and cynomolgus monkeys with an IC323 strain expressing EGFP (Hashimoto et al., 2002) and examined the tropism of wild-type MV *in vivo*. They indicated that the major target cells of wild-type MV were B and T lymphocytes and CD11c-positive, major histocompatibility complex (MHC) class-II-positive dendritic cells. This result is consistent with the fact that SLAM is a receptor for wild-type MV. Infection of ciliated epithelial cells in the trachea and lungs was also detected, suggesting the presence of another receptor for MV in epithelial cells.

Regarding the early target cells of wild-type MV, classical textbooks describe that the primary targets of MV are the epithelial cells of the respiratory tract. However, SLAM is not expressed in these epithelial cells. To examine the early target cells of wild-type and vaccine strains of MV in the lung, de Vries et al. (2010) infected cynomolgus monkeys with EGFP-expressing IC323 or vaccine strains of MV via the intratracheal or aerosol route. They found

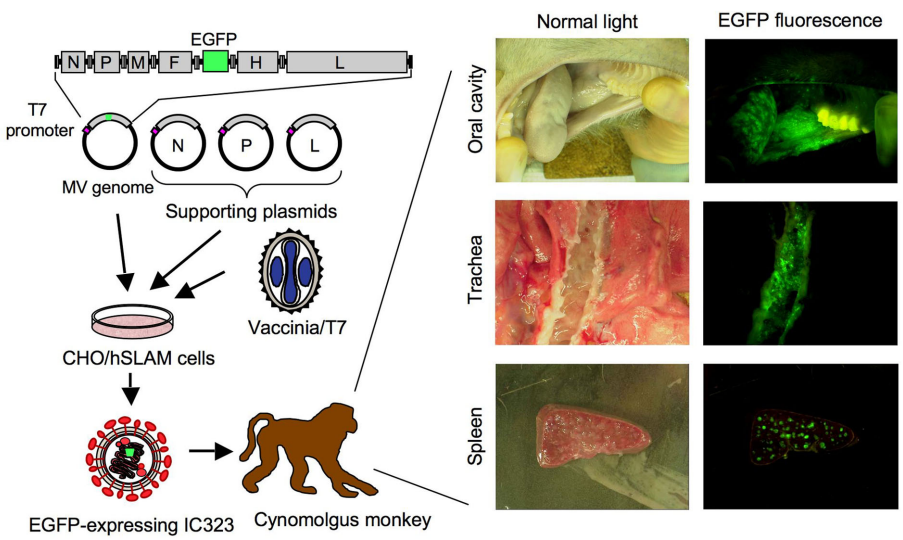

FIGURE 2 | Schematic diagram of reverse genetics of wild-type MV expressing EGFP and infection of monkeys. A plasmid carrying the full-genome cDNA of the IC-B strain and the EGFP gene under the control of the T7 promoter is introduced into CHO cells expressing human SLAM (CHO/hSLAM), along with three supporting plasmids expressing N, P, and L proteins, respectively, under the control of the T7 promoter. After infection with vaccinia virus expressing the T7 RNA polymerase, wild-type MV expressing EGFP can be recovered by mixing with B95a cells. EGFP fluorescence in tissues and organs of infected monkeys can be detected using a fluorescence microscope.

that CD11c-positive cells, which include alveolar macrophages and dendritic cells, were the major targets of both viruses. Interestingly, although viral replication and cellular tropism in the lungs were similar for the two viruses, only wild-type MV caused significant viremia, suggesting a growth defect of the vaccine strain in lymphocyte cells. Similarly, to examine the early target cells of wild-type MV, Lemon et al. (2011) infected cynomolgus monkeys with an EGFP-expressing wild-type MV based on the KS strain by aerosol infection and found that the early target cells of wild-type MV in monkeys are macrophages and dendritic cells. These studies indicated that alveolar macrophages and dendritic cells but not the epithelial cells of the respiratory tract are the early target cells of wild-type MV.

Nectin-4 is a newly identified epithelial cell receptor (EpR) for MV. To examine the effect of nectin-4-using activity of MV on disease outcomes in monkeys, an IC323 strain recognizing SLAM but not nectin-4 was generated by introducing amino acid mutations in the H protein (Leonard et al., 2008). At that time, nectin-4 had not been identified as a MV receptor, and this strain was called EpR-blind virus. When rhesus monkeys were infected with EpR-blind virus via the conjunctiva and nares, this virus induced viremia and clinical signs in infected monkeys but did not propagate in the lungs. This result indicates the importance of nectin-4 for the propagation of MV in the lungs, which is required for the subsequent exit of MV from the host. Inversely, to examine the impact of the recognition of SLAM by MV, an IC323 strain recognizing nectin-4 but not SLAM, SLAM-blind, was generated (Leonard et al., 2010). When rhesus monkeys were infected with the SLAM-blind virus, it elicited no clinical symptoms. This result indicates that SLAM recognition is necessary for MV virulence and pathogenesis.

Vaccine and laboratory-adapted strains of MV can utilize both CD46 and SLAM as cellular receptors. However, surprisingly, the impact of the CD46-using activity of vaccine and laboratory-adapted strains of MV on their tissue and organ tropism and attenuation is not well understood. As CD46 is ubiquitously expressed on all nucleated human and monkey cells, vaccine, and laboratory-adapted strains of MV may infect all tissues and organs of humans and monkeys. If so, this tropism shift may have great consequences on vaccine attenuation. In this context, de Vries et al. (2010) indicated that only CD11c-positive cells were infected with the EGFP-expressing vaccine strain via the aerosol route, suggesting that vaccine strains do not use CD46 *in vivo*. However, when the replication of vaccine and laboratory-adapted strains of MV in monkeys is limited, it will be difficult to identify infected cells in tissues. Furthermore, the infection of vaccine and laboratory-adapted strains of MV may be restricted because of mutations in the P/C/V genes, which are important for antagonizing the host IFN response (Gerlier and Valentin, 2009). Therefore, the tropism shift that solely occurs via the H protein should be evaluated using the wild-type MV expressing EGFP, which bears the H protein of a vaccine strain, such as the IC/EdH strain we developed previously (Takeuchi et al., 2002).

We recently infected cynomolgus monkeys with EGFP-expressing wild-type or IC/EdH strains and found that SLAM-expressing lymphocytes were the main targets of both strains, indicating that CD46 does not act as a receptor for vaccine and laboratory-adapted strains of MV *in vivo* (**Figure 3**; Takeuchi et al., 2012). One possible explanation for the limited expansion of the EGFP-expressing IC/EdH strain *in vivo* is the activation status of lymphocytes. It is known that stimulated lymphocytes are efficiently infected with MV and that stimulated lymphocytes express SLAM (Tatsuo et al., 2000). Thus, lymphocytes expressing SLAM may appear to be infected with both strains. The EGFP-expressing IC/EdH strain that entered into quiescent lymphocytes via the CD46 may not grow well in those cells. Alternatively, the expression level of CD46 *in vivo* may be too low to allow MV dissemination by cell–cell fusion, as it was reported that high CD46 density is required for MV-induced cell–cell fusion (Anderson et al., 2004). For whatever the reason, the growth of the EGFP-expressing IC/EdH strain was less efficient than that of the EGFP-expressing wild-type strain (Takeuchi et al., 2012), suggesting that the CD46-recognition ability of vaccine and laboratory-adapted MV strains plays a role in the MV attenuation. Further studies are required to elucidate the relationship between CD46-recognition ability and MV attenuation.

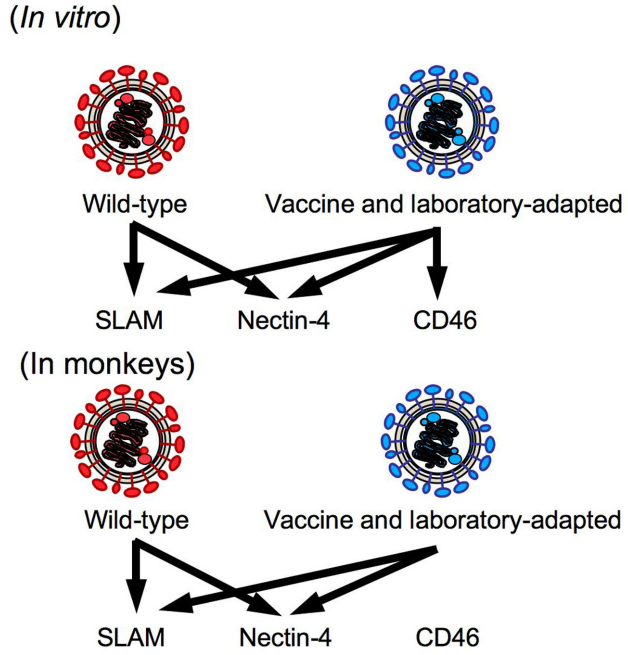

FIGURE 3 | Receptor usage of the wild-type and vaccine and laboratory-adapted strains of MV. Nectin-4 is a recently identified epithelial cell receptor for MV.

CONCLUSION

As described in this review, our IC323 strain, recovered from a plasmid carrying the full-genome cDNA of the IC-B strain, is now used as a standard wild-type MV strain in MV research. Furthermore, EGFP-expressing IC323 strains are ideal tools for the study of tissue and organ tropism of wild-type MV *in vivo*. Although the use of monkeys has several limitations, monkey models still provide the only reliable animal model for the study of the pathogenesis of MV in humans. The combination of wild-type and vaccine

MV strains generated by reverse genetics and monkey models will provide new insights into the relationship between viral gene functions or individual mutation(s) and the pathogenesis of MV.

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Wild-type measles virus is intrinsically dual-tropic

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Measles is a highly contagious disease that causes temporary and severe immunosuppression in patients. Signaling lymphocyte activation molecule (SLAM) expressed on cells of the immune system functions as a receptor for measles virus (MV). In addition to SLAM, vaccine strains of MV also use a ubiquitously expressed complement regulatory protein, CD46, as a receptor, whereas wild-type (wt) MV strains do not use this receptor. However, recent studies have indicated that SLAM is not the sole receptor for wt MV strains. These strains have an intrinsic ability to enter both immune and epithelial cells using distinct receptor binding sites in their hemagglutinin (H) protein. Recently, a clear answer was obtained through the identification of an epithelial MV receptor, nectin4, expressed at adherens junctions, thereby greatly improving our knowledge of MV receptors. It is now clear that MV specifically targets two cell types, immune cells and epithelial cells, using SLAM and nectin4, respectively. MV loses the ability to use either SLAM or nectin4 when it possesses specific mutations in the H protein. However, nectin4-blind MV still infects SLAM-positive immune cells efficiently (SLAM-tropic), and conversely, SLAM-blind MV infects nectin4-positive epithelial cells efficiently (nectin4-tropic). In this regard, MV is intrinsically dual-tropic to immune cells and epithelial cells. Although many aspects and molecular mechanisms underlying immunosuppressive effects and a highly contagious nature of MV still remain to be elucidated, analyses of physiological functions of these two receptors would provide deep insights into MV pathogenesis.

Keywords: measles virus, dual-tropic, SLAM, nectin4, receptor

MEASLES VIRUS

Measles is a highly contagious acute viral disease characterized by high fever, malaise, coryza, conjunctivitis, cough, and a maculopapular rash (Griffin, 2007). Patients with measles develop a severe and temporary immunosuppression, which is often accompanied by secondary bacterial infections (Griffin, 2007). Despite the availability of highly effective vaccines, measles-related deaths were estimated to be 164,000 worldwide in 2008 (WHO, 2009). The causative agent is measles virus (MV), which belongs to the genus *Morbillivirus* in the family *Paramyxoviridae*. The virus particle is enveloped and contains a non-segmented negative-strand RNA genome encoding six tandem linked genes, N, P/V/C, M, F, H, and L. The genome is encapsidated by the nucleocapsid (N) protein and associated with viral RNA-dependent RNA polymerases, forming a helical ribonucleoprotein complex (RNP). On the envelope, the viral particle possesses two types of viral glycoprotein spikes, the hemagglutinin (H) and fusion (F) proteins (Griffin, 2007). The H protein is responsible for binding to cellular receptors on the target host cells, and plays a key role in the determination of host cell specificity (tropism) of MV (Yanagi et al., 2009). Binding of the H protein to a receptor triggers F protein-mediated membrane fusion between the virus envelope and the host cell plasma membrane, releasing the RNP into the cytoplasm. In cells infected with MV, the H and F proteins are expressed on the cell surface and cause cell-to-cell fusion, producing syncytia.

DISCOVERIES OF CELLULAR RECEPTORS FOR MV

Basically, MV specifically infects cells expressing its receptors. Therefore, the distribution pattern of its receptors is a key determinant of which cells become infected with MV (Yanagi et al., 2009). The initial discovery of an MV receptor came in 1993 (Dorig et al., 1993; Naniche et al., 1993). Two independent studies indicated that the receptor molecule for MV is the human membrane cofactor protein (MCP/CD46), a central component of the complement system, which is expressed ubiquitously on all organs and tissues throughout the human body (Dorig et al., 1993; Naniche et al., 1993). These findings were highly welcomed from the viewpoint that MV causes a systemic infection. Meanwhile, Kobune et al. (1990) reported the isolation of lymphotropic MV strains, and subsequent studies indicated that these lymphotropic MV strains do not use MCP/CD46 as a receptor (Yanagi et al., 2009). Importantly, Kobune's isolates exhibited a high virulence in experimentally infected monkeys, whereas MCP/CD46-using classical MV isolates caused no or mild disease in monkeys (Kobune et al., 1990, 1996; Takeda et al., 1998). Hence, new two questions have arisen for MV researchers. What is the receptor for these lymphotropic strains? Which strains are the real wild-type (wt) MV strains? In 2000, using Kobune's isolates, another receptor was identified (Tatsuo et al., 2000). This receptor is signaling lymphocyte activation molecule, also known as CD150 (SLAM/CD150), which is expressed on cells of the immune system (Tatsuo et al., 2000). Subsequent studies clarified

that SLAM/CD150 is a receptor for wt MV strains circulating in patients, and that MCP/CD46 does not act as a receptor for wt MV strains (Yanagi et al., 2009). MCP/CD46 acts as a receptor only for vaccine and some laboratory MV strains (Yanagi et al., 2009). Currently, it is clear that these MV strains have acquired the ability to use MCP/CD46 as an alternative receptor to grow in laboratory cell lines lacking SLAM/CD150 expression (Yanagi et al., 2009).

Hence, it has become generally accepted that wt MV is a lymphotropic virus that specifically targets immune cells, similar to the case of human immunodeficiency virus (HIV) and human T cell lymphotropic virus type 1 (HTLV1). In 2000, a recombinant MV, IC323, was generated based on Kobune's first isolate (Takeda et al., 2000), and has greatly contributed to our understanding of the molecular bases for the pathogenesis of wt MV strains (Takeuchi et al., 2005; de Swart et al., 2007; Devaux et al., 2008, 2011; Leonard et al., 2008; Nakatsu et al., 2008; de Vries et al., 2010; Ludlow et al., 2010; Mühlebach et al., 2011; Noyce et al., 2011). At that time, only SLAM/CD150-positive cells were found to be susceptible to wt MV infections. However, it remained difficult to make a final conclusion that SLAM/CD150 is the sole receptor for wt MV, because histopathological examinations of measles patients and monkeys infected with MV have revealed considerable levels of MV protein expression in the epithelia of various organs, and histopathological changes are also evident in these epithelia (Nii et al., 1964; Lightwood and Nolan, 1970; Olding-Stenkvist and Bjorvatn, 1976; Moench et al., 1988; Craighead, 2000; **Figure 1**). In 2003, primary cultures of human small airway epithelial cells (SAECs) were shown to be susceptible to wt MV infection (Takeuchi et al., 2003). Upon MV infection, large syncytia developed in SAECs via a SLAM-independent mechanism (Takeuchi et al., 2003). After searching many cell lines, several epithelial cell lines with high susceptibility to MV infection were identified (Takeda et al., 2007; Tahara et al., 2008). Tahara et al. (2008) and Leonard et al. (2008) clearly demonstrated that wt MV infects epithelial cell lines that form tight junctions (TJs) using an unidentified receptor (Leonard et al., 2008; Tahara et al., 2008). Using these cells and recombinant IC323 expressing green fluorescent protein (IC323-EGFP; Takeda et al., 2000; Hashimoto et al., 2002), a final answer was obtained for the receptor on epithelial cells. Two groups independently demonstrated that nectin4, which is expressed at adherens junctions (AJs), acts as a receptor for MV (Mühlebach et al., 2011; Noyce et al., 2011). Interestingly, MV loses the ability to use either SLAM/CD150 or nectin4 when it possesses specific mutations in the H protein (Leonard et al., 2008; Tahara et al., 2008; **Figure 2**). Nectin4-blind MV still infects SLAM/CD150-positive immune cells efficiently (SLAM/CD150-tropic), and conversely, SLAM/CD150-blind MV infects nectin4-positive epithelial cells efficiently (nectin4-tropic; Leonard et al., 2008; Tahara et al., 2008; **Figure 2**). In this regard, MV is intrinsically dual-tropic to immune cells and epithelial cells (**Figure 2**).

There is now no doubt that SLAM/CD150 and nectin4 are the major receptors for MV. However, other molecules may further support MV infection *in vivo*, being involved in the development of measles and its neurological sequela. For example, the mechanism that the C-type lectin DC-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) acts as an attachment receptor for MV, thereby promoting MV infection of DCs, may be ideal to understand the extraordinarily high transmissibility of measles (de Witte et al., 2006, 2008). It is well known that MV causes subacute sclerosing panencephalitis (SSPE), a persistent infection of the central nervous system (CNS) with MV. This occurs with a mean latency period of 7–10 years after suffering from acute measles at a frequency of 1/5,000–1/100,000 reported cases of acute measles (Takasu et al., 2003; Bellini et al., 2005). The mechanisms underlying the spread of MV in the CNS remain to be elucidated. Although nectin4 is a possible candidate for an MV receptor in the CNS, no (or undetectable) nectin4 expression was observed in the CNS in humans (Reymond et al., 2001; Brancati et al., 2010), and some MV strains derived from SSPE patients are likely to use nectin4 inefficiently (Seki et al., 2011). Data reported by Makhortova et al. (2007) suggest that neurokinin-1, a substance P receptor, supports trans-synaptic transmission of MV by acting as a receptor for the F protein.

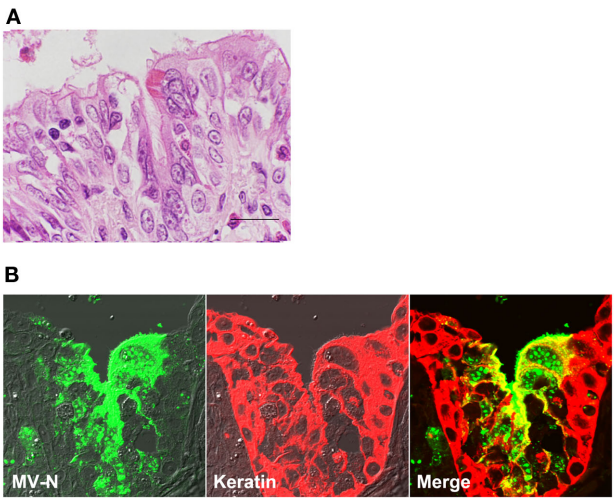

FIGURE 1 | Measles virus antigen and histopathological changes in bronchiolar epithelial cells. At 11 days post-infection, a cynomolgus monkey experimentally infected with wt MV IC323 strain was subjected to the histopathological examination. **(A)** Hematoxylin and eosin staining. Multinucleated cell were seen in the epithelial layer of the bronchiole with inflammatory infiltration of eosinophils and lymphocytes. The multinucleated cells contain eosinophilic intranuclear inclusions. Scale bar indicates 20μm. **(B)** Immunohistochemical double staining for the N protein of MV (left, green) and keratin (center, red). Multinucleated epithelial giant cells showing positive staining for the N protein are present in the bronchiole (right, yellow in merged images). Accumulation of N proteins is detected on the apical surface of ciliated epithelial cells.

SLAM/CD150

Measles virus infection causes immunosuppression in patients and is often accompanied by secondary bacterial infections. Typically, MV-induced immunosuppression is characterized by a marked lymphopenia, and an early T_H1 response followed by predominant and prolonged T_H2 response in patients, with suppression of mitogen-induced lymphocyte proliferation *ex vivo* (Griffin and

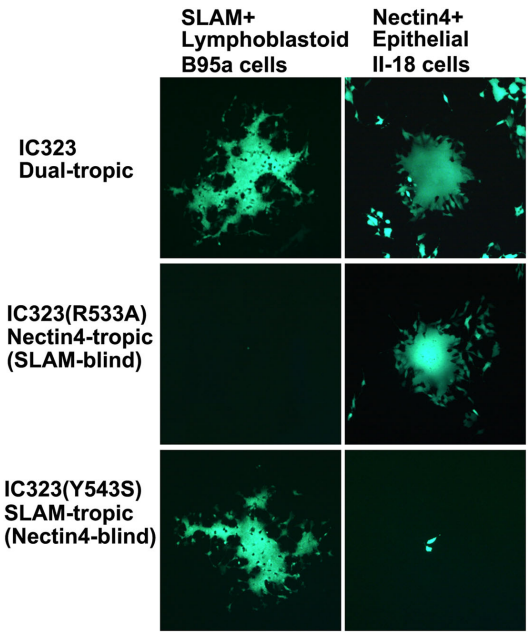

FIGURE 2 | Dual-tropic nature of wt MV. Wt MV (IC323) infects and produces syncytia in both SLAM-positive lymphoblastoid B95a and nectin4-positive epithelial II-18 cells (dual-tropic). IC323 with an R533A mutation in the H protein fails to spread in B95a cells (SLAM-blind), but replicates well in II-18 cells (nectin4-tropic). On the contrary, IC323 with a Y543S mutation in the H protein spreads in B95a cells (SLAM-tropic), but fails to spread in II-18 cells (nectin4-blind). All MVs in this figure are engineered to express EGFP.

Ward, 1993; Schneider-Schaulies and Schneider-Schaulies, 2009). Some, if not all, of these immunological observations must be attributed either directly or indirectly to the fact that MV uses SLAM/CD150 as a receptor. SLAM/CD150 is a member of the SLAM-family receptors, which belong to the immunoglobulin (Ig) superfamily (Veillette, 2010; Ma and Deenick, 2011). The SLAM-family consists of nine members (Cannons et al., 2011; Ma and Deenick, 2011). The SLAM-family receptors are type I transmembrane proteins that typically possess an extracellular region with two Ig-like domains (an amino-terminal variable (V)-like domain and a carboxy-terminal constant-2 (C2)-like domain), a transmembrane region, and a cytoplasmic region that harbors multiple tyrosine-based motifs (Detre et al., 2010; Veillette, 2010; Cannons et al., 2011; Ma and Deenick, 2011). These motifs are referred to as immunoreceptor tyrosine-based switch motifs (ITSMs; Cannons et al., 2011). The SLAM-family receptors are expressed in a broad range of immune cells and play critical roles in immunity. In general, the receptors act as self-ligands and their homophilic *trans*-interactions occur between either heterotypic or homotypic immune cells (Veillette, 2010; Ma and Deenick, 2011). SLAM/CD150 is expressed on thymocytes, subsets of B and T lymphocytes, mature dendritic cells (DCs), macrophages, and platelets, and their expression is upregulated or induced in lymphocytes and monocytes upon activation (Detre et al., 2010; Veillette, 2010; Cannons et al., 2011; Ma and Deenick, 2011).

Signaling lymphocyte activation molecule-associated protein (SAP)-family adaptors [SAP, Ewing's sarcoma-associated transcript (EAT)-2, and EAT-2-related transducer (ERT)] play important roles for the signal transductions mediated by the SLAM-family receptors (Veillette, 2010; Ma and Deenick, 2011). They are small proteins that consist of a single Src homology 2 (SH2) domain and a short carboxy-terminal region. SAP associates intracellularly with the ITSMs in the cytoplasmic region of the SLAM-family receptors via the SH2 domain (Dong and Veillette, 2010; Veillette, 2010; Ma and Deenick, 2011). SAP has the ability to bind concomitantly to the Src-family protein tyrosine kinase, Fyn, thereby coupling the SLAM-family receptors with Fyn (Dong and Veillette, 2010; Veillette, 2010; Cannons et al., 2011). Thereafter, Fyn phosphorylates tyrosine residues at the cytoplasmic region of SLAM-family receptors and other intracellular effector molecules, activating the downstream signals (Detre et al., 2010; Dong and Veillette, 2010; Cannons et al., 2011; Ma and Deenick, 2011). In another mechanism, SAP binding to the SH2 domain of SLAM-family receptors competes with the binding of other SH2 domain-containing molecules, thus modulating the SLAM-mediated signaling (Dong and Veillette, 2010; Veillette, 2010; Cannons et al., 2011). In $CD4^+$ T cells, signals via SLAM/CD150-SAP–Fyn interactions play important roles in regulating T cell receptor-mediated induction of T_H2 cytokines, such as interleukin (IL)-4 and IL-13 (Detre et al., 2010; Cannons et al., 2011; Ma and Deenick, 2011). EAT-2 also mediates the signal transduction cascades of the SLAM-family receptors via a similar but distinct mechanism to that of SAP (Cannons et al., 2011). Similar to SAP, EAT-2 also associates with the ITSMs of SLAM-family receptors through its SH2 domain, but mediates the subsequent signal cascades via its own phosphorylated tyrosine in the short carboxy-terminal region (Veillette, 2010). In general, the signals mediated by the SAP-family adaptors induce the activation and differentiation of immune cells (Veillette, 2010). However, if the SAP-family adaptors are absent, the SLAM-family receptors mediate inhibitory signals to immune cells (a switch-of-function effect; Dong and Veillette, 2010; Veillette, 2010).

Roles for SLAM/CD150 in macrophage functions, cell adhesion, and NKT cell development have also been demonstrated, although many data were obtained in mice (Dong and Veillette, 2010; Veillette, 2010; Cannons et al., 2011; Ma and Deenick, 2011). X-linked lymphoproliferative syndrome is a rare immunodeficiency disease typically caused by mutations in the SAP-encoding gene, *SH2D1A* (Veillette, 2010; Ma and Deenick, 2011). Patients with this syndrome have various functional defects and impaired differentiation of immune cells, indicating crucial roles for SAP in normal immunity (Dong and Veillette, 2010). Hypogammaglobulinemia, massive lymphoproliferative syndrome, and a fatal response to Epstein–Barr virus infection are characteristics of the disease (Veillette, 2010; Ma and Deenick, 2011). Although SLAM-family receptors have some functional redundancy, each receptor plays specific roles in a variety of immune responses (Dong and Veillette, 2010; Veillette, 2010).

NECTIN4

In general, lymphotropic viruses, such as HIV and HTLV1, can never be airborne, and are transmitted inefficiently even through

direct contact with patients. In sharp contrast, MV transmits via aerosols, and has a highly contagious nature. Therefore, this transmission style of MV cannot be easily explained by the fact that the virus uses a lymphocytic molecule, SLAM/CD150, as a receptor. The recent findings showing that MV uses nectin4, a cell adhesion molecule (CAM) expressed at the AJs of epithelia, may partly but nicely explain how and why MV transmits efficiently from a patient to other individuals. Epithelial cells are connected with one another through the formation of several specialized cell–cell junctions, such as TJs, AJs, desmosomes, and gap junctions. TJs function as a physical barrier that prevents the passage of soluble molecules through the intercellular gaps, and also blocks the lateral movement of lipids and membrane proteins across the TJ barrier, thereby acting as the border of the apical and basolateral membranes. AJs are located near the basolateral side of the TJs. They are basically formed by cadherins and nectins and intracellularly connected by actin filaments. Nectin4 is a member of the nectin family, which consists of four members (nectin1, 2, 3, and 4; Takai et al., 2008a). Nectin1 and nectin2 were originally identified as poliovirus receptor-related protein (PRR)-1 and PRR-2, respectively, and subsequently shown to support the entry of some herpes viruses (Takai et al., 2008a,b). Similar to the SLAM-family members, nectins are also type I transmembrane proteins that belong to the Ig superfamily (Takai et al., 2008a,b). In general, they possess an extracellular region with three Ig-like domains (an amino-terminal V-like domain and two C-like domains), a transmembrane region, and a cytoplasmic region with a short afadin-binding motif (Takai et al., 2008a,b). The consensus motif was reported to be E/A-X-Y-V for nectin1, 2, and 3, while nectin4 does not have this motif but still binds to afadin (Reymond et al., 2001; Takai et al., 2008a). Reymond et al. (2001) proposed a new consensus motif, K/R-X-X-Y/L-V, for all four nectins. Afadin is an actin filament (F-actin)-binding protein, and supports nectins to interact and co-operate with cadherins, other CAMs, and intracellular signaling molecules (Takai et al., 2008a).

Nectins are expressed as dimers, and interact *in trans* with other nectin dimers expressed on neighboring cells (Takai et al., 2008a,b). All nectins show homophilic interactions, while heterophilic interactions are also observed between specific nectins, such as those between nectin1/nectin3 and nectin 2/nectin3 (Takai et al., 2008a,b). Some nectin-like molecules also interact with nectins (Takai et al., 2008a,b). Nectin4 shows homophilic interactions as well as heterophilic interactions with nectin1 (Reymond et al., 2001; Takai et al., 2008a). The Ig V-like domain is used for the *trans*-interaction (Reymond et al., 2001; Fabre et al., 2002). Nectins play key roles in the initiation of AJ formation, and regulate various physiological functions of epithelial cells, such as contact inhibition of cell movement and proliferation, survival, differentiation, and cell polarization (Takai et al., 2008a,b).

Although nectin1 and nectin2 are expressed in a broad range of tissues, the expression of nectin3 and nectin4 is more specific (Reymond et al., 2001). Reymond et al. (2001) and Brancati et al. (2010) showed that human nectin4 is expressed mainly in the placenta and to lesser extents in the trachea, prostate, lung, and stomach. In addition, Brancati et al. (2010) demonstrated nectin4 expression in human keratinocytes, suprabasal nucleated layers of the epidermis, and non-keratinized structures of hair. Some levels of expression in epithelial cells of the tonsil, oral mucosa, esophagus, and nasopharynx have also been reported (www.proteinatlas.org). Although, in many cases, nectin4 is expressed in low or undetectable levels in normal human tissues, many cancer cells are highly positive for nectin4. Thus, it has been proposed that nectin4 is a new tumor-associated marker (Fabre-Lafay et al., 2007; Takano et al., 2009; Derycke et al., 2010). These observations may provide a rationale for the use of MV as an oncolytic agent (Mühlebach et al., 2011). In humans, mutations in the *PVRL4* gene encoding nectin4 cause ectodermal-dysplasia-syndactyly syndrome (EDSS), in which patients have affected skin and skin appendages, such as hair, teeth, and nails (Brancati et al., 2010; Jelani et al., 2011).

RELEVANCE OF SLAM/CD150 AND NECTIN4 TO MV PATHOGENESIS

The pathology of measles can now be drawn with these two receptors (**Figure 3**). Although nectin4-expressing epithelial cells can be the initial targets of MV, no or very limited infection of epithelia was observed in monkeys experimentally infected with MV at the early days after infection (Ludlow et al., 2010; Lemon et al., 2011). Instead, MV initiates its infection via SLAM-mediated entry into alveolar macrophages and DCs in the lung or respiratory tracts (de Witte et al., 2008; de Vries et al., 2010; Lemon et al., 2011). These infections may allow MV to penetrate into the human body and reach the lymphoid organs or tissues, where SLAM/CD150-expressing cells are abundant (Corry et al., 1984; Lehmann et al., 2001). After initial replication in these lymphoid organs or tissues, MV or MV-infected lymphocytes can easily enter the bloodstream. Subsequently, a dramatic MV infection is observed in all lymphoid organs, such as the spleen, thymus, appendix, tonsil, and lymph nodes, throughout the body (Moench et al., 1988; Kobune et al., 1996; de Swart et al., 2007; de Vries et al., 2010). At the time when the MV infection of lymphoid organs reaches its peak, MV infection of epithelia, such as squamous stratified epithelia of the tongue and buccal mucosa and ciliated epithelia of the trachea, becomes evident (Nii et al., 1964; Olding-Stenkvist and Bjorvatn, 1976; Moench et al., 1988; de Swart et al., 2007). This epithelial infection is probably led by MV-infected immune cells and initiated through the basolateral side, since monkeys infected with MV often show infectious foci in the epithelia with MV-infected lymphoid or myeloid cells in the subepithelial cell layers of the trachea, bronchus, and tongue (de Vries et al., 2010; Ludlow et al., 2010). The H protein expressed on MV-infected immune cells that migrate through the epithelial cell layer likely recognizes nectin4 expressed at AJs, triggering F protein-mediated membrane fusion between the MV-infected immune cells and the target epithelial cells. Mühlebach et al. (2011) demonstrated a correlation between nectin4 expression and MV infection in epithelia *in vivo*. Importantly, MV has a mechanism that further facilitates virus shedding in the airway. In epithelia, MV selectively releases progeny virus particles from the apical membrane to the luminal side of the respiratory tract (Leonard et al., 2008; Tahara et al., 2008). Leonard et al. (2008) showed that MV genetically engineered to use SLAM/CD150, but not nectin4 (nectin4-blind or SLAM/CD150-tropic), does not shed progeny viruses into the respiratory tract,

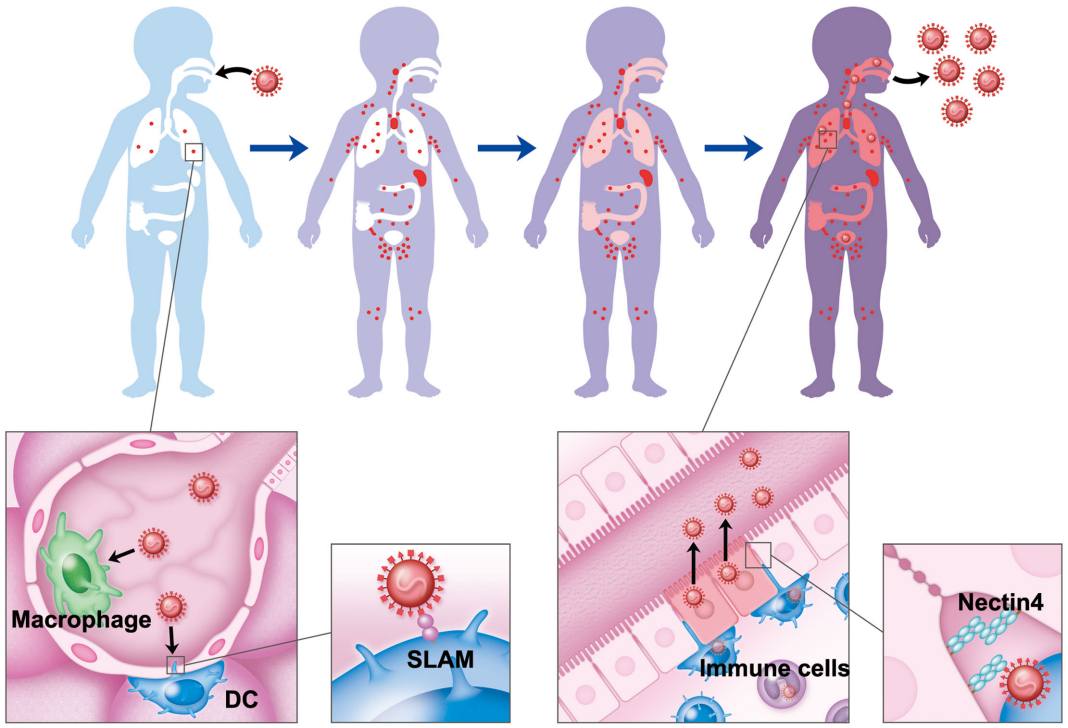

FIGURE 3 | Pathology of measles. MV initiates its infection via SLAM-mediated entry into alveolar macrophages and DCs in the lung. After initial replication in regional lymph nodes, MV enters the bloodstream and subsequently spreads to all lymphoid organs, such as the spleen, thymus, appendix, tonsil, and lymph nodes, throughout the body. When the MV infection of the lymphoid organs reaches its peak, MV-infected immune cells transfer MV to epithelial cells through the basolateral side. At this step, MV uses nectin4 expressed at AJs as a receptor. In the epithelia, MV selectively releases its progeny virus particles from the apical membrane to the luminal side of the respiratory tract.

although it does show systemic infection of lymphoid organs, similar to the case for wt MV.

CONCLUDING REMARKS

Membrane cofactor protein/CD46 was first identified as a receptor for MV (Dorig et al., 1993; Naniche et al., 1993). However, our current knowledge of MV receptors has been totally transformed. In 2000, it was shown that SLAM/CD150 expressed on cells of the immune system, but not MCP/CD46, is a real receptor for wt MV. However, recent studies further showed that SLAM/CD150 is not the sole receptor for MV. MV has an intrinsic ability to enter not only immune cells but also epithelial cells. In 2011, a clear answer was obtained through the identification of the epithelial MV receptor nectin4, which is expressed at AJs, thereby partly explaining why MV exhibits its highly contagious nature (Mühlebach et al., 2011; Noyce et al., 2011). Recent studies on MV receptors greatly advanced our understanding of MV pathogenesis. However, many aspects and molecular mechanisms underlying immunosuppressive effects and a highly contagious nature of MV still remain to be elucidated. Analyses of physiological roles of MV receptors, SLAM/CD150, and nectin4, would provide deep insights into MV pathogenesis.

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Exploitation of lipid components by viral and host proteins for hepatitis C virus infection

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Hepatitis C virus (HCV), which is a major causative agent of blood-borne hepatitis, has chronically infected about 170 million individuals worldwide and leads to chronic infection, resulting in development of steatosis, cirrhosis, and eventually hepatocellular carcinoma. Hepatocellular carcinoma associated with HCV infection is not only caused by chronic inflammation, but also by the biological activity of HCV proteins. HCV core protein is known as a main component of the viral nucleocapsid. It cooperates with host factors and possesses biological activity causing lipid alteration, oxidative stress, and progression of cell growth, while other viral proteins also interact with host proteins including molecular chaperones, membrane-anchoring proteins, and enzymes associated with lipid metabolism to maintain the efficiency of viral replication and production. HCV core protein is localized on the surface of lipid droplets in infected cells. However, the role of lipid droplets in HCV infection has not yet been elucidated. Several groups recently reported that other viral proteins also support viral infection by regulation of lipid droplets and core localization in infected cells. Furthermore, lipid components are required for modification of host factors and the intracellular membrane to maintain or up-regulate viral replication. In this review, we summarize the current status of knowledge regarding the exploitation of lipid components by viral and host proteins in HCV infection.

Keywords: HCV, hepatitis, lipid droplets, host factor

INTRODUCTION

Hepatitis C virus (HCV) is a major causative agent of chronic liver disease including steatosis, cirrhosis, and hepatocellular carcinoma. Epidemiological studies indicate that HCV is also associated with extrahepatic manifestations including type 2 diabetes mellitus, B-cell non-Hodgkin lymphoma, mixed cryoglobulinemia, and Sjögren's syndrome (Jacobson et al., 2010). It has been estimated that there are 170 million patients worldwide, of whom most are infected with HCV. Combination therapy with pegylated interferon (PEG-IFN) and ribavirin has been the standard treatment but it fails to cure ∼50% of treated patients (Soriano et al., 2009).

Hepatitis C virus belongs to the genus *Hepacivirus* of the family Flaviviridae. The viral genome of HCV is characterized by a single positive strand RNA with a nucleotide length of 9.6 kb and it encodes a single polypeptide (**Figure 1**). This polyprotein is cleaved by host and viral proteases into structural and non-structural proteins (Harada et al., 1991; Hijikata et al., 1991; Grakoui et al., 1993a,b). Structural proteins, including the core protein and two envelope proteins, and the viroporin p7 are located within one-third of the N-terminal, while the remaining viral proteins are classified as non-structural proteins which form a replication complex with host factors (Grakoui et al., 1993c). HCV core protein is cleaved by signal peptide cleavage and then released from E1 (Santolini et al., 1994). After cleavage by signal peptidase (SP), the C-terminal transmembrane region of the core protein is further cleaved by signal peptide peptidase (SPP; Hussy et al., 1996; McLauchlan et al., 2002). The nucleocapsid, composed of matured core proteins and the viral genome, is surrounded by an envelope composed of host lipids and viral envelope proteins (Wakita et al., 2005). The life cycle of HCV is shown in **Figure 2**. The viral envelope proteins play a role in the binding to host receptors and membrane fusion for uncoating. Recently, several groups reported that the viral particle binds to a very low-density lipoprotein (VLDL), including apolipoprotein E (apoE), which is required for the binding step (Andre et al., 2002; Nielsen et al., 2006; Chang et al., 2007; Benga et al., 2010) as described below. The virus infects hepatocytes via entry factors known as receptors and co-receptors. The viral particle complex composed of the enveloped nucleocapsid and VLDL including apoE (Merz et al., 2011), is reported to bind to heparin sulfate (HS; Barth et al., 2003) and the low-density lipoprotein (LDL) receptor (LDLR; Agnello et al., 1999), although Albecka et al. (2012) recently reported that LDLR is required for optimal replication of the HCV genome rather than entry of the infectious viral particle. Other host factors may be involved in apoE-mediated entry. The HCV viral particle is transferred to the scavenger receptor class B type I (SR-BI; Scarselli et al., 2002; Bartosch et al., 2003) and CD81 (Pileri et al., 1998) through E2 binding and then enters cells with claudin-1 (CLDN1; Evans et al., 2007) and occludin (OCLN; Ploss et al., 2009) by endocytosis. The Niemann–Pick C1-like 1 cholesterol absorption receptor has recently been reported to be an HCV cell entry factor that is involved in the entry step between post-binding and

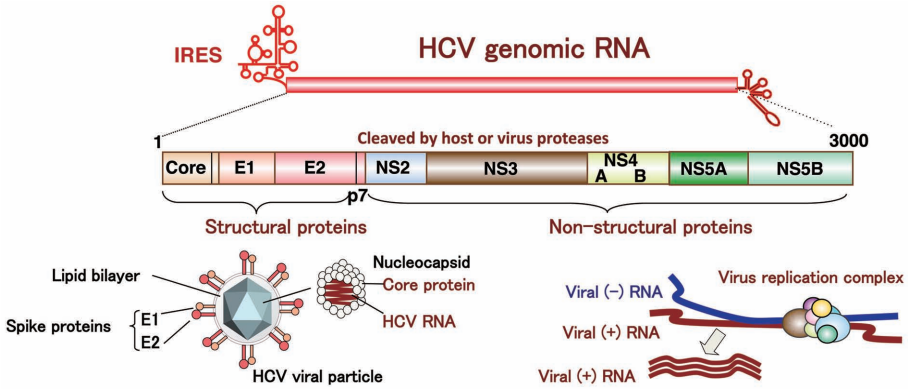

FIGURE 1 | Structure of HCV. HCV RNA encodes a polyprotein composed of about 3,000 amino acids. The core protein, and two envelope proteins are classified as structural protein, while NS2, NS3, NS4A, NS4B, NS5A, and NS5B are non-structural proteins.

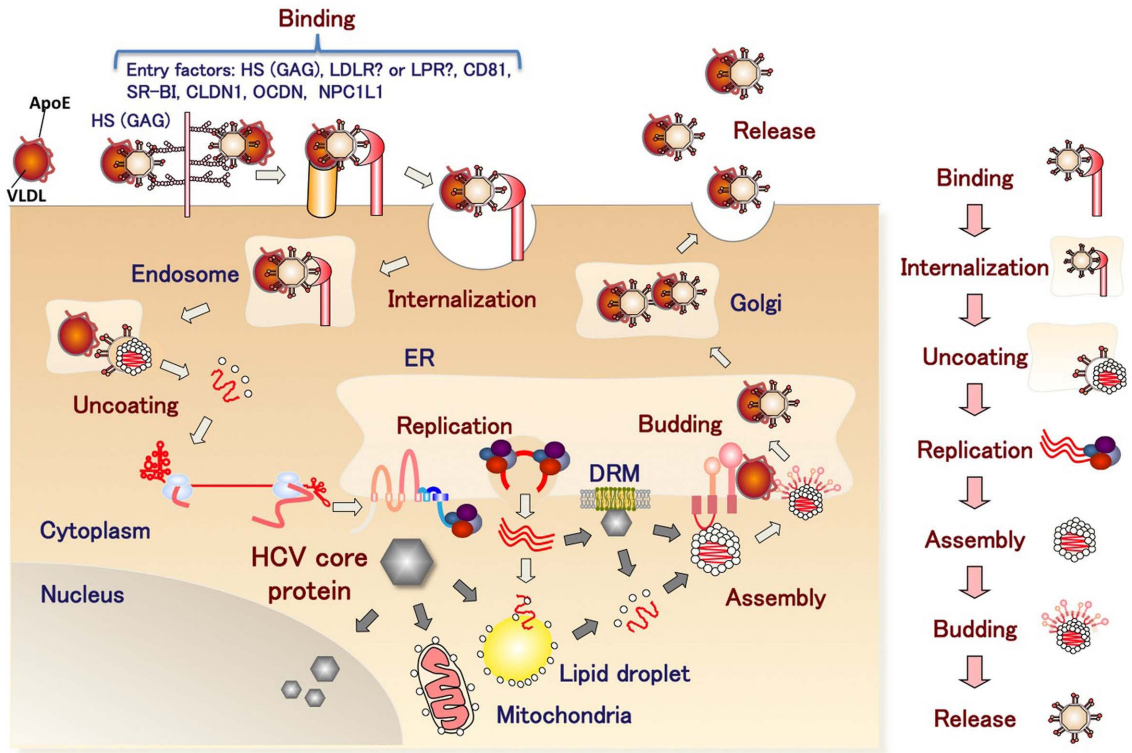

FIGURE 2 | The infection cycle of HCV. The HCV complex with VLDL binds to entry factors on the surface of hepatocytes and then enters cells by endocytosis. After uncoating, viral replication is carried out in the convoluted membrane structure called the membranous web. The viral nucleocapsid egresses into the lumen side of the ER and binds to VLDL. The HCV complex with VLDL is released from the infected cells.

pre-fusion (Sainz et al., 2012). The viral envelope fuses with the host plasma membrane in an endosome under a low pH condition (Takikawa et al., 2000; Hsu et al., 2003; Blanchard et al., 2006; Codran et al., 2006; Meertens et al., 2006; Tscherne et al., 2006). The capsid protein and viral genome are expected to be released into the cytoplasm of infected cells. The viral replication, assembly, and budding are summarized in **Figure 3** on the basis of current information. The viral genome is translated dependent on own internal ribosome entry site (Tsukiyama-Kohara et al., 1992) and transcribed by the translated and processed NS3 to NS5B (Lohmann et al., 1999). The viral protein NS4B induces a convoluted membrane structure (termed a membranous web) with host lipid components and proteins, in which the viral replication is carried out (Egger et al., 2002; Gosert et al., 2005; Ferraris et al., 2010). The newly synthesized viral positive stranded RNA genome is released from the membranous web and passes to the

core protein via NS5A (Masaki et al., 2008). The core protein is translocated on the surface of the lipid droplet or endoplasmic reticulum (ER) membrane for efficient formation of viral particles, and then encloses the synthesized viral genome to form a capsid near the membranous web (Miyanari et al., 2007; Boson et al., 2011). The capsids are enclosed by an endoplasmic membrane containing the viral envelope proteins E1 and E2 and are then released into ER lumen side, since intracellular envelope proteins are categorized as high-mannose type glycoproteins and the viral particle composed of core proteins and envelope proteins egresses into the lumen side of the intracellular compartment associated with lipid droplets (Miyanari et al., 2007; Vieyres et al., 2010). The viral particle is secreted through a host secretion pathway, although the mechanism by which HCV particles are secreted in infected cells remains poorly understood.

Although no effective vaccine for HCV has been developed, antiviral drugs targeting to the viral and host factors have been reported recently. The HCV replicon system was reported for a screening system based on cultured cells (Lohmann et al., 1999) and has been improved by modification of cell lines and marker genes and introduction of adaptive mutations in the region of the viral RNA genome for high efficiency of viral replication (Blight et al., 2000; Krieger et al., 2001; Lohmann et al., 2001; Ikeda et al., 2002; Pietschmann et al., 2002). The complete infectious cycle of HCV in cultured cells was established in a highly permissive cell line by using the genotype 2a strain JFH1 or its chimeric recombinant virus (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). A system based on the cultured cell line has been an exclusive drug-screening system for finding antiviral compounds that interfere with the process of viral RNA replication under intracellular conditions. NS3 forms a complex with cofactor NS4A (Failla et al., 1994, 1995; Koch et al., 1996). This interaction stabilizes NS3 and retains it on the ER where it acquires the ability of a protease against viral polyprotein (Wolk et al., 2000) and host protein IPS-1/MAVS (Foy et al., 2005; Loo et al., 2006), which is a molecule downstream of the RIG-I sensor molecule (Sumpter et al., 2005; Loo et al., 2008). NS3 serine protease activity is a target of the direct acting HCV antiviral drugs known as telaprevir and boceprevir, which are available clinically by combination therapy with PEG-INF and ribavirin (Hofmann and Zeuzem, 2011). The RNA helicase activity of NS3 and NS5B RNA-dependent RNA polymerase are also used for drug-screening in particular (Hicham Alaoui-Ismaili et al., 2000; Dhanak et al., 2002; Borowski et al., 2003; De Francesco et al., 2003; Boguszewska-Chachulska et al., 2004; Maga et al., 2005; Najda-Bernatowicz et al., 2010). Combination therapy using several compounds targeting host and viral factors may be able to completely eradicate the virus and suppress the pathogenicity induced by HCV infection.

Liver steatosis, which is characterized by accumulation of lipid droplets in hepatocytes, is significantly associated with the incidence of hepatocellular carcinoma in HCV-infected patients (Ohata et al., 2003). Severe liver steatosis has been frequently found in patients infected with the genotype 3a virus (Rubbia-Brandt et al., 2000; Adinolfi et al., 2001). Successful clearance of HCV reduces steatosis in genotype 3a patients, suggesting an association between genotype 3a and severe steatosis. Furthermore, HCV core protein derived from genotype 1 also induced liver steatosis in mouse and cultured cells (Barba et al., 1997; Moriya et al., 1997; Hope and McLauchlan, 2000). Lipid droplets containing triglycerides and cholesteryl ester are increased in cells expressing core protein and are surrounded by the core protein (Hope and McLauchlan, 2000). Non-structural proteins associate with the lipid droplets surrounded by HCV core proteins to supply the synthesized viral genome for viral assembly (Miyanari et al., 2007). Other lipid components are reported to be involved in formation of viral particles and the viral RNA replication as described below. This review mainly summarizes the viral and host factors that are associated with lipid metabolism with regard to HCV replication and pathogenicity.

THE ROLE OF VLDL IN HCV INFECTION

Hepatitis C virus replicates in a convoluted membrane structure as a membranous web (Egger et al., 2002; Gosert et al., 2005; Ferraris et al., 2010) and assembles in the area of the ER membrane-associated with lipid droplets surrounded by the core protein (Miyanari et al., 2007). The LDLR has also been proposed to function as one of entry factors described above for HCV entry, in which interaction between LDLR and HCV particles is facilitated though interaction of the virus with host lipoprotein components (Monazahian et al., 1999; Chang et al., 2007; Huang et al., 2007; Miyanari et al., 2007; Gastaminza et al., 2008). HCV RNA containing particles derived from infected human serum were fractionated in densities with a value of 1.03–1.25 g/ml (Thomssen et al., 1992, 1993). The HCV RNA particles of the fraction with a density of lower than 1.06 g/ml possessed infectivity against chimpanzees, while HCV RNA derived from fractions with a higher density showed poor infectious ability (Bradley et al., 1991; Hijikata et al., 1993). The infectious HCV particles form a LDL–virus complex in the sera of human patients (Andre et al., 2002). An LDL–virus complex was found in the fractions with very low to low buoyant densities (1.03–1.25 g/ml), which varied with the stage of infection (Pumeechockchai et al., 2002; Carabaich et al., 2005). HCV particles prepared from infected human serum forms a complex with lipoproteins designated as lipo-viro-particles (LVP; **Figure 3**; Andre et al., 2002; Nielsen et al., 2006). LVP includes triglycerides, HCV RNA, core protein, and apolipoproteins B and E (Andre et al., 2002), which are components of VLDLs and LDL (Brodsky et al., 2004).

Very low-density lipoprotein is formed with a hydrophobic particle composed of triglycerides and cholesteryl ester that is surrounded by a surface coat containing phospholipid, free cholesterol, and two dominant lipoproteins, apoB and apoE (review to see Havel, 2000). Both apoB and apoE were found in a low-density fraction of HCV RNA particles (Andre et al., 2002; Chang et al., 2007). HCV virions could also be precipitated with antibodies against apoB or apoE (Andre et al., 2002; Chang et al., 2007). ApoB and microsomal triglyceride transfer protein (MTP) are required for HCV assembly and production, since knockdown of apoB or a specific antibody to MTP could inhibit HCV production (Huang et al., 2007; Gastaminza et al., 2008). However, another report suggests that knockdown of apoB or antibodies to apoB exhibited no significant effect on HCV infectivity and production (Jiang and Luo, 2009). The monoclonal antibodies against apoE neutralized HCV infection in cultured cells (Chang et al., 2007; Jiang and Luo,

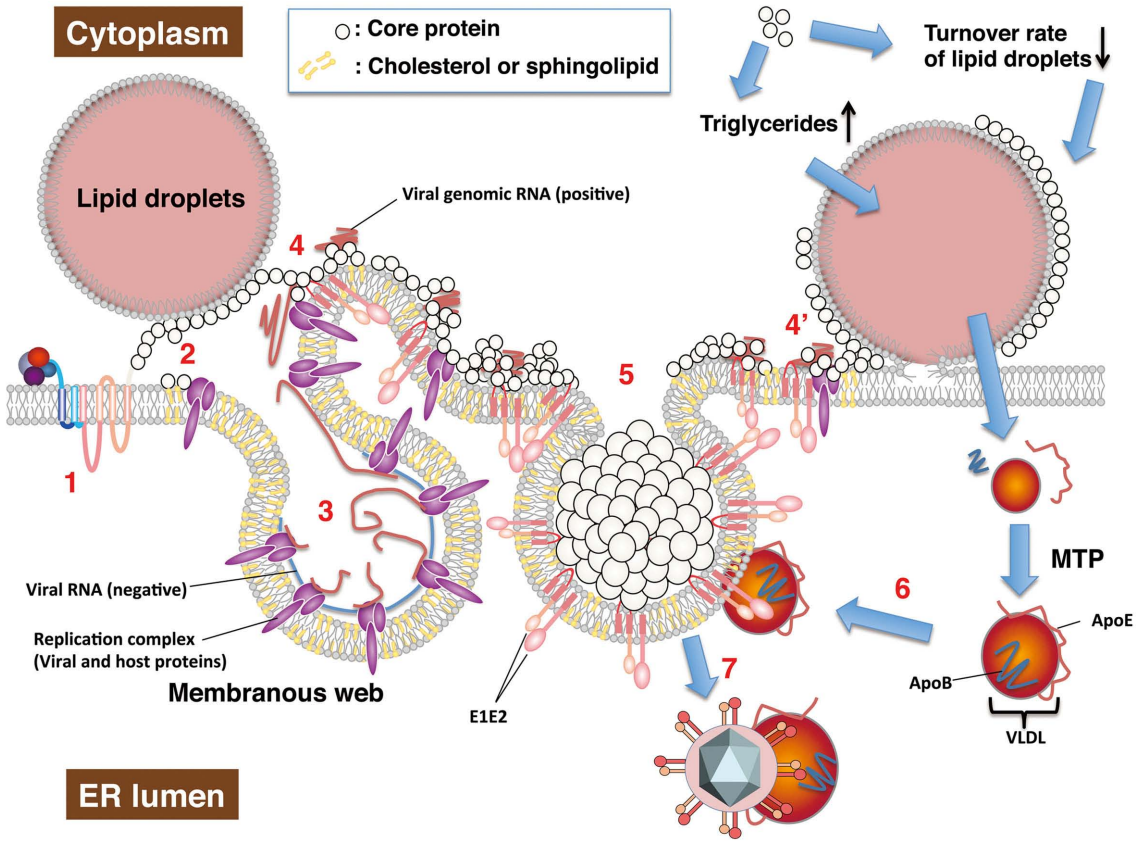

FIGURE 3 | Hepatitis C virus RNA replication and particle production on lipid droplets and the ER membrane. HCV RNA replication and viral particle production are thought to be carried out on the lipid droplets and ER membrane. The viral proteins take advantage of host lipid metabolism and intracellular compartments to produce viral components as described in the text. Each step is described below. (1) A newly synthesized or uncoated vial genomic RNA is translated to a viral polyprotein followed by a cleaving polyprotein. (2) Matured non-structural proteins form replication a complex with host factors. The core protein is translocated to lipid droplets by DGAT1. In cells infected with the highly infectious strain (e.g., Jc1), the core protein may be retained on ER membrane by cooperating with non-structural proteins. (3) NS4B is involved in the convoluted membrane structure dubbed the membranous web. Negative and positive strands of viral RNA are synthesized by the replication complex based on each other. (4) The positive strand of genomic RNA is enclosed with the core proteins, cooperating non-structural proteins. Envelope proteins, NS2, and p7 may determine recruitment of the core protein from lipid droplets to the ER membrane along microtubules. Lipid droplets hemi-fused with ER membrane (Wolins et al., 2006) may be involved in recruitment of core protein on the ER membrane (4′). (5) The core proteins enclose the viral RNA genome, and are then surrounded by envelopes. Non-structural proteins and other factors may support formation of nucleocapsid and budding. (6) VLDL binds to viral particles to form LVP before or after budding. (7) The viral particles egress into the ER lumen with VLDL including ApoE. The core protein upregulates synthesis or storage of triglycerides and cholesteryl ester, cooperating with host factors including DGAT1, and so on.

2009), while knockdown of apoE markedly reduced HCV infectivity and infectious viral production without affecting viral entry and replication (Chang et al., 2007; Berger et al., 2009; Jiang and Luo, 2009). Hishiki et al. (2010) suggested that the isoforms 3 and 4, but not 2, of apoE are critical for HCV infectivity dependent of affinity to LDLR. Furthermore, NS5A could interact with apoE in infected cells and colocalization of both proteins supports the notion of intracellular interaction in infected cells (Benga et al., 2010). The C-terminal alpha-helix region spanning from residue 205 to 280 was critical for NS5A–apoE interaction and viral production (Cun et al., 2010). ApoE included in LVP may directly bind to LDLR or LDLR-related proteins in hepatocytes (**Figure 2**), since apoE is a ligand for all members of the LDLR gene family (see review described by Herz et al., 2009). These results suggest that apoE is an essential host factor for HCV entry.

LOCALIZATION OF THE CORE PROTEIN ON BOTH ER AND LIPID DROPLETS IN INFECTED CELLS

Hepatitis C virus core protein is located at the N-terminus of the HCV polyprotein (**Figure 1**). The HCV core protein is cleaved from a precursor polyprotein by a SP, releasing it from an envelope E1 protein. Then, the C-terminal transmembrane region of the core protein is further processed by a SPP (McLauchlan et al., 2002). The intramembrane processing of the HCV core protein by SPP is critical for the production of infectious viral particles (Okamoto et al., 2008). The C-terminal end of the mature HCV core protein expressed in insect and human cell lines was determined to be Phe^{177} (Ogino et al., 2004; Okamoto et al., 2008). Randall et al. (2007) reported that the introduction of an siRNA targeted to SPP (called HM13) reduced the production of infectious HCV particles, suggesting that SPP is required for HCV

particle production. Our previous report (Okamoto et al., 2008) showed that the production of HCV in cells persistently infected with the JFH1 strain was impaired by treatment with an SPP inhibitor and that JFH1 viruses bearing SPP-resistant mutations in the core protein failed to propagate in a permissive cell line. These data suggest that intramembrane processing of HCV core protein by SPP is required for viral propagation. Matured core protein was found in a detergent-resistant membrane fraction, which was distinct from the classical lipid rafts (Matto et al., 2004). Our data also suggest that cleavage of HCV core protein by SPP is required for localization of HCV core protein in detergent-resistant membrane fractions including cholesterol and sphingolipid (**Figure 3**, step 4 and 5). Detergent-resistant membrane fractions may be derived from the membranous web where the viral replication complex synthesizes the viral RNA genome, since the replication complex is fractionated in lipid raft fractions including Vesicle-associated membrane protein-associated protein (VAP)-A, cholesterol, and sphingolipid (**Figure 3**, step 3; Shi et al., 2003; Aizaki et al., 2004; Gao et al., 2004; Sakamoto et al., 2005). Furthermore, an HCV core protein mutation resistant to SPP results in delayed localization of HCV core protein on lipid droplets and reduction of virus production (Targett-Adams et al., 2008). These reports suggest that cleavage of HCV core protein by SPP is required for its suitable intracellular localization for the viral assembly. Sequence analysis of the core protein suggests that high hydrophobicity is found in the region from amino acid residues 119 to 174, which is called domain 2 (Hope and McLauchlan, 2000). Domain 2 is critical for localization of the core protein on lipid droplets and shares common features with the core protein of GBV-B, but not of other viruses belonging to the Flaviviridae family (Hope et al., 2002). When three hydrophobic amino acids, Leu139, Val140, and Leu144, in the most hydrophobic peak in domain 2 were replaced with Ala to reduce hydrophobicity, the triple mutations in the core protein led to resistance to SPP cleavage, dislocation of the detergent-resistant membrane, and a reduction in virus production (Okamoto et al., 2004, 2008). Furthermore, comparative analysis between JFH1 and Jc1 suggest that binding strength of domain 2 of core for lipid droplets determines efficiency of virus assembly (Shavinskaya et al., 2007). These results suggest that hydrophobicity of domain 2 in the core protein is required for lipid droplet localization, SPP cleavage, and virus production.

Host lipid biogenesis is responsible for replication and assembly. HCV core protein contributes to the accumulation and production of host lipid components and is detected on the surface of lipid droplets (Hope and McLauchlan, 2000). The core protein is translocated into the lipid droplets near the replication complex and encloses newly synthesized viral RNA to form the nucleocapsid (**Figure 3**, step 2–4 or 4′), egresses into the lumen side of the ER, then is surrounded with host lipid components and viral envelope proteins (**Figure 3**, step 5; Miyanari et al., 2007). HCV core protein interacts with diacylglycerol acyl transferase 1 (DGAT1), which is required for the trafficking of core protein to lipid droplets (**Figure 3**, step 2; Herker et al., 2010). However, the translocation of the core protein to lipid droplets may not be required for efficient production of viral particles. The recombinant virus Jc1 exhibits a higher virus titer than the JFH1 strain (Lindenbach et al., 2006; Pietschmann et al., 2006). The core protein of the Jc1 strain is hardly detected on lipid droplets in infected cells and is mainly localized on ER membranes, together with envelope protein E2 (Miyanari et al., 2007; Shavinskaya et al., 2007; Boson et al., 2011). Expression of p7 increases the ER localization of core protein in the absence of envelope proteins (Boson et al., 2011). However, Miyanari et al. (2007) reported that the core protein of the Jc1 strain was mainly localized with envelope proteins on ER in cells transfected with a complete viral genome, but on lipid droplets in cells that were transfected with the viral genome lacking envelope protein genes. Expression of envelope proteins and p7 may determine intracellular localization of the core protein with regard to viral assembly (**Figure 3**, step 2 and 4 or 4′).

NS2 has been reported to be involved in the assembly process of HCV particles (Jones et al., 2007; Jirasko et al., 2008; Dentzer et al., 2009). NS2, which is composed of three transmembrane regions and a cytoplasmic domain in order after p7 (Lorenz et al., 2006), is known as the autoprotease of which C-terminal cytoplasmic domain is involved in *cis* cleavage at the NS2–NS3 junction (Santolini et al., 1995; Yamaga and Ou, 2002; Lorenz et al., 2006). Genetic interaction was implied between the N-terminal region of NS2 and the upstream structural proteins, since the first transmembrane of NS2 was identified as a genetic determinant for infectivity by construction of chimeric HCV with various genotypes (Pietschmann et al., 2006). Analyses by co-immunoprecipitation and imaging microscopy for interaction between NS2 and other viral proteins in cultured cells suggest that NS2 interacts with p7 and E2 on the ER-derived dotted structure closed to lipid droplets that are surrounded by HCV core protein (Popescu et al., 2011). NS2 also interacts with NS3/4A to recruit the core protein from lipid droplets to the cytoplasmic motile puncta along microtubules (Counihan et al., 2011). HCV p7 is a short hydrophobic protein composed of 63 amino acids and is encoded between the structural and non-structural proteins (Carrere-Kremer et al., 2002). The cytoplasmic loop of p7 is located between the N-terminal and C-terminal transmembrane regions (Carrere-Kremer et al., 2002). HCV p7 is known as a viroprotein that forms homooligomerize to be a ion channel, which is then involved in assembly and release of virus particle in infected cells by modulating pH equilibration in intracellular vesicles (Carrere-Kremer et al., 2002; Jones et al., 2007; Steinmann et al., 2007; Wozniak et al., 2010). Mutations of conserved amino acids required for ion channel activity impaired the production of infectious virus (Jones et al., 2007). However, recruitment of HCV core protein from lipid droplets to the ER assembly site was independent of the ion channel activity of HCV p7 (Boson et al., 2011). HCV p7 enhanced ER localization of the core protein without other viral proteins regardless of viral genotype, although compatibilities between two transmembrane regions of p7 and the first transmembrane domain of NS2 are responsible for ER localization of core protein and infection (Boson et al., 2011). The second transmembrane region of p7, rather than the first, is critical for compatibilities with NS2 regarding recruitment of core protein to the ER assembly site, although both transmembrane regions of p7 are important to sustain infectivity (Boson et al., 2011). These reports speculate that localization of the core protein on lipid droplets may contribute to suppression of virus production and maintenance of persistent HCV infection, while localization of the

core protein on ER may positively support virus production under the fulminant condition.

REGULATION OF HOST LIPID METABOLISM BY THE CORE PROTEIN

The mechanisms by which the core protein can induce liver diseases and extrahepatic manifestations are unknown. Liver steatosis, which is one of the characteristics associated with persistent HCV infection, develops by accumulation of triglyceride-rich lipids in hepatocytes. However, the precise functions of HCV proteins in the development of fatty liver remain unknown due to the lack of an adequate system to investigate the pathogenesis of HCV. HCV core protein expression has been shown to induce lipid droplets in cell lines and hepatic steatosis and hepatocellular carcinoma in transgenic mice (Barba et al., 1997; Moriya et al., 1997; Hope and McLauchlan, 2000). The lipid composition of the core-transgenic mouse is similar to that of a hepatitis C patient (Koike et al., 2010; Miyoshi et al., 2011). These reports suggest that HCV core protein plays an important role in the development of various types of liver failure, including steatosis and hepatocellular carcinoma. Biosynthesis of triglycerides is mainly regulated by the sterol regulatory element-binding protein (SREBP)-1c. It has been reported that many genes regulated by SREBPs were induced during the early stage of HCV infection in the livers of chimpanzees (Bigger et al., 2004). Our study has demonstrated that the core protein enhances the binding activity of the LXRα–RXRα complex to the *srebp-1c* promoter in a PA28γ-dependent manner, resulting in upregulation of SREBP-1c and its regulating genes (Moriishi et al., 2007). The activation may be mediated by the direct interaction between the core protein and RXRα (Tsutsumi et al., 2002). Another mechanism is thought to be suppression of lipid secretion. Reduced serum levels of cholesterol and apolipoprotein B have been reported in patients with severe hepatitis C and core-transgenic mice (Perlemuter et al., 2002). The MTP regulates the assembly and secretion of VLDLs consisting of apolipoprotein E, cholesterol, and triglycerides. In core-transgenic mice, MTP-specific activity is significantly decreased (Perlemuter et al., 2002). In addition, DGAT1, which plays an important role in trafficking core protein from lipid droplets to the ER membrane (Herker et al., 2010), was reported to delay the turnover of lipid droplets that are coated by the core protein (Harris et al., 2011; **Figure 3**). Furthermore, increases in saturated and monounsaturated fatty acids enhance HCV RNA replication (Kapadia and Chisari, 2005). The core protein can enhance the production of reactive oxygen species (ROS) by induction of induced nitric oxide synthetase (iNOS) or by damage to the mitochondrial electron transport system, contributing to the emergence of hepatocellular carcinoma (Moriya et al., 2001; Okuda et al., 2002; Nunez et al., 2004), suggesting that accumulation of lipids hastens the occurrence of hepatocellular carcinoma by enhancing ROS production. The core protein is reported to be degraded by PA28gamma-dependent, but ubiquitin-independent, proteasome activity, and directly binds to PA28gamma (Moriishi et al., 2003; Suzuki et al., 2009). PA28gamma knockdown diminished liver steatosis, hepatocellular carcinoma, and insulin resistance induced by HCV core protein in the mouse liver (Moriishi et al., 2007). After our reports, several groups found that PA28gamma plays an important role in cell cycling by degradation of SRC-3, p16, p19, and p53 (Li et al., 2006; Chen et al., 2007; Zhang and Zhang, 2008). Furthermore, HCV propagation in a cell culture system is potently suppressed by PA28gamma knockdown, regardless of cell growth (Moriishi et al., 2010). One possibility is that E6AP-dependent ubiquitination of the core protein in cytoplasm is competitively suppressed by peptide fragments deduced from nuclear core protein. However, there is still the possibility of an indirect effect of PA28gamma, since potent reduction of PA28gamma, but not intermediate reduction, can induce nuclear accumulation of HCV core protein in cultured cells and the mouse liver, but both potent and intermediate reductions could suppress viral production (Moriishi et al., 2007, 2010; Cerutti et al., 2011). Further study will be required to clarify the mechanism by which PA28gamma regulates core-induced liver diseases and the HCV life cycle.

NS3/4A AND LIPID DROPLETS

The NS3 also cleaves the host adaptor proteins IPS-1/MAVS and TRIF to modulate TLR and RIG-I signaling, resulting in inhibition of type I interferon production (Ferreon et al., 2005; Li et al., 2005a,b; Cheng et al., 2006; Loo et al., 2006). It is speculated that NS3 suppresses the activation of host innate immunity induced by HCV RNA and then contributes to persistent infection with HCV. NS3/4A may be responsible for not only the replication, but also the virus assembly and production by interaction with viral and host proteins on a region close to lipid droplets/ER assembly site. NS3/4A interacts with NS2 cooperating with p7 and E2 to recruit the core protein from lipid droplets to the cytoplasmic motile puncta along microtubules (Boson et al., 2011; Counihan et al., 2011; Popescu et al., 2011). HCV NS3/4A also interacts with host protein Y-box-binding protein-1 (YB-1) and influences the equilibrium between viral replication and infectious particle production (Chatel-Chaix et al., 2011). Knockdown of YB-1 impaired HCV RNA replication, regardless of the viral genotype, but did not affect NS3/4A autoprocessing and MAVS cleavage (Chatel-Chaix et al., 2011). JFH1 infection allowed YB-1 to translocate to lipid droplets containing core protein and NS3 (Chatel-Chaix et al., 2011), although knockdown of YB-1 enhanced the production of viral infectious particles (Chatel-Chaix et al., 2011). YB-1 may cooperate with NS3/4A to negatively regulate the steps after replication and to positively regulate viral replication.

NS5A AND CYCLOPHILINS

The peptide bond *cis*/*trans* isomerase converts between *cis* and *trans* peptide bonds leading to correct folding of the protein substrate. Peptidyl prolyl *cis*/*trans* isomerase (PPIase) includes the families of cyclophilin (Fischer et al., 1989), FK506-binding proteins (FKBP; Siekierka et al., 1989a,b) and parvulins (Rahfeld et al., 1994), and the secondary amide peptide bond *cis*/*trans* isomerase (Schiene-Fischer et al., 2002). Cyclophilin and FKBP are categorized as immunophilins, which are targeted by the immunosuppressants cyclosporin and FK506, respectively (Liu et al., 1991). Some cyclophilins and FKBP8 were shown to interact with NS5B and/or NS5A and to regulate HCV replication (Watashi et al., 2005; Okamoto et al., 2006), suggesting that immunophilins could lead to promising therapies for chronic hepatitis C, as discussed below.

Cyclosporin A and its derivatives, which target cyclophilins, were shown to impair HCV RNA replication and to exhibit efficacy in hepatitis C patients (Watashi et al., 2003; Ishii et al., 2006). Inoue et al. (2003) reported cyclosporin A treatment of HCV in a clinical trial. Cyclosporin derivatives lacking the ability to interact with cyclophilin lost their inhibitory effect on HCV replication (Watashi et al., 2005). Cyclophilin B is reported to be a 20-kDa secreted neurotropic factor for spinal cord cells in chick embryos (Spik et al., 1991), and is secreted into human milk and blood (Spik et al., 1991; Allain et al., 1994). Cyclophilin B specifically interacts with NS5B, the HCV RNA-dependent RNA polymerase around the ER of the HCV replicon cells, and promotes NS5B's association with viral RNA (Watashi et al., 2005). Cyclosporin A (CsA) was shown to disrupt the interaction between NS5B and cyclophilin B (Watashi et al., 2005). Treatment with cyclosporin A and knockdown of cyclophilin B suppressed the replication of HCV. However, several groups reported that interaction between NS5A and cyclophilin A is more important for HCV replication than interaction between NS5B and cyclophilin B. There is a growing consensus that cyclophilin A in particular is a crucial factor during HCV replication. A number of point mutations in both NS5A and NS5B have been reported to be associated with *in vitro* resistance to cyclophilin A (Yang et al., 2008; Chatterji et al., 2009; Kaul et al., 2009). Direct interaction between cyclophilin A and NS5B or NS5A has been observed (Yang et al., 2008). Several CsA-analogs, i.e., NIM811 (Ma et al., 2006), DEB025, and SCY-635 (Hopkins et al., 2010), are currently in preclinical and clinical development. DEB025 disrupts the interaction between NS5A and cyclophilin A and suppresses cyclophilin A isomerase activity. Although experimental differences in cell lines and replicons may affect employment of cyclophilins in HCV replication, the main molecule targeted by the cyclosporin analogs used clinically so far seems to be cyclophilin A.

The treatment with CsA has been associated with increased susceptibility to atherosclerosis and the development of hyperlipidemia (reviewed by Kockx et al., 2010). Treatment with CsA upregulated activity of cholesteryl ester transfer protein and suppressed lipoprotein lipase activity (Tory et al., 2008). Upregulation of cholesteryl ester by cholesteryl ester transfer protein could lead to accumulation of lipoprotein with cholesteryl ester. The report by Anderson et al. (2011) suggests that cyclophilin A and cyclophilin 40 are important for not only viral replication, but also the release of infectious viral particles. NIM811 treatment suppresses virus production and viral RNA replication (Goto et al., 2006). NIM811 treatment led to enlargement of lipid droplets and apoB crescent formation in replicon cells, but not naïve Huh7 cell line, while decreasing apoB secretion and the number of lipid droplets, rendering NS5A dislocation with apoB (Anderson et al., 2011). Knockdown of cyclophilins A and 40 in replicon cells showed the similar changes in lipid droplets size and apoB localization, comparing with NIM811 treatment (Anderson et al., 2011). Cyclophilins A and 40 may regulate lipid trafficking in the presence of HCV proteins to support secretion of viral particles.

NS5A/B AND MEMBRANE-ASSOCIATED PROTEINS

Host lipids are well known to be essential components in the viral life cycle, including the assembly, budding, and replication of various viruses (Chen et al., 2005; Giese et al., 2006; Mannova et al., 2006; Oomens et al., 2006). In the case of HCV, several types of lipids are required for the HCV life cycle. Saturated and monounsaturated fatty acids, but not polyunsaturated fatty acids, enhance HCV RNA replication (Kapadia and Chisari, 2005), suggesting that lipid biogenesis is involved in HCV replication. HCV particles bind to lipoprotein receptors for entry (Agnello et al., 1999; Scarselli et al., 2002) and are believed to exist with lipoproteins in the serum of infected patients (Thomssen et al., 1992). There is also evidence that HCV uses the VLDL assembly and secretion pathway for maturation and secretion of viral particles (Huang et al., 2007; Gastaminza et al., 2008). Cholesterol and sphingolipids are employed for virion maturation and infectivity, since depletion of cholesterol or down-regulation of sphingomyelin reduces infectivity (Aizaki et al., 2008). Accumulation of lipid components in the liver leads to liver steatosis, and is associated with progression to liver fibrosis and hepatocellular carcinoma, as described above.

Hepatitis C virus replication is suppressed by an inhibitor of geranylgeranyl transferase I, but not by that of farnesyl transferase (Ye et al., 2003). Geranylgeranylate is known as an intermediate found in the mevalonate pathway and is covalently bound to various cellular proteins that are associated with plasma or the intracellular membrane (Horton et al., 2002). Immunoprecipitation analysis revealed that NS5A interacts with FBL2 (Wang et al., 2005a). The F-box motif is located in the N-terminus of FBL2, followed by 11 leucine-rich repeats (Ilyin et al., 1999) and the CAAX motif, which is thought to be modified by geranylgeranylation (Wang et al., 2005a). The F-box motif is generally essential for the formation of the ubiquitin ligase complex (Ilyin et al., 1999), suggesting that FBL2 regulates the ubiquitination of host or viral proteins through the interaction with NS5A. Another possibility is that FBL2 retains the viral replication complex by interacting with NS5A (**Figure 3**, step 3).

Screening of a genome-wide siRNA library revealed phosphatidylinositol 4-kinase III alpha (PI4KA) and COPI vesicle coat complex as a human gene associated with HCV replication (Bigger et al., 2004; Borawski et al., 2009; Li et al., 2009; Tai et al., 2009; Trotard et al., 2009; Vaillancourt et al., 2009; Reiss et al., 2011). Phosphatidylinositol 4-phosphate, which is associated with oxysterol binding protein (OSBP) and CERT (Peretti et al., 2008; Banerji et al., 2010) as described below, is increased by HCV infection (Bigger et al., 2004; Hsu et al., 2010; Reiss et al., 2011; Tai and Salloum, 2011). PI4KA is co-localized with NS5A and double stranded RNA in the replication platform composed of detergent-resistant lipid components, known as a membranous web, and is critical for HCV replication at posttranslational stages in the membranous web (Berger et al., 2009). NS5A can interact with PI4KA (Berger et al., 2011; Lim and Hwang, 2011; Reiss et al., 2011) and recruit PI4KA to the membranous web (Berger et al., 2009; Tai et al., 2009; Reiss et al., 2011; Tai and Salloum, 2011). Furthermore, PI4KA, but not phosphatidylinositol 4-kinase III beta, induces the membranous web structure under the non-replicative condition (Berger et al., 2011; Lim and Hwang, 2011; Reiss et al., 2011). Biosynthesis of phosphatidylinositol 4-phosphate by PI4KA that is recruited by NS5A in the membranous web may be required for HCV replication and can be an endogenous biomarker of the membranous web (**Figure 3**, step 3).

Vesicle-associated membrane protein-associated proteins were originally identified as proteins that bind to vesicle-associated membrane protein (VAMP) in the nematode *Aplysia* and were designated as VAMP-associated protein 33 kDa (later renamed VAP-A; Skehel et al., 1995). Furthermore, one homolog and its splicing variant were reported as VAP-B and -C, respectively (Nishimura et al., 1999). VAP is classified as a type II membrane protein, and is composed of three functional domains: major sperm protein (MSP), which occupies the N-terminal half region, the coiled-coil domain, and the transmembrane domain. VAP-A shares 60% identity with VAP-B, while VAP-C is the splicing variant of VAP-B that lacks the coiled-coil and transmembrane domains (Nishimura et al., 1999). GST pull-down and immunoprecipitation analyses revealed that NS5A and NS5B interact with human VAP-A/B and that the N-terminal MSP domain and the coiled-coil domain of VAP-A/B are responsible for the binding to NS5B and NS5A, respectively (Tu et al., 1999; Hamamoto et al., 2005). In addition, systematic RNAi screening revealed that 62 target host genes are involved in HCV RNA or proteins including VAP-A/B (Randall et al., 2007). Several reports suggest that HCV replication takes place on the membranous web (Shi et al., 2003; Gao et al., 2004; Sakamoto et al., 2005). NS4B is predominantly associated with a lipid-raft-like detergent-resistant fraction equivalent to the membranous web, and both NS5A and NS5B were co-localized in a similar fraction in the presence of NS4B (Sakamoto et al., 2005). VAP-A was also localized in a detergent-resistant fraction, suggesting that it plays an important role in HCV replication because the dominant negative mutant of VAP-A suppressed the replication of HCV RNA (Gao et al., 2004). VAP-B forms a homodimer and heterodimer with VAP-A, and knockdown of VAP-A or VAP-B led to substantial suppression of HCV replication (Hamamoto et al., 2005). These findings suggest that VAP-A and -B positively regulate HCV replication by binding to NS5A/B.

The physiological function of VAPs was reported to be trafficking of ceramide and cholesterol between ER and the Golgi apparatus. Several VAP-interacting proteins share the FFAT motif (two phenylalanines in an acidic tract), which has the consensus amino acid sequence EFFDAxE, as determined by a comparison among oxysterol binding proteins, OSBP-related proteins (ORPs; Loewen et al., 2003), and the ceramide transport protein CERT (Hanada et al., 2003; Kawano et al., 2006), contributing to the regulation of lipid metabolism. OSBP binds and transports cholesterol or hydroxycholesterol from ER to the Golgi (Ridgway et al., 1992; Wang et al., 2005b), while CERT binds and transports ceramide from ER to the Golgi, where the ceramide is converted to sphingolipids (Kumagai et al., 2005). Altering the sphingomyelin/ceramide ratio of the plasma membrane can effect HCV entry via the cell surface expression of CD81 (Voisset et al., 2008). OSBP mediates HCV secretion while binding to NS5A and VAP-A (Amako et al., 2009). Inhibition of CERT function effectively suppressed HCV release regardless of RNA replication (Aizaki et al., 2008). Phosphorylation of CERT and OSBP by protein kinase D negatively regulates VAPs binding to CERT and OSBP resulting in an effect on HCV infection (Amako et al., 2011). HCV NS5A may allow VAP-A/B to provide ceramide and cholesterol to replication complexes for upregulation of virus propagation (**Figure 3**, step 3).

The VAP-B-splicing variant VAP-C interacts with NS5B via the short form of the MSP domain and then suppresses the HCV replication by disrupting binding of other VAPs to NS5B (Kukihara et al., 2009). Expression of VAP-C is observed in various tissues except for the liver, suggesting that tissue distribution of VAP-C determines the tropism of HCV infection (Kukihara et al., 2009). These findings suggest that VAP-C negatively regulates HCV replication by inhibiting the interaction between VAP-A/B and NS5B. Furthermore, expression of VAP-C was negligible in B cells prepared from chronic hepatitis C patients, in whom B cells included HCV particles (Ito et al., 2010), and expression of the full HCV genome in B cells induced B-cell lymphoma in a conditional transgenic mouse (Kasama et al., 2010), suggesting that HCV infection increases the chance of developing B-cell lymphomas via dysregulation of lipid metabolism.

CONCLUSION

This review summarizes several recently reported viral and host factors that exploit lipid components to support HCV infection. The mechanism by which HCV proteins cooperate with host factors to exploit lipid components and to regulate lipid metabolism in the infection has not been elucidated completely. The aim of identifying host factors is effective and stable therapy; targeting the host factors might be done to prevent the emergence of resistant viruses. Cyclosporin analogs will be used clinically in the near future. Wide screening and proteomics analyses have revealed novel host factors that are required for HCV replications over the past decade. The mechanism by which HCV infection induces formation of membranous web in infected cells has been unknown yet, although NS4B is involved in formation of membranous web (Egger et al., 2002; Gosert et al., 2005; Ferraris et al., 2010). We also found several host proteins to be NS4B-associating host factors by proteomics analysis based on the TargetMine program (Tripathi et al., 2010). Further study will be required to identify the prominent factors essential for lipid metabolism that are associated with each step in the HCV life cycle and to develop effective and stable therapies for hepatitis C.

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Molecular mechanism of hepatitis C virus-induced glucose metabolic disorders

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Hepatitis C virus (HCV) infection causes not only intrahepatic diseases but also extrahepatic manifestations, including metabolic disorders. Chronic HCV infection is often associated with type 2 diabetes. However, the precise mechanism underlying this association is still unclear. Glucose is transported into hepatocytes via glucose transporter 2 (GLUT2). Hepatocytes play a crucial role in maintaining plasma glucose homeostasis via the gluconeogenic and glycolytic pathways. We have been investigating the molecular mechanism of HCV-related type 2 diabetes using HCV RNA replicon cells and HCV J6/JFH1 system. We found that HCV replication down-regulates cell surface expression of GLUT2 at the transcriptional level. We also found that HCV infection promotes hepatic gluconeogenesis in HCV J6/JFH1-infected Huh-7.5 cells. HCV infection transcriptionally up-regulated the genes for phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase (G6Pase), the rate-limiting enzymes for hepatic gluconeogenesis. Gene expression of PEPCK and G6Pase was regulated by the transcription factor forkhead box O1 (FoxO1) in HCV-infected cells. Phosphorylation of FoxO1 at Ser319 was markedly diminished in HCV-infected cells, resulting in increased nuclear accumulation of FoxO1. HCV NS5A protein was directly linked with the FoxO1-dependent increased gluconeogenesis. This paper will discuss the current model of HCV-induced glucose metabolic disorders.

Keywords: HCV, diabetes, gluconeogenesis, GLUT2, FoxO1, JNK, NS5A

INTRODUCTION

Hepatitis C virus (HCV) is a positive-sense, single stranded RNA virus that belongs to the genus *Hepacivirus* of the family *Flaviviridae*. The approximately 9.6-kb HCV genome encodes a unique open reading frame that is translated into a polyprotein of about 3,000 amino acids, which is cleaved by cellular signalases and viral proteases to generate at least 10 viral proteins, such as core, envelope 1 (E1) and E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Choo et al., 1991; Lemon et al., 2007).

Hepatitis C virus is the main cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. More than 170 million people worldwide are chronically infected with HCV (Poynard et al., 2003). Persistent HCV infection causes not only liver diseases but also extrahepatic manifestations. It is well established that HCV perturbs the glucose metabolism, leading to insulin resistance and type 2 diabetes in predisposed individuals. Several epidemiological, clinical, and experimental data suggested that HCV infection serves as an additional risk factor for the development of diabetes (Mason et al., 1999; Negro and Alaei, 2009; Negro, 2011). HCV-related glucose metabolic changes and insulin resistance and diabetes have significant clinical consequences, such as accelerated fibrogenesis, increased incidence of hepatocellular carcinoma, and reduced virological response to interferon (IFN)-α-based therapy (Negro, 2011). Therefore, it is very important to clarify the molecular mechanism of HCV-related diabetes. However, the precise mechanisms are poorly understood.

Experimental data suggest a direct interference of HCV with the insulin signaling pathway. Transgenic mice expressing HCV core gene exhibit insulin resistance (Shintani et al., 2004; Koike, 2007). In this transgenic mice model, both tyrosine phosphorylation of the insulin receptor substrate (IRS)-1 and IRS-2 are decreased. These decreases are recovered when the proteasome activator PA28γ is deleted, suggesting that the HCV core protein suppresses insulin signaling through a PA28γ-dependent pathway (Miyamoto et al., 2007). Several other reports also showed a link of the HCV core protein with insulin resistance (Kawaguchi et al., 2004; Pazienza et al., 2007).

Hepatocytes play a crucial role in maintaining plasma glucose homeostasis by adjusting the balance between hepatic glucose production and utilization via the gluconeogenic and glycolytic pathways, respectively. Gluconeogenesis is mainly regulated at the transcriptional level of the glucose 6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) genes, whereas glycolysis is mainly regulated by glucokinase (GK). Gluconeogenesis and glycolysis are coordinated so that one pathway is highly active within a cell while the other is relatively inactive. It is well known that increased hepatic glucose production via gluconeogenesis is a major feature of type 2 diabetes (Clore et al., 2000).

To identify a novel mechanism of HCV-related diabetes, we have been investigating the effects of HCV on glucose production in hepatocytes using HCV RNA replicon cells (Lohmann et al., 1999) and HCV J6/JFH1 cell culture system (Lindenbach et al., 2005; Wakita et al., 2005; Bungyoku et al., 2009). We previously reported that HCV replication suppresses cellular glucose uptake through down-regulation of cell surface expression of glucose transporter 2 (GLUT2; Kasai et al., 2009). Furthermore, we

recently reported that HCV promotes hepatic gluconeogenesis via an NS5A-mediated, forkhead box O1 (FoxO1)-dependent pathway, resulting in increased cellular glucose production in hepatocytes (Deng et al., 2011). This paper discusses our current model for HCV-induced glucose metabolic disorders.

HCV REPLICATION DOWN-REGULATES CELL SURFACE EXPRESSION OF GLUT2

The uptake of glucose into cells is conducted by the facilitative glucose carrier, glucose transporters (GLUTs). GLUTs are integral membrane proteins that contain 12 membrane-spanning helices. To date, a total of 14 isoforms have been identified in the GLUT family (Wu and Freeze, 2002; Macheda et al., 2005; Godoy et al., 2006). Glucose is transported into hepatocytes by GLUT2. We previously reported that HCV J6/JFH1 infection suppresses hepatocytic glucose uptake through down-regulation of surface expression of GLUT2 in human hepatoma cell line, Huh-7.5 cells (Kasai et al., 2009). We also demonstrated that GLUT2 expression in hepatocytes of the liver tissues from HCV-infected patients was significantly lower than in those from patients without HCV infection. Our data suggest that HCV infection down-regulates GLUT2 expression at transcriptional level. We are currently analyzing transcriptional control of human GLUT2 promoter in HCV replicon cells as well as in HCV J6/JFH1-infected cells.

HCV INFECTION PROMOTES HEPATIC GLUCONEOGENESIS

Then we analyzed hepatic glucose production and expression of transcription factors using HCV replicon cells and HCVcc system in order to clarify a role of HCV infection in glucose metabolic changes. Hepatic glucose production is usually regulated by gluconeogenesis and glycolysis. Therefore, we examined whether HCV infection induces gluconeogenesis or glycolysis. We found that the PEPCK and G6Pase genes were transcriptionally up-regulated in J6/JFH1-infected cells (**Figure 1**). On the other hand, the GK gene was transcriptionally down-regulated in HCV-infected cells. We obtained similar data in HCV replicon cells (both in subgenomic replicon cells and full-genomic replicon cells). When HCV replication was suppressed by IFN treatment, the up-regulation of PEPCK and G6Pase gene expression as well as the down-regulation of GK gene expression were canceled. From these results, HCV infection selectively up-regulates PEPCK and G6Pase genes, whereas HCV infection down-regulates GK gene (Deng et al., 2011).

Both HCV replicon cells and HCV-infected cells produced greater amounts of glucose than the control cells. IFN treatment canceled the enhanced glucose production in HCV replicon cells as well as in HCV-infected cells. G6P is an important precursor molecule that is converted to glucose in the gluconeogenesis pathway (**Figure 1**). Our metabolite analysis showed that a significantly higher level of G6P was accumulated in HCV-infected cells than in the control cells, suggesting that HCV indeed promotes hepatic gluconeogenesis to cause hyperglycemia. There is a trend toward an increase in gluconeogenesis in HCV-infected cells (**Figure 1**).

HCV SUPPRESSES FoxO1 PHOSPHORYLATION AT Ser319, LEADING TO THE NUCLEAR ACCUMULATION OF FoxO1

It has been reported that G6Pase, PEPCK, and GK are regulated by certain transcription factors, including FoxO1 (Hirota et al., 2008), hepatic nuclear factor 4α (HNF-4α; Hirota et al.,

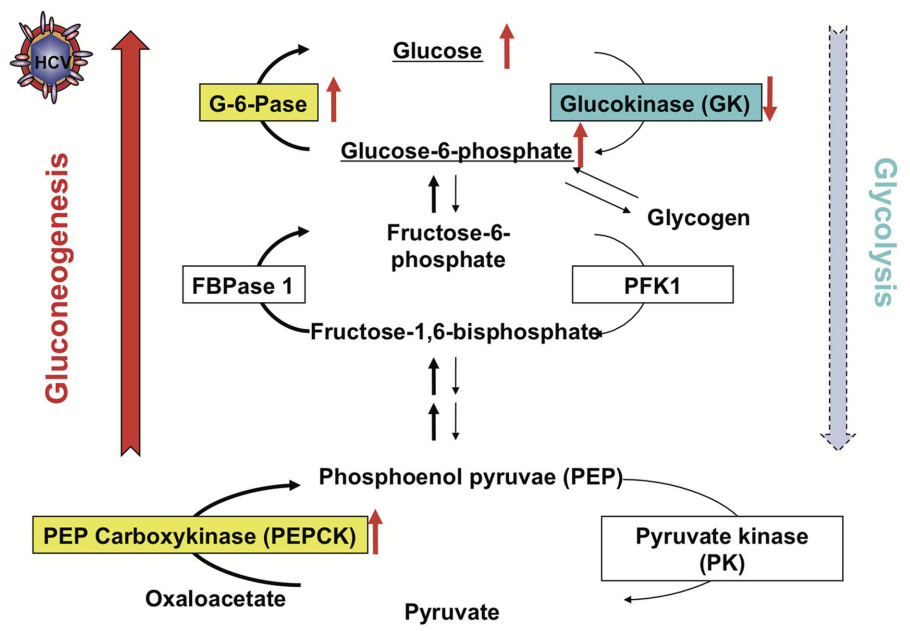

FIGURE 1 | Regulation of Gluconeogenesis and Glycolysis in the HCV-infected cells. HCV infection promotes gluconeogenesis via transcriptional up-regulation of the genes for PEPCK and G6Pase, the rate-limiting enzymes for hepatic gluconeogenesis, and transcriptional down-regulation of the gene for GK, the rate-limiting enzyme for hepatic glycolysis.

2008), Krüppel-like factor 15 (KLF15; Takashima et al., 2010), and cyclic AMP (cAMP) response element binding protein (CREB; Rozance et al., 2008). While we were analyzing these factors in both HCV replicon cells and HCV J6/JFH1-infected cells, we found the involvement of the FoxO1 in the transcriptional activation of G6Pase and PEPCK (Deng et al., 2011). It is known that the FoxO1 enhances gluconeogenesis through the transcriptional activation of various genes, including G6Pase and PEPCK (Gross et al., 2008). The function of FoxO1 is regulated by post-translational modifications, including phosphorylation, ubiquitylation, and acetylation (Tzivion et al., 2011). The phosphorylated form of FoxO1 is exported from the nucleus to the cytosol, resulting in loss of its transcriptional activity (**Figure 2**). Phosphorylation status of FoxO1 at Ser319 is critical for FoxO1 nuclear exclusion (Zhao et al., 2004). Although the total amounts of FoxO1 protein were unchanged, FoxO1 phosphorylation at Ser319 was markedly suppressed in HCV-infected cells compared to that in the mock-infected cells. It is known that the FoxO1 is phosphorylated by the protein kinase Akt and is exported from the nucleus to the cytosol, resulting in loss of its transcriptional activity (Tzivion et al., 2011). The majority of FoxO1 was accumulated in the nuclear fraction in HCV-infected cells, whereas in control cells FoxO1 was distributed in both the nuclear and cytoplasmic fractions. Akt phosphorylation was enhanced in HCV-infected cells, although the protein levels of total Akt protein were comparable, which is consistent with the report by Burdette et al. (2010). Our findings suggest an interesting scenario in which the HCV-mediated suppression in FoxO1 phosphorylation is caused by an unknown mechanism independent of Akt activity.

HCV-INDUCED JNK ACTIVATION IS INVOLVED IN THE SUPPRESSION OF FoxO1 PHOSPHORYLATION

It is known that the stress-sensitive serine/threonine kinase JNK regulates FoxO at multiple levels (van der Horst and Burgering, 2007; Karpac and Jasper, 2009). We demonstrated that HCV infection induces phosphorylation and activation of JNK in a time-dependent manner, which is similar to that observed for the suppression of FoxO1 phosphorylation. As a result, c-Jun, a key substrate for JNK, got phosphorylated and activated in HCV-infected cells. The JNK inhibitor SP600125 clearly prevented the phosphorylation of c-Jun, and concomitantly recovered the suppression of FoxO1 phosphorylation in HCV-infected cells, suggesting that HCV activates the JNK/c-Jun signaling pathway, resulting in the nuclear accumulation of FoxO1 by reducing its phosphorylation status. The detailed mechanisms of HCV-induced suppression of FoxO1 phosphorylation via the JNK/c-Jun signaling pathway remain to be explored. There are at least two possibilities. The JNK/c-Jun signaling pathway (1) suppresses a protein kinase, or (2) activates a protein phosphatase to reduce phosphorylation of FoxO1.

HCV-INDUCED MITOCHONDRIAL REACTIVE OXYGEN SPECIES PRODUCTION IS INVOLVED IN INCREASED GLUCOSE PRODUCTION THROUGH JNK ACTIVATION

Hepatitis C virus infection increases mitochondrial reactive oxygen species (ROS) production (Deng et al., 2008). *N*-acetyl cysteine (NAC; a general antioxidant) clearly prevented the phosphorylation of JNK, and concomitantly canceled the suppression of FoxO1 phosphorylation in HCV-infected cells, suggesting that

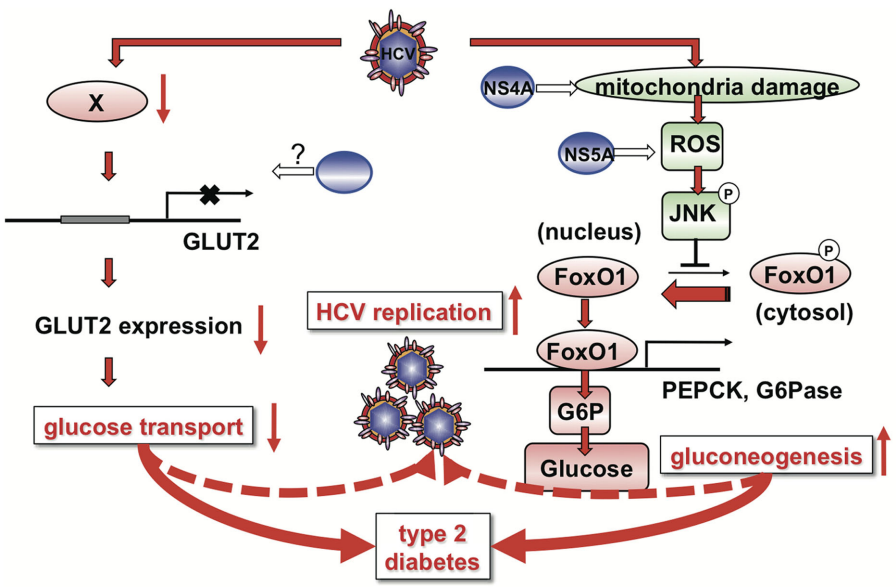

FIGURE 2 | A proposed mechanism of HCV-induced glucose metabolic disorders. HCV infection down-regulates cell surface expression of GLUT2 in hepatocytes at the transcriptional level. HCV down-regulates a transcription factor involved in GLUT2 gene expression through an unknown mechanism. HCV infection induces mitochondria damage and ROS production, leading to JNK activation. HCV NS4A protein is involved in mitochondrial damage. HCV NS5A protein is involved in ROS production. HCV-induced ROS production causes JNK activation, resulting in the decreased phosphorylation and nuclear accumulation of FoxO1. Nuclear accumulation of FoxO1 up-regulates gene expression of PEPCK and G6Pase, leading eventually to increased glucose production by gluconeogenesis. High glucose levels in the hepatocytes may confer an advantage in efficient replication of HCV.

HCV-induced ROS production is involved in the JNK activation. There was no significant difference in HCV RNA replication or infectious virus release between SP600125- or NAC-treated HCV-infected cells and non-treated HCV-infected cells. These results suggest that ROS-mediated JNK activation plays a key role in the suppression of FoxO1 phosphorylation, nuclear accumulation of FoxO1, and enhancement of glucose production in HCV-infected cells (Deng et al., 2011).

HCV NS5A IS INVOLVED IN THE ENHANCEMENT OF GLUCOSE PRODUCTION

Then we sought to determine which HCV protein(s) is involved in the enhancement of glucose production. Transient expression of NS5A protein in Huh-7.5 cells significantly promoted the gene expression levels of G6Pase and PEPCK determined by real time quantitative RT-PCR. Promoter assay revealed that the level of PEPCK promoter activity was significantly higher in NS5A-expressing cells than in the control cells. Our results suggest that NS5A activate both the PEPCK promoter and the G6Pase promoter, leading to an increase in glucose production (Deng et al., 2011). The study by Banerjee et al. (2010) suggests that the HCV core protein modulates FoxO1 and FoxA2 activation and affects insulin-induced metabolic gene regulation in human hepatocytes. Our results, however, suggest that the HCV core protein is not significantly involved in the increased gluconeogenesis (Deng et al., 2011). The difference between these two studies needs to be explored.

There were previous reports suggesting that ROS production is induced in NS5A-expressing cells (Dionisio et al., 2009) or in hepatocytes of NS5A transgenic mice (Wang et al., 2009). We therefore sought to determine whether NS5A contributes to increased hepatic gluconeogenesis through the induction of ROS production. NS5A-expressing cells displayed a much stronger signal of ROS than in control cells. NS5A-expressing cells promoted phosphorylation level at Ser63 of c-Jun and suppressed FoxO1 phosphorylation at Ser319, suggesting that NS5A mediates JNK/c-Jun activation and FoxO1 phosphorylation suppression. These results suggest that NS5A play a role in the HCV-induced enhancement of hepatic gluconeogenesis through JNK/c-Jun activation and FoxO1 phosphorylation suppression.

CONCLUSION AND FUTURE PERSPECTIVES

Taken together, we propose a model of HCV-induced glucose metabolic disorders as shown in **Figure 2**. HCV infection down-regulates cell surface expression of GLUT2 in hepatocytes at the transcriptional level. HCV down-regulates a transcription factor involved in GLUT2 gene expression through an unknown mechanism. As GLUT2 is a facilitative GLUT, it ensures large bidirectional fluxes of glucose in and out the cell due to its low affinity and high capacity (Leturque et al., 2009). Down-regulated cell surface expression of GLUT2 results in disruption of bidirectional transport of glucose in hepatocytes. Even in the fasting state, down-regulation of GLUT2 may result in low glucose uptake of hepatocytes, causing hyperglycemia. In the fed state, glucose secretion from hepatocytes may be suppressed due to low level cell surface expression of GLUT2, as GLUT2 is a bidirectional transporter.

Hepatitis C virus infection induces mitochondria damage and ROS production, leading to JNK activation. HCV NS4A protein is involved in mitochondrial damage (Nomura-Takigawa et al., 2006). HCV NS5A protein is involved in ROS production (Dionisio et al., 2009; Wang et al., 2009; Deng et al., 2011). HCV-induced ROS production causes JNK activation, which results in the decreased phosphorylation and nuclear accumulation of FoxO1 by an unidentified mechanism. Nuclear accumulation of FoxO1 up-regulates gene expression of PEPCK and G6Pase, leading eventually to increased glucose production by gluconeogenesis (Deng et al., 2011).

These two pathways, HCV-induced down-regulation of GLUT2 expression and up-regulation of gluconeogenesis, may contribute to development of type 2 diabetes in HCV-infected patients at least to some extent. HCV-induced down-regulation of GLUT2 expression and up-regulation of gluconeogenesis may result in high concentration of glucose in HCV-infected hepatocytes. As suggested in a recent study, low glucose concentration in the hepatocytes inhibits HCV replication (Nakashima et al., 2011). Therefore, high glucose levels in the hepatocytes may confer an advantage in efficient replication of HCV.

Our understanding of HCV-induced glucose metabolic disorders will require much more work to fully unfold this pathway. Further investigation including the mechanism of HCV-induced GLUT2 downregualtion, JNK-mediated decreased phosphorylation of FoxO1, and the possible effect(s) of the dysregulation of hepatic gluconeogenesis on the HCV life cycle and host cells are currently under way.

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Peripheral B cells as reservoirs for persistent HCV infection

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Hepatitis C virus (HCV) is an enveloped positive-stranded RNA virus of approximately 9.6 kb that belongs to the Flaviviridae family (Suzuki et al., 2007). HCV infection is a global health problem affecting nearly 200 million people (Lauer and Walker, 2001). The infection causes prolonged and persistent disease in over half of viral carriers that often leads to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (Afdhal, 2004). Recent studies have suggested that HCV infects not only hepatocytes but also peripheral mononuclear lymphocytes, particularly B cells, which express CD81, a widely expressed tetraspanin molecule. CD81 has been shown to interact with the E2 region of HCV envelope proteins (Pileri et al., 1998) and is thus regarded as one of the key molecules involved in HCV infection. HCV infection of B cells is the likely cause of various B cell dysregulation disorders. Herein, we propose that HCV uses peripheral B cells as reservoirs for persistent infection, which are in turn responsible for HCV pathogenesis.

Although the liver is considered the primary and main target of HCV infection, extrahepatic manifestations such as mixed cryoglobulinemia, a systemic immune complex-mediated disorder characterized by B cell proliferation that may evolve into overt B cell non-Hodgkin's lymphoma (B-NHL), are often recognized among patients persistently infected with HCV (Agnello et al., 1992; Zuckerman et al., 1997). Epidemiological evidence strongly suggests a close association between chronic HCV infection and B-NHL occurrence (Turner et al., 2003; de Sanjose et al., 2008). A pathogenic role of HCV in B cell disorders has been further demonstrated by studies in which the clinical resolution of B cell dysfunctions was observed after successful regimens of anti-HCV treatment (Mazzaro et al., 1996; Agnello et al., 2002). Based on the aforementioned data, Antonelli et al. (2008) postulated a role of B cells in HCV pathogenesis. In accordance with this notion, our recent study clearly demonstrated that HCV infects and may replicate in the peripheral $CD19^+$ B cells of chronic hepatitis C (CHC) patients (Ito et al., 2010a). In order to determine how HCV evades antiviral innate immune responses that are normally induced in B cells, we analyzed expression levels of IFN-β in peripheral B cells of CHC patients because type I IFN plays a critical role in the antiviral innate immune response. We found that HCV infection failed to trigger antiviral immune responses, such as IFN-β production, in B cells of CHC patients (Ito et al., 2010b). This suggests that HCV evades antiviral innate immune responses in peripheral B cells and uses these cells as reservoirs for its persistent infection in the host.

The idea that B cells may serve as HCV reservoirs was advocated by Muller et al. (1993). Several subsequently published papers also favored the notion of HCV lymphotropism (Ducoulombier et al., 2004; Blackard et al., 2006; Pal et al., 2006). **Figure 1** illustrates the possible process of HCV infection in B cells based on previous studies using human hepatocyte-derived cell lines (Burlone and Budkowska, 2009; Georgel et al., 2010). Among B cell subsets, memory B cells are assumed to be the main reservoirs of HCV infection primarily because of their long lifespans. In support of this notion, our recent study indicated that $CD19^+$ $CD27^+$ cells (i.e., memory B cell phenotype) express a high amount of CXCR3, a chemokine receptor, and are recruited to the inflammatory site in the liver of CHC patients where IFN-γ-inducible protein-10, a CXCR3 ligand, is highly produced (Mizuochi et al., 2010; **Figure 1**). This unique strategy seems to

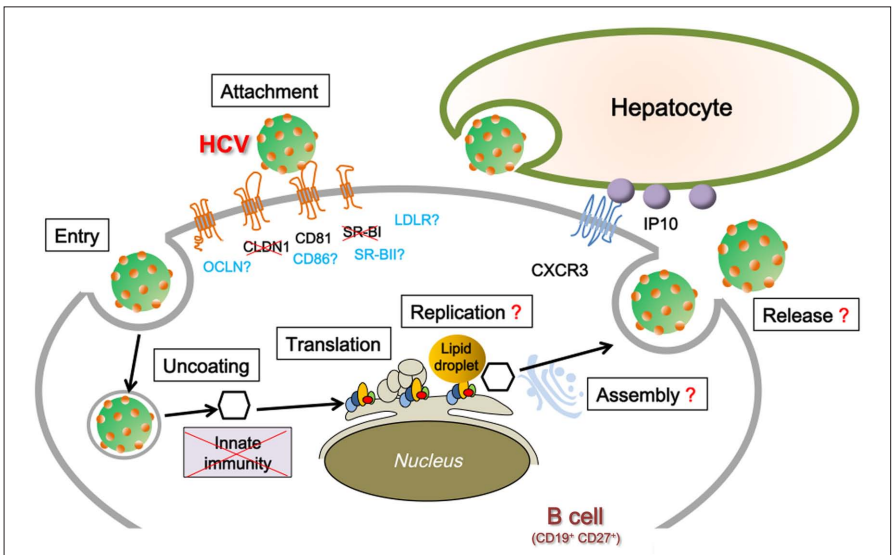

FIGURE 1 | Proposed lifecycle of HCV in B cell. The life cycle of the hepatitis C virus (HCV) has several specific steps. Virus entry is mediated by the direct interaction of envelope glycoproteins with co-receptors, presumably E2–CD81 binding. CLDN1 and SR-BI are not expressed in B cell. Innate immunity response against HCV is suppressed by HCV infection. Some HCV-encoding proteins, i.e., core and NS3, have been proven to be expressed in B cells. HCV RNA replication was examined by nested PCR and *in situ* hybridization. Mechanisms of virus assembly, vesicle fusion, and virion release in B cell remain unknown. LDLR, low-density lipoprotein receptor; SR-BI, scavenger receptor class B type I; SR-BII, scavenger receptor class B type II; CLDN1, claudin-1; OCLN, occludin; CXCR3, chemokine (C-X-C motif) receptor 3; IP-10, interferon gamma-induced protein-10.

be beneficial for HCV in securing sites for persistent infection. HCV may search for reservoir sites in cellular compartments other than hepatocytes in case the liver becomes unsuitable for HCV replication, perhaps because of cellular destruction caused by the host immune response and/or by irrelevant conditions for successful virus replication, such as the development of cirrhosis or hepatocellular carcinoma.

At least two important issues remain to be investigated. First, how do HCV-infected B cells evade "acquired/adaptive" immune responses represented by cytotoxic T cells (CTL)? In peripheral blood, the frequencies of HCV-specific $CD8^+$ lymphocytes with persistent HCV infection are lower than those with acute HCV infection. Furthermore, the CTL response to the HCV antigen is impaired in chronic HCV patients (Lechner et al., 2000). Interestingly, the percentage of CTL in peripheral blood is lower than that in the liver, which may be advantageous for persistent HCV infection in B cells. Because the peripheral B cells of CHC patients express the HCV core as well as NS3 antigens (Ito et al., 2010a), both of which encode functional CTL epitopes (Hiroishi et al., 2010), it is possible that HCV-infected B cells are eliminated by CTL to some extent. However, the fact that substantial amounts of HCV-infected B cells are found in CHC patients suggests incomplete elimination by CTL by an inhibitory mechanism, i.e., HCV E2-mediated inhibition of IL-2/IFN-γ secretion (Petrovic et al., 2011). Second, do HCV-infected B cells produce infectious HCV? Stamataki et al. (2009) demonstrated that the infectious JFH-1 strain of HCV can bind B cells but fails to establish productive infection. On the other hand, Inokuchi et al. (2009) recently demonstrated the presence of negative-stranded HCV RNA, a marker of viral replication, in B cells from 4 of 75 (5%) CHC patients. These results support the notion that HCV replicates in B cells and suggest that infectious HCV are produced in B cells. We have currently been investigating this intriguing issue by using an *in vitro* assay system.

In conclusion, lymphoid reservoirs of HCV infection may play a role in viral persistence and thereby be involved in its pathogenesis. Infection and replication of HCV in peripheral B cells should be regarded as a considerable impediment to the treatment of CHC patients undergoing various antiviral regimens. From a therapeutic viewpoint, it may be beneficial to eliminate peripheral B cells in CHC patients by administering anti-B cell antibodies, such as rituximab, along with combination chemotherapy of peg-IFN-α and ribavirin, which eliminate circulating HCV in the blood. Together, this could lead to a synergistic effect on HCV clearance in CHC patients.

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Scavenger receptor B2 as a receptor for hand, foot, and mouth disease and severe neurological diseases

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Enterovirus 71 (EV71) is one of the major causative agents of hand, foot, and mouth disease (HFMD). Infection with EV71 is occasionally associated with severe neurological diseases such as acute encephalitis, acute flaccid paralysis, and cardiopulmonary failure. Because cellular receptors for viruses play an important role in cell, tissue, and species tropism, it is important to identify and characterize the receptor molecule. Recently, cellular receptors and host factors that stimulate EV71 infection have been identified. Several lines of evidence suggest that scavenger receptor class B, member 2 (SCARB2) plays critical roles in efficient EV71 infection and the development of disease in humans. In this review, we will summarize the findings of recent studies on EV71 infection and on the roles of SCARB2.

Keywords: EV71, SCARB2, HFMD, neurological disease

INTRODUCTION

Human enteroviruses (HEVs) are a large family of human pathogens belonging to the Picornaviridae family, and these viruses can cause a variety of diseases. HEVs are classified into four groups, species A (HEV-A) to species D (HEV-D). HEV-A is composed of at least 16 members of different serotypes: Coxsackievirus (CV) A2, CVA3, CVA4, CVA5, CVA6, CVA7, CVA8, CVA10, CVA12, CVA14, CVA16, Enterovirus 71 (EV71), EV76, EV89, EV90, and EV91 (Pallansch and Roos, 2007). The number of isolated and characterized HEV-A viruses is continually increasing (Oberste et al., 2007, 2008). Members of HEV-A are known to be causative agents of hand, foot, and mouth disease (HFMD), herpangina, respiratory disease, meningitis, and polio-like flaccid paralysis (Pallansch and Roos, 2007). EV71 and CVA16 are the major causative agents of HFMD. HFMD is normally a mild disease, but HFMD caused by EV71 is sometimes associated with severe neurological diseases such as acute fatal encephalitis, polio-like acute flaccid paralysis, and neurogenic pulmonary edema (Schmidt et al., 1974; Chumakov et al., 1979a,b; Melnick, 1984; Ho et al., 1999; Chan et al., 2000). Recently, large outbreaks of EV71 associated with severe neurological diseases have occurred repeatedly in the Asia-Pacific region (Ho et al., 1999; Komatsu et al., 1999; Ahmad, 2000; Chan et al., 2000; McMinn et al., 2001a,b; Fujimoto et al., 2002; Wang et al., 2002; De et al., 2011). EV71 has become a serious public health concern (Qiu, 2008).

Virus infection is initiated by attachment of the virus to a cellular receptor at the surface of a susceptible cell. Cellular receptors for viruses therefore play important roles in determining the cell, tissue, and species tropism and pathogenicity of viruses (Bergelson, 2010). Thus, the identification and characterization of the cellular receptor for EV71 are important steps in the elucidation of the pathogenicity of EV71. Recently, several research groups identified cellular receptors and host factors that enhance EV71 infection (Nishimura et al., 2009; Yamayoshi et al., 2009; Yang et al., 2009, 2011; Han et al., 2010). Herein, we will summarize the importance of scavenger receptor class B, member 2 (SCARB2) as a receptor for EV71.

IDENTIFICATION OF SCARB2 AS A RECEPTOR FOR EV71

Monolayer cultures of primate cells such as RD cells and Vero cells are susceptible to EV71 strains, and these cells are often used for the isolation of EV71 from clinical specimens (Mizuta et al., 2005, 2009). Mouse cells, such as L929 cells, are generally not highly susceptible to EV71 infection. Only residual virus antigen-positive cells are observed, even if the mouse cells are infected at a high multiplicity of infection. This species-specific restriction of EV71 infection is due to the absence on mouse cells of the cellular receptor that permits viral entry into cells. Yamayoshi et al. (2009) employed a strategy to identify the human EV71 receptor; this strategy is outlined in **Figure 1**. These researchers transfected human genomic DNA into mouse L929 cells and succeeded in selecting two L929 cell lines, Ltr051 and Ltr246, that became susceptible to EV71 among approximately 70,000 transformed cells. The integrated human gene(s) are expected to encode the EV71 receptor.

One of the transformed cell lines, Ltr051, was highly susceptible to EV71, with an infection efficiency similar to that of RD cells, whereas the other cell line, Ltr246, was susceptible to EV71 but with a lower efficiency. By microarray analysis of the RNAs expressed in the transformant cells, it was shown that Ltr051 cells carried the gene for human *SCARB2*. L929 cells that expressed SCARB2 constitutively (L-SCARB2 cells) were susceptible to all EV71 strains tested, irrespective of the sub-genogroup. EV71 infection in RD cells was inhibited both by anti-SCARB2 antibodies and by soluble SCARB2. The human and monkey cell lines that are EV71-susceptible cells expressed SCARB2. These results suggest that SCARB2 plays a critical role in the EV71 infection pathway. In addition, Yamayoshi et al. (2009) found that CVA16 also uses SCARB2 as a receptor, suggesting that SCARB2 serves as a receptor for other HEV-As that cause HFMD.

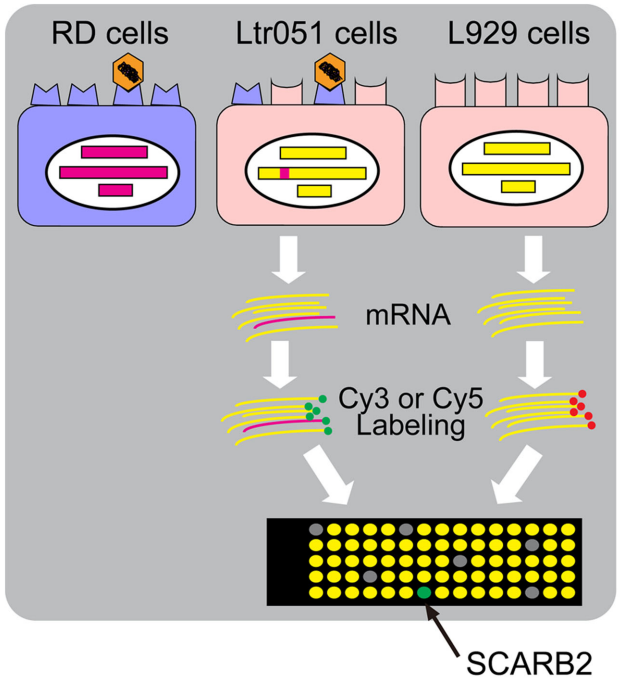

FIGURE 1 | The strategy to identify the human EV71 receptor, SCARB2. Mouse L929 cells became susceptible to EV71 when a human genomic DNA (red) was integrated into the genome (Ltr051 cells). RNA was prepared from Ltr051 cells and parental L929 cells and labeled with Cy3 or Cy5, respectively. The labeled RNAs were competitively hybridized to a microarray for human transcriptome analysis. The RNA species that were expressed in the Ltr051 cells but not in L929 cells were selected as candidates for the receptor mRNA. Each candidate was expressed in mouse L929 cells to examine the EV71 susceptibility of the transfected cells. SCARB2 expression alone was sufficient to achieve susceptibility.

The other cell line, Ltr246, is susceptible to a subset of EV71 strains but not all strains. Despite all of the efforts undertaken to identify the gene that is integrated in this cell line and that supports EV71 infection, this gene has not yet been identified. Other research groups have proposed that molecules act as receptors or other entities to enhance EV71 infection (see Mini Review by Nishimura and Shimizu, submitted). However, infection mediated by P-selectin glycoprotein ligand-1 (PSGL-1) or by an unknown molecule expressed in Ltr246 cells is not as efficient as infection mediated by SCARB2. Infection with EV71 in L929 cells expressing PSGL-1 was successful only for a subset of EV71 strains (Miyamura et al., 2011). SCARB2 seems to play the most important role in EV71 infection *in vitro* and *in vivo* because SCARB2 serves as a receptor for all EV71 strains and is expressed in the sites of EV71 replication *in vivo*.

STRUCTURE AND FUNCTION OF SCARB2

Scavenger receptor class B, member 2 (also known as Lysosomal Integral Membrane Protein II, LGP85 or CD36b like-2) is composed of 478 amino acids and belongs to the CD36 family, which includes CD36 and scavenger receptor B, member 1 (SR-BI and its splicing variant SR-BII; Calvo et al., 1995; Eskelinen et al., 2003). SCARB2 is one of the most abundant proteins in the lysosomal membrane and participates in membrane transportation and the reorganization of the endosomal/lysosomal compartment (Kuronita et al., 2002; Eskelinen et al., 2003; Blanz et al., 2010). SCARB2 shuttles between these compartments and the plasma membrane (**Figure 2B**, left; Eskelinen et al., 2003). SCARB2 is a type III double-transmembrane protein with a large extracellular domain (when it is present at the cell surface) and short cytoplasmic domains at the amino- and carboxy-terminus (**Figure 2A**; Calvo et al., 1995). SCARB2 is expressed in a variety of tissues, including neurons in the CNS. SCARB2 deficiency in mice causes ureteric pelvic junction obstruction, deafness, and peripheral neuropathy, and SCARB2 deficiency in humans causes action myoclonus–renal failure syndrome (AMRF; Gamp et al., 2003; Berkovic et al., 2008).

Mouse SCARB2 exhibits 85.8% amino acid identity with human SCARB2 (**Figure 2A**). Using chimeric mutants that include human and mouse SCARB2 sequences, Yamayoshi and Koike (2011) mapped the region that was important for efficient EV71 binding and infection. L929 cells expressing chimeras that carried amino acids 142–204 from the human sequence were susceptible to EV71, whereas chimeras that carried the mouse sequence in this region were not susceptible. It was shown that this region is also critical for binding to the virion. This region of the SCARB2 protein exhibits 76.2% amino acid identity between the human and mouse sequences. Removal of the carbohydrate moiety of the recombinant soluble SCARB2 protein by PNGase F treatment did not abolish the binding of the virus to the receptor, suggesting that the protein moiety of human SCARB2 plays a critical role in binding. Recently, Chen et al. (2012) identified critical residues required for human SCARB2 binding to EV71, which was comprised of residues 144–151 in a highly variable region among species. On the viral proteins, they showed that amino acids lined on the wall of the canyon (the EF loop of VP1) were important for SCARB2 binding and viral infectivity (Chen et al., 2012). To elucidate the mode of interaction of EV71 and SCARB2 more precisely, crystallographic analysis will be needed.

MECHANISM OF EV71 INFECTION IN RD CELLS

Hussain et al. (2011) have performed a screen of host factors required for EV71 entry into RD cells using an siRNA library. They found that the repression of genes associated with clathrin-mediated endocytosis, including AP2A1, ARRB1, CLTC, CLTCL1, SYNJ1, ARPC5, PAK1, ROCK1, and WASF1, resulted in significant inhibition of EV71 infection. They observed both co-localization of EV71 with clathrin using an immunofluorescence assay and virions in clathrin-coated pits by electron microscopy. EV71 entry into cells was inhibited when a dominant-negative mutant of Eps15, which binds to AP-2, was expressed and when cells were treated with drugs that selectively inhibit clathrin-dependent endocytosis (chlorpromazine and cytochalasin B). Entry was not inhibited by drugs that inhibit caveolae-dependent endocytosis and macropinocytosis. Hussain et al. also showed that EV71 infection was abolished when cells were treated with drugs that inhibit acidification of the endosome (Bafilomycin A1 and concanamycin A). Taken together, these results suggest that the mechanism of EV71 infection is that which is summarized in **Figure 2B** (right).

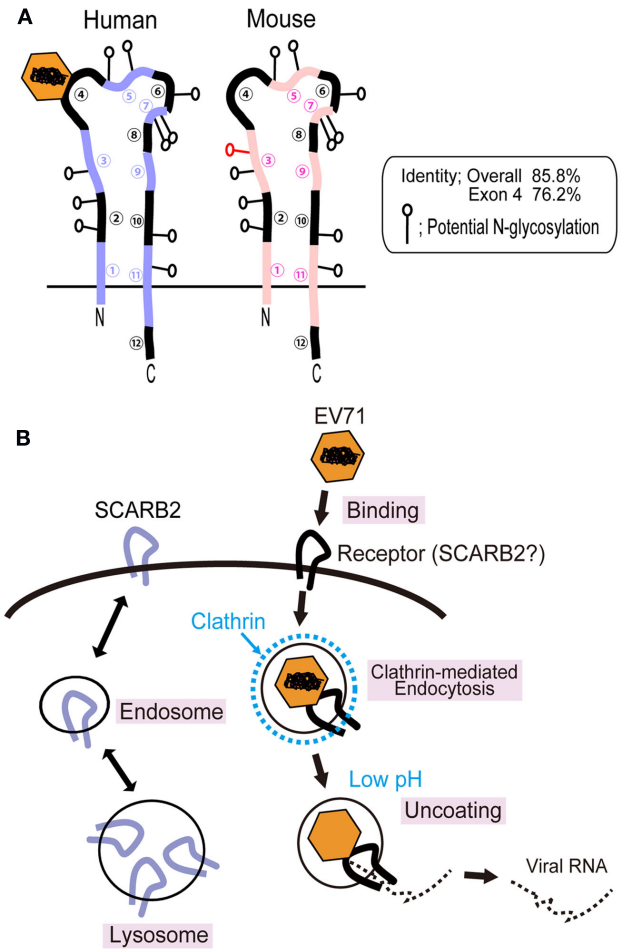

FIGURE 2 | Scavenger receptor class B, member 2 and EV71 infection in RD cells. (A) Comparison of the structures of human and mouse SCARB2. The human and mouse SCARB2 proteins are type III transmembrane proteins consisting of 478 amino acids. The amino acid identity of human SCARB2 to mouse SCARB2 is 85.8%. Because mouse SCARB2 does not serve an efficient receptor, it is possible to identify the virus binding site using chimeric receptors including mouse and human sequences. The 63-amino-acid region that is encoded in exon 4 of the human *SCARB2* gene plays an essential role in EV71 binding and infection. **(B)** Mechanism of EV71 infection in RD cells. The SCARB2 protein is located in the lysosomal and endosomal compartments and shuttles to the plasma membrane. This protein is able to bind EV71 when present at the cell surface. Hussain et al. (2011) have shown that EV71 infection is dependent on the clathrin-mediated endocytosis pathway by knockdown of the expression of proteins that participate in clathrin-mediated endocytosis. EV71 infection is also inhibited when cells are treated with drugs that block acidification of the endosome.

EV71 infection has been shown to be sensitive to the disruption of clathrin-mediated endocytosis and the acidic endosomal pH. Infection of RD cells with EV71 is largely blocked by anti-SCARB2 antibody or soluble SCARB2 (Yamayoshi et al., 2009), suggesting that the SCARB2-dependent pathway is the main pathway of EV71 infection in RD cells. SCARB2 is also known to be internalized by clathrin-dependent endocytosis (Le Borgne et al., 2001; Rodionov et al., 2002). The evidence has not directly shown that SCARB2 is involved in this pathway. The involvement of SCARB2 in this pathway should be confirmed experimentally.

It is known that the poliovirus receptor (CD155) and the major group rhinovirus receptor (intercellular adhesion molecule-1) are able to bind poliovirus and rhinovirus, respectively, and induce a conformational change that leads to the uncoating of the viral genome. Chen et al. (2012) reported that SCARB2, but not PSGL-1, induced a conformational change from native 160S virions to 135S particles. The change was enhanced under an acidic environment (pH 5.6; Chen et al., 2012). It should be further determined whether SCARB2 induces a conformational change to empty capsids and whether VP4 is released during SCARB2-mediated conformational change.

ANIMAL MODEL OF EV71 INFECTION

To study the neuropathogenicity of EV71, experiments using live animals are essential. The most reliable animal model is the monkey model (Hashimoto et al., 1978; Chumakov et al., 1979b; Hashimoto and Hagiwara, 1982; Hagiwara et al., 1983, 1984; Nagata et al., 2002, 2004; Arita et al., 2005, 2007; Zhang et al., 2011) because the species barrier caused by receptor differences is not a critical problem. The localization patterns of EV71-induced lesions in monkeys after intraspinal and intravenous inoculation were highly consistent with those observed in humans with severe EV71 encephalitis at autopsy (Lum et al., 1998; Wang et al., 1999; Chan et al., 2000; Shieh et al., 2001). Infected monkeys showed acute flaccid paralysis, which is a sign of involvement of the pyramidal tract, and tremor and ataxia, which are signs of involvement of the extrapyramidal tract. Histopathological changes were observed in the cerebellar and pontine vestibular nuclei, and in the spinal cord. Although the monkey is an excellent model for study of EV71 neuropathogenicity, monkey models have disadvantages with respect to handling, ethics, and cost. Experiments to identify the virulence determinants in the EV71 genome are limited to one report (Arita et al., 2005).

EV71, like other CVAs, is able to infect suckling mice. Some investigators use the suckling mouse model (Chumakov et al., 1979b; Chen et al., 2004; Ong et al., 2008). A problem with this system is that mice lose susceptibility to EV71 as they age (Yu et al., 2000; Chua et al., 2008). To circumvent this problem, some research groups have isolated mouse-adapted EV71 (Wang et al., 2004, 2011; Chua et al., 2008). Khong et al. (2012) found that 2-week-old mice deficient in type I and type II interferon receptors are susceptible to EV71 strains that were not artificially adapted to mice. Because mouse SCARB2 and PSGL-1 do not function as EV71 receptors in mice (Nishimura et al., 2009; Yamayoshi et al., 2009; Yamayoshi and Koike, 2011), EV71 infection in suckling mice is mediated by an unknown mechanism distinct from the SCARB2- and PSGL-1-mediated mechanisms. Indeed, EV71 exhibits different tissue tropism in suckling mice than in humans. In addition to infecting the CNS, EV71 replicates efficiently in the muscle of mice, unlike in humans (Chen et al., 2004; Wang et al., 2004; Chua et al., 2008; Ong et al., 2008).

In poliovirus, critical nucleotides or amino acids that influence the neurovirulence have been reported (Evans et al., 1985; Omata et al., 1986; Kawamura et al., 1989; Westrop et al., 1989). To identify such neurovirulence determinants in EV71, attempts were made

to determine nucleotide changes that influence the virulence level in suckling mice (Arita et al., 2008; Chua et al., 2008; Li et al., 2011; Huang et al., 2012). Some virulence determinants were mapped to the capsid region. Because the viral capsid is involved in the binding to the receptor and in other steps of infection, such as uncoating and stabilization of the virion, it is difficult to claim that amino acid changes in the capsid region are responsible for adaptation or virulence.

One of the strategies that can be used to overcome the problems discussed above is to generate transgenic mice that express the human EV71 receptor(s). Transgenic mice expressing human PVR, CD155, are susceptible to poliovirus and are used for the study of poliovirus pathogenicity (Ren et al., 1990; Koike et al., 1991; Horie et al., 1994; Abe et al., 1995). To this end, transgenic mice that express human PSGL-1 driven by the CMV promoter were generated (Liu et al., 2011). The expression of human PSGL-1 by this method was not sufficient to cause disease. It is obvious that the CMV promoter does not mimic the native PSGL-1 promoter in humans. Transgenic mice that express PSGL-1 with a distribution identical to that in human tissues are desirable. It seems that human SCARB2 expression in mice is necessary for the development of disease. A transgenic mouse model that develops disease that resembles the severe neurological diseases observed in humans will greatly contribute to the study of EV71 pathogenicity.

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Cellular receptors for human enterovirus species A

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Human enterovirus species A (HEV-A) is one of the four species of HEV in the genus *Enterovirus* in the family *Picornaviridae*. Among HEV-A, coxsackievirus A16 (CVA16) and enterovirus 71 (EV71) are the major causative agents of hand, foot, and mouth disease (HFMD). Some other types of HEV-A are commonly associated with herpangina. Although HFMD and herpangina due to HEV-A are common febrile diseases among infants and children, EV71 can cause various neurological diseases, such as aseptic meningitis and fatal encephalitis. Recently, two human transmembrane proteins, P-selectin glycoprotein ligand-1 (PSGL-1) and scavenger receptor class B, member 2 (SCARB2), were identified as functional receptors for EV71 and CVA16. In *in vitro* infection experiments using the prototype HEV-A strains, PSGL-1 and SCARB2 could be responsible for the specific receptors for EV71 and CVA16. However, the involvement of both receptors in the *in vitro* and *in vivo* infections of clinical isolates of HEV-A has not been clarified yet. To elucidate a diverse array of the clinical outcome of HEV-A-associated diseases, the identification and characterization of HEV-A receptors may provide useful information in understanding the HEV-A pathogenesis at a molecular level.

Keywords: human enterovirus species A, enterovirus 71, receptor, PSGL-1, SCARB2

INTRODUCTION

The genus *Enterovirus* within family *Picornaviridae*, non-enveloped viruses with a single-stranded RNA genome of positive polarity, is comprised of more than 100 serotypes (Pallansch and Roos, 2007). Human enteroviruses (HEVs) are presently classified into four species, HEV species A, B, C, and D (HEV-A, B, C, and D). At present, coxsackievirus A2 (CVA2), CVA3, CVA4, CVA5, CVA6, CVA7, CVA8, CVA10, CVA12, CVA14, CVA16, enterovirus 71 (EV71), EV76, EV89, EV90, EV91, and EV92 have been classified as HEV-A (Oberste et al., 2004, 2005, 2008). In addition, four types of simian enteroviruses are classified as HEV-A (Oberste et al., 2007). Although most enterovirus infections are asymptomatic, particular clinical manifestations are associated with specific types of enteroviruses (Pallansch and Roos, 2007). CVA2, CVA4, CVA5, CVA6, and CVA10 are commonly associated with herpangina. EV71 and CVA16 are major causative agents of hand, foot, and mouth disease (HFMD), a common febrile disease occurring mainly in young children, characterized by skin rash involving palms and soles, and ulcers on oral mucosa. Recently, HFMD outbreaks mainly due to CVA6 have been reported (Fujimoto et al., 2012). Clinical manifestations of HFMD caused by EV71, CVA6, and CVA16 are usually mild and self-limited. However, EV71 infection causes a diverse range of neurological diseases, such as aseptic meningitis, acute flaccid paralysis, brainstem encephalitis, and neurogenic pulmonary edema, and may result in long-term neurological sequelae, mainly in infants and young children (Alexander et al., 1994; McMinn, 2002; Modlin, 2007).

Recently, two human transmembrane proteins, P-selectin glycoprotein ligand-1 (PSGL-1; Nishimura et al., 2009) and scavenger receptor class B, member 2 (SCARB2; Yamayoshi et al., 2009), were identified as functional receptors for EV71 and CVA16 (Patel and Bergelson, 2009). In addition, annexin II (Yang et al., 2011), sialic acid (SA; Yang et al., 2009), and dendritic cell (DC)-specific ICAM3-grabbing non-integrin (DC-SIGN; Lin et al., 2009b) were found to be cellular factors involved in the early stages of EV71 infection. This review summarizes our current understanding of the EV71/CVA16 receptors and their role in HEV-A infection.

P-SELECTIN GLYCOPROTEIN LIGAND-1

Patients with severe EV71-associated encephalitis and neurological pulmonary edema showed a significant depletion of T cells and high levels of proinflammatory cytokines (Lin et al., 2003; Wang et al., 2003), suggesting the possible involvement of lymphocytes in EV71 infection and the immunopathogenesis. Therefore, we generated a cDNA library from Jurkat T cells and used it for expression cloning to identify a receptor on lymphocytes that specifically binds to EV71. Finally we identified PSGL-1 as a functional EV71 receptor on Jurkat T cells (Nishimura et al., 2009).

P-selectin glycoprotein ligand-1 is a sialomucin leukocyte membrane protein expressed as a homodimer of disulfide-linked subunits and it can bind to three different selectins (P, E, and L; Sako et al., 1993; Laszik et al., 1996; Somers et al., 2000). The tissue distribution of PSGL-1 is restricted to myeloid, lymphoid, and dendritic lineages, and platelets. PSGL-1 is also expressed on DCs of lymph nodes and macrophages in the intestinal mucosa (Laszik et al., 1996), which could be the primary sites of EV71 replication. PSGL-1 plays critical roles in the tethering and rolling of leukocytes for the recruitment of cells from blood vessels to the sites of acute inflammation upon stimulation by infection.

We found that some representative EV71 strains bind to PSGL-1 but other strains did not (Nishimura et al., 2009). According to their PSGL-1 binding capability, we classified the EV71 isolates as PSGL-1 binding strains (EV71-PB) and PSGL-1-non-binding strains (EV71-non-PB). The replication of EV71-PB in Jurkat T

cells was inhibited by anti-PSGL-1 monoclonal antibody (KPL1), indicating that EV71-PB replicated in Jurkat cells in a PSGL-1-dependent manner. On the other hand, EV71 replicated in non-leukocyte cells (such as RD cells) expressing little or no PSGL-1, and the replication was not affected by KPL1. Therefore we conclude that EV71 does not use PSGL-1 as the major cellular receptor on RD cells and other receptor(s), including SCARB2 or annexin II, may be responsible for EV71 infection in non-leukocyte cells expressing little or no PSGL-1.

Post-translational modifications of the N-terminal region of PSGL-1 contribute the efficient binding to selectins and chemokines (Pouyani and Seed, 1995; Sako et al., 1995; Wilkins et al., 1995; Liu et al., 1998; Hirata et al., 2004). In this region, there are a potential *O*-glycosylation residue (T57) and three potential tyrosine sulfation sites (Y46, Y48, and Y51). We demonstrated that tyrosine sulfation, not *O*-glycosylation, of the N-terminal region of PSGL-1 facilitates its binding to EV71-PB and viral replication in Jurkat T cells (Nishimura et al., 2010).

Coxsackievirus A16 is genetically and antigenically related to EV71 and is a major causative agent of HFMD as well as EV71 (Oberste et al., 2004). The inoculation of L-PSGL-1.1 cells, mouse L929 cells stably expressing human PSGL-1, with the prototype CVA16-G-10 strain induced faint cytopathic effects (CPE) (Nishimura et al., 2009). The replication of CVA16-G-10 was partially inhibited by KPL1 in L-PSGL-1.1 cells. This result indicated that the prototype CVA16 strain may use human PSGL-1 and another unidentified receptor(s) to infect L-PSGL-1.1 cells. CVA16-G-10 replication in Jurkat cells was not apparently inhibited by KPL1 (Nishimura et al., 2009), but significantly inhibited by a sulfation inhibitor, sodium chlorate (Nishimura et al., 2010). Therefore some sulfated molecules other than PSGL-1 might be involved in the replication of CVA16 in Jurkat cells. CVA16-G-10 would use unidentified receptor(s) to infect Jurkat T cells (Nishimura et al., 2009; Patel and Bergelson, 2009).

To investigate the PSGL-1-dependent replication phenotype of HEV-A, we tested the replication of 10 prototype HEV-A strains in L-PSGL-1.1 cells in the presence or absence of KPL1 (**Figure 1A**). On day 6 post-inoculation, there was no significant replication of CVA4, CVA5, CVA6, or CVA8 in L-bsd cells (blasticidin-resistant control L929 cells) or L-PSGL-1.1 cells. Although higher viral titers were found for CVA2 and CVA7 in L-PSGL-1.1 cells compared with those in L-bsd cells, replication was not affected by KPL1. These results suggest that CVA2 and CVA7 may infect to L-PSGL-1.1 cells in an alternative pathway via PSGL-1 or glycosylated PSGL-1, without the interaction between EV71-PB and the N-terminal region of PSGL-1 recognized by KPL1. We could not demonstrate any PSGL-1-dependent replication of the CVA3, CVA10, CVA12, and CVA14 strains in L-PSGL-1.1 cells, because they replicated even in PSGL-1 negative L-bsd cells as previously reported for certain HEV-A field isolates (Nadkarni and Deshpande, 2003; Yamayoshi et al., 2009).

Coxsackievirus A7 and CVA14 infection induced CPE in L-PSGL-1.1 cells, but not in L-bsd cells (**Table 1**). On the other hand, CVA7 and CVA14 induced CPE in L-Empty cells (puromycin-resistant control L929 cells; **Table 1**; Yamayoshi et al., 2009). The difference in the CPE induction by some HEV-A strains might be due to the maintenance or cultivation conditions of the mouse L929-derived cells regardless of the receptor expression of PSGL-1 or SCARB2. Some strains of HEV-A are able to infect mouse L929 cells regardless of expression of PSGL-1 or SCARB2 (Nadkarni and Deshpande, 2003; Yamayoshi et al., 2009). It is therefore impossible to determine receptor usage of HEV-A by simply investigating the susceptibility of mouse L929 cells expressing the putative cellular receptor. Receptor usage of HEV-A should be determined carefully by showing several lines of evidence such as acquisition of susceptibility by expressing a putative receptor in non-susceptible cells, loss of susceptibility by knocking down of the receptor in susceptible cells, and direct binding of the virus to the receptor, etc.

L-PSGL-1.1 cells did not support PSGL-1-dependent replication of the HEV-B and HEV-C strains (**Figure 1B**); however, the prototype EV70 strain (HEV-D) replicated in L-PSGL-1.1 cells more efficiently than in L-bsd cells. Although EV70 replication was not affected by KPL1 (**Figure 1B**), we cannot exclude the possibility that EV70 utilizes α2,3-linked SA, which could be a receptor for EV70 (Nokhbeh et al., 2005).

Recently we reported that that four out of five EV71-PB strains replicated poorly in L-PSGL-1.1 cells (Miyamura et al., 2011). We found that EV71 variants, which were propagated once in L-PSGL-1.1 cells, have several possible adaptive mutations, including a putative amino acid determinant of the adaptive phenotype in L-PSGL-1.1 cells at VP2-149 (Miyamura et al., 2011). The results suggest that adaptive mutations, along with a PB phenotype, may facilitate efficient PSGL-1-dependent replication of the EV71 variants in L-PSGL-1.1 cells. It is possible that HEV-A strains other than EV71 also require adaptive mutations for efficient replication in L-PSGL-1.1 cells.

SCAVENGER RECEPTOR CLASS B, MEMBER 2

Yamayoshi et al. (2009) identified SCARB2 (also known as lysosomal integral membrane protein II, or CD36b like-2) as an EV71 receptor on RD cells, widely used for isolation of EV71 from clinical specimens. They transfected EV71-non-susceptible mouse L929 cells with the genomic DNA of RD cells and selected two cell clones that were susceptible for EV71 infection. By a transcriptome analysis, SCARB2 was identified as an EV71 receptor on RD cells.

Scavenger receptor class B, member 2 is a heavily *N*-glycosylated type III transmembrane protein consists from 478 amino acids and belongs to the CD36 family of scavenger receptor proteins (Fujita et al., 1992; Calvo et al., 1995). SCARB2 has a N-terminal transmembrane domain, a ∼400 amino acid lumeral domain, a C-terminal transmembrane domain, and a C-terminal cytoplasmic tail of ∼20 amino acids (Fujita et al., 1992). SCARB2 involves in an enlargement of early endosomes and late endosomes/lysosomes and an impairment of endocytic membrane out of the enlarged compartments (Kuronita et al., 2002). SCARB2 deficiency caused ureteric pelvic junction obstruction, deafness, and peripheral neuropathy in mice (Gamp et al., 2003). SCARB2 is expressed ubiquitously in human tissues (Eskelinen et al., 2003); therefore, it might be involved in systemic EV71 infections (Yamayoshi et al., 2009).

Human SCARB2 has 10 potential *N*-glycosylation sites (Fujita et al., 1992). But the carbohydrate chains of human SCARB2 are not essential for the interaction between EV71 and human SCARB2 (Yamayoshi and Koike, 2011). Experiments using a

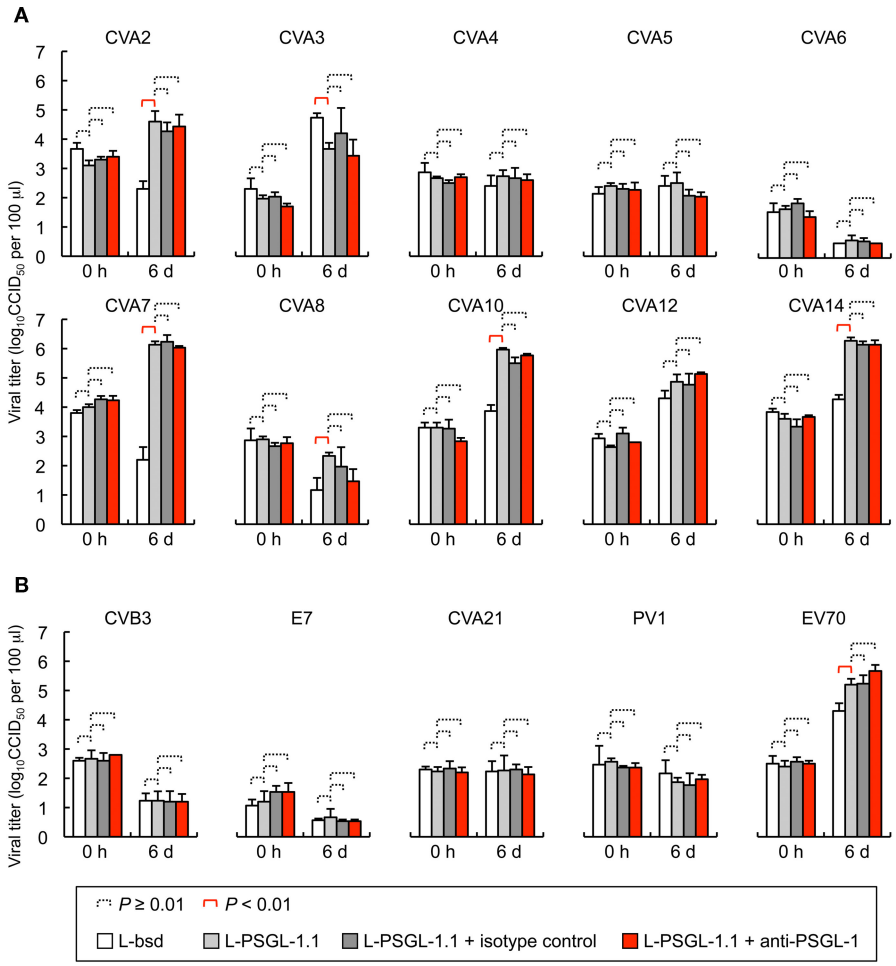

FIGURE 1 | Human enteroviruses replication in L-PSGL-1.1 cells. (A) Replication of the HEV-A strains (**Table 1**) in L-PSGL-1.1 cells in the presence or absence of anti-PSGL-1 mAb (KPL1) or an isotype control. Cells were inoculated with viruses at 10 $CCID_{50}$/cell for 1 h, washed, and incubated in the medium, as described previously (Nishimura et al., 2009). Cells were incubated at 34˚C. For mAb inhibition, the cells were pretreated with 10 µg/ml mAb for 1 h, washed, and maintained in the medium with 10 µg/ml mAb. At the indicated time [just after infection (0 h) and 6 days postinfection (6 d)], the infected cells and supernatants were freeze-thawed and viral titers were determined by $CCID_{50}$ titration using RD cells. The titers are expressed as the mean and error bars indicate SD of triplicate analyses. The mean viral titers were compared using Student's *t*-test. *P* values < 0.01 were considered statistically significant. **(B)** Viral replication of HEV-B, C, and D in L-PSGL-1.1 cells. Replication of two HEV-B [CVB3-Nancy and echovirus 7 (E7)-Wallace] and two HEV-C strains [CVA21-Coe and poliovirus 1 (PV1)-Sabin 1], and one HEV-D (EV70-J670/71) strains in L-PSGL-1.1 cells in the presence or absence of KPL1 or an isotype control. The titers are expressed as the mean and error bars indicate SD of triplicate analyses. The mean viral titers were compared using Student's *t*-test. *P* values < 0.01 were considered statistically significant.

series of chimeric proteins between human and mouse SCARB2 identified that the amino acids 142–204 of human SCARB2 (encoded by human *SCARB2* exon 4) are responsible for EV71 binding and infection (Yamayoshi and Koike, 2011).

Mouse L929 cells expressing human SCARB2 in the presence of puromycin (L-SCARB2 cells) permitted the replication of all EV71 strains tested, including the non-PB strains (Yamayoshi et al., 2009). CVA16 induced CPE in L-SCARB2 cells, whereas CVA2, CVA3, CVA4, CVA5, CVA6, CVA8, and CVA12 did not. CVA16 grew efficiently in L-SCARB2, whereas CVA2, CVA3, CVA4, CVA5, CVA6, CVA8, and CVA12 did not (**Table 1**). Yamayoshi et al. (2009) concluded that CVA16 also infect L-SCARB2 cells in a SCARB2-dependent manner and that infection with most other HEV-A is not dependent upon SCARB2. CVA7, CVA10, and CVA14 induced CPE in both L-Empty cells and L-SCARB2 cells (Yamayoshi et al., 2009). They could not determine whether the CPE induced by these viruses were due to human SCARB2–mediated infection.

ANNEXIN II

Yang et al. (2011) identified annexin II as an EV71 VP1-binding protein on RD cells. Using a recombinant VP1 protein of EV71 fused with a calmodulin-binding peptide, they tried to identify VP1-binding proteins from the total cellular proteins of RD cells. A virus-overlay protein-binding assay followed by a mass spectrometry analysis identified annexin II as a VP1-binding protein.

Annexin II is a member of the annexin family – the multifunctional phospholipid-binding proteins. Annexin II on the surface of endothelial cells acts as a profibrinolytic coreceptor for both

Table 1 | Induction of CPE by the HEV-A strains.

Serotype	Strain	Accession No.	L-bsd[1]	L-PSGL-1.1	L-Empty[2,3]	L-SCARB2[3]
CVA2	Fleetwood	AY421760	–	–	–	–
CVA3	Olson	AY421761	–	–	–	–
CVA4	JR[4]	AB457644	–	–	–	–
CVA5	Swartz	AY421763	–	–	–	–
CVA6	Gdula	AY421764	–	–	–	–
CVA7	Parker	AY421765	–	+	+	+
CVA8	Donovan	AY421766	–	–	–	–
CVA10	Kowalik	AY421767	+	+	+	+
CVA12	Texas-12	AY421768	–	–	–	–
CVA14	G-14	AY421769	–	+	+	+
CVA16	G-10	U05876	–	+	–	+

[1] *Blasticidin-resistant L929 cells (a negative control for L-PSGL-1.1 cells).*

[2] *Puromycin-resistant L929 cells (a negative control for L-SCARB2 cells).*

[3] *Yamayoshi et al. (2009).*

[4] *Prototype CVA4 strain (high point) is unavailable from ATCC, therefore we used an in-house reference strain of CVA4, the JR strain.*

plasminogen and tissue plasminogen activator facilitating the generation of plasmin (Kim and Hajjar, 2002). The interaction to annexin II was specific to EV71; CVA16 did not bind to annexin II in the virus-overlay protein-binding assay (Yang et al., 2011).

SIALIC ACID

Sialic acid is usually found as terminal monosaccharides on the glycan chains of glycolipids and glycoproteins (Varki and Varki, 2007). Coxsackievirus A24 variant (CVA24v) uses SA-containing glycoconjugates as attachment receptors on corneal cells (Nilsson et al., 2008). Yang et al. (2009) hypothesized that SA would be important for EV71 infection, as the transmission route of EV71 and CVA24v is fecal-oral and/or droplet-aerosol route. EV71 infection to DLD-1 intestinal cells was inhibited by an *O*-glycan synthesis inhibitor, but not by an *N*-glycan synthesis inhibitor. Sialidase treatment decreased EV71 replication in DLD-1 cells. Furthermore, DLD-1 cells co-cultured with SA-linked galactose significantly reduced the EV71 infection. Thus Yang et al. (2009) concluded that SA-linked glycans are EV71 receptors on DLD-1 cells. Recently, Neu5Acα2,3Gal disaccharides on PSGL-1 were reported as a candidate receptor of CVA24v (Mistry et al., 2011). It is unknown whether other enteroviruses, including HEV-A, recognize SA-containing glycans as the entry receptors.

DENDRITIC CELL-SPECIFIC ICAM3-GRABBING NON-INTEGRIN

Dendritic cells play crucial roles in antiviral immunity by functioning as professional antigen-presenting cells to prime T cells and by secreting cytokines to modulate immune responses. In a mouse model of EV71 infection, DCs from the brains of EV71-infected, but not of uninfected, mice expressed viral antigen and primed T cells efficiently (Lin et al., 2009a). Lin et al. (2009b) reported that EV71 infection enhances mouse DCs to elicit protective immune response and also found that EV71 infects human immature DCs and that viral entry is partially inhibited by anti-DC-SIGN antibody. However, the direct interaction between EV71 and DC-SIGN is still unclear. It is essential to characterize the role of DC-SIGN and other receptors for EV71 in DCs for understanding the host immunological responses and immunopathogenesis of HEV-A including EV71.

CONCLUSION

Identification of PSGL-1 and SCARB2 as the cellular receptors for EV71 and CVA16 has advanced our understanding of the early stages of HEV-A infections at the molecular level. However, further experiments using clinical HEV-A isolates are necessary to clarify the general role of PSGL-1 and SCARB2 in HEV-A infection and their pathogenesis. Most of the prototype (laboratory-adapted) HEV-A strains other than EV71 and CVA16 may use unidentified receptor(s) to infect susceptible human cells such as RD cells. Characterization of the identified and unidentified HEV-A receptors is essential to understand the mechanism of HEV-A infection and development of a diverse array of the clinical outcomes of HEV-A-associated diseases.

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Mouse hepatitis virus receptor as a determinant of the mouse susceptibility to MHV infection

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In this review, we report that the receptor of mouse hepatitis virus (MHV), carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), is an important determinant of mouse susceptibility to MHV infection. This finding was revealed by using mouse strains with two different allelic forms of the MHV receptor, *Ceacam1a* and *Ceacam1b*. Although previous studies indicated that susceptibility is determined by a single gene, *Ceacam1*, our recent work in gene-replaced mice with chimeric *Ceacam1* pointed toward the involvement of other host factors (genes) in the susceptibility. Studies on mouse susceptibility to MHV, as well as the factors involved in their susceptibility, are overviewed.

Keywords: MHV, coronavirus, receptor, MHV receptor, mouse, MHV resistant gene, gene-replacement

INTRODUCTION

Mouse hepatitis virus (MHV) is a murine coronavirus which causes a wide range of diseases in the mouse and rat, including hepatitis, enteritis, respiratory diseases, and encephalomyelitis in the central nervous system (Wege et al., 1982). There are also great differences in tissue tropism and virulence among various strains and isolates of MHV (Hirano et al., 1981; Wege et al., 1982). Complex factors, such as age and immunological status of the host animal, dose, and route of virus inoculation, affect disease outcomes (Taguchi et al., 1977; Hirano et al., 1981). Naturally occurring cases of MHV infections in mouse colonies are generally enteric infections that are not highly pathogenic to immunocompetent adult mice, while virus infections are mostly of a persistent nature (Ishida et al., 1978; Homberger, 1997). Viruses shed in the feces from persistently infected mice are a source of acute and chronic fatal diseases encountered in mice prone to MHV infection, e.g., diarrhea of suckling mice and wasting syndrome in immunodeficient nude mice (Hirano et al., 1975; Tamura et al., 1977; Ishida et al., 1978). Thus, the asymptomatic infection of adult mice is a major problem in animal facilities where MHV-free mice are used for experiments and evokes serious problems in terms of maintaining animals of sufficient quality to allow scientists to perform experiments with high reproducibility and reliability.

It is well known that there is a difference in susceptibility to MHV infection among mouse strains. A number of works have reported differing susceptibilities to MHV infection among mouse strains from 1960 to the present (Bang and Warwick, 1960; Taguchi et al., 1976; Stohlman and Frelinger, 1978; Knobler et al., 1981). Bang and Warwick (1960) showed that the C3H inbred strain was resistant to MHV-2 infection, while Princeton outbred mice were deemed susceptible. They further showed that susceptibility was determined by a single gene, expressed in macrophages (Bang and Warwick, 1960). Stohlman and Frelinger (1978) reported, using a neurotropic MHV, JHMV strain, that SJL mice are resistant, while others are susceptible. By cross and backcross studies, they indicated that susceptibility is dominant, as determined by a major gene and a gene marginally influencing its susceptibility (Stohlman and Frelinger, 1978). Thereafter, Smith et al. (1984) identified the locus of the gene that determines the susceptibility of mice to MHV infection to be on chromosome 7. From those findings, it was postulated that mouse susceptibility to MHV infection is dominant and determined mostly by a single gene located on chromosome 7. On the basis of those findings, Holmes and her colleagues identified an MHV receptor.

IDENTIFICATION OF MHV RECEPTOR

Holmes and co-workers tried to find a receptor protein by using susceptible BALB/c and resistant SJL/J mice (Boyle et al., 1987). They prepared cell membrane fractions from MHV target tissues, intestines, and liver, of those mice and showed, by using a viral overlay protein blot assay (whereby virions bind to the protein on a membrane filter prepared by Western blotting), that BALB/c mice express a molecule of 110–120 kDa on cell membranes to bind MHV virus particles. However, in the same fraction of SJL mice, they failed to find the protein that would bind MHV (Boyle et al., 1987; Williams et al., 1990). Accordingly, they speculated that the protein was a receptor for MHV and determines mouse susceptibility. Thereafter, they isolated the protein and analyzed the partial amino acid sequence of the protein, which suggested to them that the protein is similar to a carcinoembryonic antigen, CEA (Williams et al., 1991). They finally isolated a cDNA clone and identified the protein to be a biliary glycoprotein of the CEA family (Dveksler et al., 1991), now called carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1; Beauchemin et al., 1999). The expression of this protein in non-permissive cells converted them into cells that were permissive to MHV infection,

a finding that indicated this protein serves as an MHV receptor (Dveksler et al., 1991). These findings suggested to them that the susceptibility of the mouse to MHV infection is determined by the presence or absence of this molecule. Soon after these findings were published, they reported that the CEACAM1 counterpart is expressed in the SJL (Dveksler et al., 1993b); however, they also reported that the CEACAM1 counterpart found in SJL is also functional as a receptor for MHV when expressed in non-permissive cells, although the receptor functionality is less efficient compared with the CEACAM1 found in BALB/c (Dveksler et al., 1993b). Holmes and co-workers explained that the relatively small difference in receptor function between BALB/c CEACAM1 and SJL CEACAM1 could result in the very large biological differences in the multiple cycle infections that are required to cause disease in animals (Dveksler et al., 1993b). There is a slight difference in amino acid sequences between CEACAM1 found in most mouse strains and CEACAM1 expressed in SJL in the MHV-binding region (Rao et al., 1997; Beauchemin et al., 1999); these are allelic forms and called CEACAM1a for BALB/c type and CEACAM1b for SJL type (Beauchemin et al., 1999). In contrast to the hypothesis by Holmes and co-workers, Yokomori and Lai (1992) expressed CEACAM1a derived from C57BL/6 and CEACAM1b from SJL mice in MHV-non-permissive cells and found no significant difference in viral growth between cells expressing CEACAM1a and those with CEACAM1b, which may mean that the susceptibility difference observed between those two mouse strains may not be attributable to MHV receptor protein.

STRUCTURE AND MHV-BINDING ACTIVITY OF CEACAM1s

There are at least four different types of CEACAM1 isoforms derived by alternative splicing (Beauchemin et al., 1999). CEACAM1 belongs to an immunoglobulin (Ig) superfamily and is composed of an ectodomain, transmembrane domain (TM), and cytoplasmic tail (Cy). The ectodomain consists of four Ig constant region-like domains, i.e., the N, A1, B, and A2 domains from the N terminus of the molecule (Dveksler et al., 1991; Beauchemin et al., 1999). Alternative splicing generates the molecules with two ectodomains, composed of N and A2, while those four- and two-domain forms have either long or short Cy, which results in the four different forms of CEACAM1 (Beauchemin et al., 1999). Also as described above, there are two allelic forms in CEACAM1, 1a, and 1b, and, thus, at least eight totally different MHV receptor molecules exist (Beauchemin et al., 1999). Recently, some CEACAM1 molecules deleting TM and Cy were found in intestinal epithelium and intestinal secretions, and those neutralize MHV (Terahara et al., 2009).

The molecule on MHV virion that binds to the receptor is the spike (S) protein with ca. 180–200 kDa class I fusion glycoprotein (Bosch et al., 2003). One spike is composed of a trimer of the S protein. The S protein of most MHV is cleaved in the middle of the molecule, and the N terminal subunit is called the S1 and C terminal subunits S2, respectively (Sturman et al., 1985). N terminal 330 amino acids in S1 are responsible for receptor binding (Kubo et al., 1994), and S2 is critical for envelope–cell membrane fusion, namely, cell entry (Bosch et al., 2003).

The N domain of CEACAM1 alone is sufficient to bind MHV; a soluble form consisting of the N domain alone works efficiently to bind to MHV (Dveksler et al., 1993a; Miura et al., 2004). Also, the N domain alone can neutralize viruses and induce conformational change in the S protein. Moreover, the binding of the N domain to the S protein of MHV induces the fusion activation of the S protein (Taguchi and Matsuyama, 2002; Miura et al., 2004). However, when the N domain alone is expressed on the membrane (which has both TM and Cy), this molecule fails to work as a functional receptor (Dveksler et al., 1993a; Miura et al., 2004). This may be due to its short molecule that is buried among various cell surface molecules and thus fails to have a chance to attach to the virions (Dveksler et al., 1993a). We have compared the virus-binding activity of CEACAM1a and CEACAM1b. Soluble CEACAM1a and CEACAM1b composed of N and A2 domains prepared on nitrocellulose paper were allowed to attach to an MHV virion (viral protein blot assay). By this method, CEACAM1a binds to virions more than 300-fold efficiently when compared with CEACAM1b (Ohtsuka et al., 1996). We also examined the neutralization activity of the two soluble proteins, finding that, in this assay, CEACAM1a is highly reactive and neutralizes MHV more than 300-fold efficiently than does CEACAM1b. These results are in agreement with the observations of the Holmes lab (Boyle et al., 1987). However, when those proteins are expressed in MHV-non-permissive BHK cells, CEACAM1a exhibits only a 10- to 30-fold higher receptor function when compared with that of CEACAM1b, and, again, this finding is similar to those obtained by the Holmes lab (Boyle et al., 1987). Accordingly, from these findings, we concluded that the small difference in receptor function between CEACAM1a and CEACAM1b will be amplified after several viral growths, as also postulated by Holmes and her collaborators. As for the receptor binding site in the N domain of CEACAM1, Gallagher and co-workers reported six contiguous amino acids from 38 to 43 in the N domain different between CEACAM1a and CEACAM1b play an important role in the differences seen in receptor function (Rao et al., 1997). Others showed that amino acids 34–52 are involved in virus-binding (Wessner et al., 1998).

ANALYSIS OF THE ROLE OF CEACAM1 IN THE MHV SUSCEPTIBILITY OR RESISTANCE OF MICE

The above data suggested to us that CEACAM1 is an important factor to determine the susceptibility of mice and could be a product of the gene responsible for susceptibility mapped in a locus on chromosome 7 by Smith et al. (1984). This idea is strengthened by the fact that the MHV receptor *Ceacam1* gene is mapped to the same region on chromosome 7 (Robbins et al., 1991). If the CEACAM1 receptor is a determinant of susceptibility, and susceptibility is dominant over resistance, then mice with CEACAM1a/CEACAM1a and CEACAM1a/CEACAM1b are susceptible and only mice with a CEACAM1b/CEACAM1b phenotype, such as SJL mice, are resistant. To assess this possibility, we have mated BALB/c and SJL to generate F1 mice, and then we obtained mice backcrossed to SJL. We also produced F2 mice from BALB/c and SJL mice. By using these mice, we examined the relationship between the mouse genotype of *Ceacam1* and its susceptibility to MHV. Of more than 120 backcrossed and F2 mice, those with *Ceacam1a*/*Ceacam1a* and *Ceacam1a*/*Ceacam1b* were fully susceptible, while those with *Ceacam1b*/*Ceacam1b* were resistant (Ohtsuka and Taguchi, 1997). These experimental results are well in accordance with the hypothesis, i.e., that mouse susceptibility to MHV is determined by a receptor gene. We have also

studied the relationship between MHV susceptibility and CEACAM1 allelic forms by using wild mice (Ohtsuka et al., 2001). The study showed that most of subspecies of wild mice distributed worldwide express both or either CEACAM1a and CEACAM1b. Among those mice, CEACAM1a-expressing ones showed high susceptibility to MHV, while those with CEACAM1b were not highly susceptible, being in good agreement to the result and hypothesis obtained by mouse cross and backcross experiment shown above.

It has been reported in studies using gene-knockout mice that CEACAM1a is a critical factor for mouse susceptibility to MHV infection (Blau et al., 2001; Hemmila et al., 2004). Beauchemin and co-workers produced mice whose *Ceacam1* gene is partially disrupted or mice in which the *Ceacam1a* gene was deleted and showed that those mice showed a reduced susceptibility and no susceptibility to MHV infection, respectively (Blau et al., 2001; Hemmila et al., 2004). These findings clearly show that CEACAM1 is an important factor in determining the susceptibility to MHV. However, these studies did not explain the difference of susceptibility to MHV infection observed between BALB/c with CEACAM1a and SJL with CEACAM1b receptor protein.

ANALYSIS OF GENE-REPLACED MICE

If the differing susceptibilities between CEACAM1a-expressing mice and SJL with CEACAM1b are determined by CEACAM1, then MHV-susceptible mice with CEACAM1a are converted to SJL type-resistant mice, when the *Ceacam1a* mouse gene is replaced with a *Ceacam1b* gene. We have produced C57BL/6 (B6) mice whose original *Ceacam1a* gene is replaced by a *Ceacam1b* gene (Hirai et al., 2010). Since it has been revealed that the chimeric CEACAM1a, which is replaced with 1–70 amino acids of the N terminus of N domain with CEACAM1b counterpart, functioned as CEACAM1b, suggesting that there is a critical region in 1–70 amino acids to determine the difference of receptor functionality between CEACAM1a and CEACAM1b (Wessner et al., 1998). Thus, we produced B6 mice whose N terminal region (1–70 amino acids) is replaced by CEACAM1b (we call it CEACAM1ba) to see the mouse susceptibility is determined by CEACAM1. CEACAM1ba has two N-linked glycosylation sites in the N domain as CEACM1b, while CEACAM1a has three sites, showing that chimeric CEACAM1ba is more like CEACAM1b rather than CEACAM1a. Those gene-replaced mice expressed the chimeric protein in the tissues or cells where CEACAM1a and CEACAM1b were expressed in B6 and SJL mice, respectively. It was also revealed that there is no significant difference in the expression level of CEACACM1a and CEACAM1ba in B6 and chimeric B6 mice (Hirai et al., 2010). Additionally, the chimeric CEACAM1 did not react with the CEACAM1a-specific monoclonal antibody, CC1 as reported previously (Wessner et al., 1998). We then examined the susceptibility of mice having a chimeric *Ceacam1*. The gene-replaced mice, when compared to B6 mice, were resistant to a lethal dose of MHV-A59 infection, a finding similar to those in SJL mice that were resistant to the infection. However, when we examined virus growth in B6, SJL, and the gene-replaced mice, high titer of MHV were detected in the liver and other target tissues of B6 mice, while low levels of virus titer were recorded in SJL mice, findings which are similar to the results we have thus far obtained. Interestingly, no virus growth was detected in the mice having gene-replacement with chimeric CEACAM1, indicating the gene-replaced mice showed a much higher resistance to MHV infection than did the SJL mice. Since the virus-binding domain is derived from SJL, the chimeric mouse susceptibility should be similar to that in SJL mice, if our hypothesis is correct. However, at the moment, we have no convincing explanation on the difference in susceptibility of SJL and chimeric mice with identical MHV-binding site on CEACAM1.

Two other molecules, CEACAM2 (Nedellec et al., 1994) and pregnancy-specific glycoprotein (PSG; Chen et al., 1995), have so far been reported to work as MHV receptor. These proteins, however, seem unlikely to function as an MHV receptor in the mouse, since CEACAM1 knockout mice produced in Beauchemin lab showed complete resistance to MHV-A59 infection (Hemmila et al., 2004),. Moreover, as there are no substantial differences in amino acid sequences in those two receptor proteins expressed in B6 and SJL, there is little possibility that those proteins are involved in the differences of MHV susceptibility between SJL and chimeric mice. One point to be further studied is the effect of FVB Cre mouse that is used to exclude neomycin resistant gene from initially produced chimeric mouse (Hirai et al., 2010). To exclude the FVB mouse genes, we performed the backcross of neomycin deleted mouse to B6 mouse by 11 generations, however, some FVB mouse gene still remaining in backcrossed mice could have some unexpected effects on the chimeric mice. It is important to study whether any of FVB mouse genes are involved in the high resistance of chimeric mice.

We did observe that peritoneal macrophages from chimeric mice, which express chimeric CEACAM1ba, were resistant to MHV, while chimeric CEACAM1 expressed in cultured cell lines, such as BHK, showed MHV susceptibility, slightly lower than that in cells expressing CEACAM1b. From these findings, it could be postulated that some cellular factor(s) other than CEACAM1 could modify animal susceptibility. This idea appears to be in accordance with findings by Stohlman and Frelinger (1978), who postulated two factors to determine mouse strain differences in susceptibility to MHV infection. Studies to elucidate such factors are in progress.

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Cell receptors for influenza A viruses and the innate immune response

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The interaction of the hemagglutinin (HA) of the influenza A viruses (IAV) with the cell surface is a key factor for entry of the virus and productive infection of the cell. This glycoprotein has affinity for sialic acids (SA), and different strains present specificity for SA bound through α2,3 or α2,6 linkages to the underlying sugar chain, which is usually related with host and cell tropism. Nucleic acid recognizing receptors (mainly RIG-I and Toll-like receptors) are the most extensively studied pattern recognition receptors for IAV. However, due to the ability of the HA of avian, swine, or human influenza viruses to bind differently linked SA and also to the high levels and variability of glycosylations of their major virion glycoprotein components, HA and NA, IAV interacting proteins on the cell surface could also play an important role in initiating different signaling pathways to elicit the immune response in infected cells. But, at present, these processes are not well understood. In this mini-review we discuss how the interactions of IAV with cell surface receptors on immune cells might be important for the induction of specific innate immune responses and as a result, for pathogenicity in humans.

Keywords: influenza virus, receptors, sialic acids, innate immune response

INTRODUCTION

Influenza viruses are an important threat to human health and global economy, causing an annual average of 36,000 deaths and over 200,000 hospitalizations during the 1990s (CDC, 2010). In addition, due to the high frequency of mutations and recombination of their genome (antigenic drift and shift), and their airborne transmission, they have a high potential to become pandemic viruses. Influenza viruses belong to the family Orthomyxoviridae, and include the types, A, B, and C, from which influenza A viruses (IAV) are the responsible for most of the disease burden (Mubareka and Palese, 2011). IAV have a segmented negative stranded RNA genome, consisting on 8 segments that encode up to 12 proteins (Palese and Shaw, 2007; Wise et al., 2009).

The major glycoproteins present on the surface of the virion are the hemagglutinin (HA) and the neuraminidase (NA). To date, 16 HA and 9 NA subtypes have been described, and the combination of different subtypes of these proteins results in different subtypes of IAV (Mubareka and Palese, 2011). The main roles of the HA are to mediate the interaction of the viral particle with the cell components on the surface and to promote the fusion of the viral and endosomal membranes, leading to the release of the ribonucleoproteins (RNP) into the cytoplasm. The interaction of influenza viruses with their receptors is necessary for subsequent internalization of the virus. Some studies demonstrated that IAV enter the cell via clathrin mediated endocytosis, although under certain conditions they can also enter in a caveolin and clathrin independent manner (Lakadamyali et al., 2004, 2006; De Vries et al., 2011).

Here we review and discuss how the interaction of IAV with their receptors on the cell surface of immune cells, such as dendritic cells (DCs) and macrophages, might influence infection and promote specific innate immune responses, with important implications in the outcome of the disease, either promoting viral clearance or generating exacerbated local immune responses resulting in acute infection and increased pathogenesis.

RECEPTOR SPECIFICITY OF THE IAV

The circulation of IAV is species-specific, and therefore, transmission among different species is usually not observed. It is known that the receptor specificity has an important role in maintaining the host species barrier, although other proteins, like PB2, also need to be adapted for efficient infection of other hosts different than the original host (Almond, 1977; Neumann and Kawaoka, 2006). Sialic acids (SA, *N*-acetylneuraminic acid) are the primary receptors for virus attachment to cell surfaces, binding to a pocket at the distal tip of the HA of IAV (Weis et al., 1988). SA consist of nine carbon sugars frequently attached through α2,3 or α2,6 (SAα2,3 or SAα2,6) linkages to underlying sugar chains of glycoproteins in the cell membrane. It is known that avian and human viruses differ in their SA binding affinity, since HA from human isolates usually bind SAα2,6, while the avian isolates usually have affinity

Abbreviations: CLRs, C-type lectin receptors; DCs, dendritic cells; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin; EGFR, epidermal growth factor receptor; HA, hemagglutinin; HPAIV, high pathogenic influenza avian viruses; IAV, influenza A virus; NA, neuraminidase; NLRs, Nod-like receptors; PI3K, phosphatidylinositol 3-kinases; PRRs, pattern recognition receptors; RLRs, RIG-I-like receptors; RNP, ribonucleoprotein; RTK, receptor tyrosine kinases; SA, sialic acids; TLRs, Toll-like receptors.

for SAα2,3 (Rogers and Paulson, 1983). Related to this, human upper respiratory tract present mainly SAα2,6, which is believed to act as one of the main barriers for transmission of avian IAV to humans (Shinya et al., 2006; Van Riel et al., 2006; Nicholls et al., 2007).

High pathogenic avian influenza viruses (HPAIV) H5N1 are currently a pandemic threat, and almost 600 people have been reported to be infected as of November 15, 2011 (WHO, 2011), resulting in severe pneumonia with a lethal outcome in 60% of those reported cases. Humans infected with H5N1 present unusually high serum concentrations of chemokines and pro-inflammatory cytokines, which are thought to contribute to disease severity (Peiris et al., 2004; De Jong et al., 2006; Maines et al., 2008; Sirinonthanawech et al., 2011). Although no sustained human-to-human transmission has been established yet, possibly due to the low levels of SAα2,3 present in the human airways, HPAIV have been shown to be able to infect and replicate efficiently in cells in the lower region of the respiratory tract, including type II pneumocytes and alveolar macrophages, where the avian-like virus receptor is predominant (Shinya et al., 2006; Van Riel et al., 2006; Nicholls et al., 2007). Therefore, the receptor specificity of influenza viruses is important not only for the host tropism, but also for tissue tropism in humans, since it determines the airway region and cell types that support active viral replication, what might contribute to the severity of the disease observed in humans infected with HPAIV. Moreover, a recent work by Watanabe et al. (2011) showed that a single amino-acid change in the HA (D222G) of a H1N1 2009 pandemic IAV, which is determinant for the receptor binding preference modification from SAα2,6 to mixed SAα2,3 and SAα2,6, correlated with severe infection in macaques and higher replication in human lung tissue. It is believed that modifications in the receptor specificity of avian H5N1 influenza virus would be necessary to allow human-to-human direct transmission (Enserink, 2007; Tumpey et al., 2007). However, some studies indicate that a change from SAα2,3 to SAα2,6 binding preference is not enough to increase transmission efficiency in ferrets (Maines et al., 2006; Chen et al., 2012), which are a suitable animal model for the study of influenza since they are susceptible to human influenza viruses (Matsuoka et al., 2009), suggesting that several molecular changes, not only in the HA gene but also in other segments, would be required to confer efficient fitness in humans and become pandemic.

The development of glycan arrays has provided highly valuable information regarding the receptor binding specificity of the HA of numerous IAV isolates (Stevens et al., 2006a,b). These assays allow researchers to determine the SAα2,3 or SAα2,6 glycans that interact with the viral HA or whole virus, by using a broad panel of synthetic polymers. Therefore, we now know that not only the SAα2,3 or SAα2,6 linkages, but also the underlying sugar chain features, like other modifications such as sulfation, fucosylations, or additional sialylation of the sialyl-receptors are determinant for the binding specificity of the HA, being very variable among the different IAV subtypes and isolates (Stevens et al., 2006a), which could be determinant not only in the host and cell tropism, but also in differential recognition by cell receptors.

SIALYLATED GLYCANS AS RECEPTORS: INVOLVEMENT IN THE IMMUNE RESPONSE TO IAV AND PATHOGENESIS

In the last few years, numerous studies have provided increasing evidence for a role for the viral HA–SA interaction in the induction of innate immunity and pathogenesis. SA are structural components of the cell surface, and they have important implications in the immune responses (Crocker and Varki, 2001; Videira et al., 2008). On the other hand, DCs are essential players in the induction of innate immunity, as well as in the initiation of adaptive responses. Upon sensing invading pathogens via several pattern recognition receptors (PRRs), they differentiate to mature DCs and migrate to the secondary lymphoid organs, where they present the processed antigen to T-lymphocytes. Importantly, human DCs express SAα2,3 and SAα2,6 on their surface (Videira et al., 2008; Ramos et al., 2011), and the levels of expression of sialyltransferases and therefore, the SA levels on the surface, are modulated during differentiation and maturation processes (Bax et al., 2007; Videira et al., 2008). Additionally, it has also been shown that the elimination of the SA from the surface using a sialidase induced the maturation and activation of human DCs (Crespo et al., 2009), and that endogenous neuraminidase activity is necessary for the immune response to bacterial infections (Amith et al., 2009; Stamatos et al., 2010). These last studies reinforce the idea of a role for SA in immunity, and also could have implications in the immune responses against IAV, as the IAV NA is one important component of viral particles. One of the first reports suggesting the importance of HA–SA interaction in eliciting innate immune responses was published by Miller and Anders (2003), who showed that inactivated IAV are able to induce type I IFN production in mouse spleen cells in a SA-dependent manner. Herein, it is important to mention that the induction of type I IFN in immune cells by inactivated viruses via TLR7 could also be dependent on HA–SA interaction, since a more efficient SA-dependent entry of inactivated viruses would result in an enhanced recognition of viral ssRNA by TLR7 in the endosomes (Diebold et al., 2004).

Several studies have been performed comparing cytokine responses between avian H5N1 viruses and human H1N1 in immune and epithelial cells, and most were able to demonstrate that the H5N1 IAV induced the strongest production of cytokines and chemokines, as compared to H1N1 IAV (Cheung et al., 2002; Chan et al., 2005; Lee et al., 2008, 2009; Cheng et al., 2010). However, the viral factors responsible for this effect were not clearly determined, due to high disparity in the viral segments among the strains compared, and it is likely that a combination of them (HA, NS1, PB1-F2. . .) could account for these differences. In order to elucidate if differences in receptor specificity of the IAV are important for this hyper-induction of pro-inflammatory cytokines, we recently reported that recombinant viruses bearing the HA and NA from a HPAIV H5N1, which differed only in two amino-acids that were shown to modify receptor specificity, induced different cytokine profiles in human DCs, macrophages, and primary bronchial–epithelial cells (Ramos et al., 2011). Using these viruses with identical genes with the exception of two amino-acids in the HA we could clearly analyze the contribution of this important factor to the immune responses in human immune cells. Specifically, the recombinant IAV with avian-type receptor specificity (226Q/228G) induced higher levels of type I IFN, TNF-α, IP-10,

RANTES, and IL-6 than the one with increased human-type receptor specificity (226L/228S), indicating that the differential binding of the IAV to their receptors in the cell surface could contribute for the hyper-cytokinemia observed in HPAIV patients. These data indicate that recognition of IAV by cell surface receptors in immune cells could occur differently depending on their binding preference, as proposed in the model shown in **Figure 1**. In this hypothetical model, IAV could be detected by separate unknown receptors differently sialylated (either SAα2,3 or SAα2,6; **Figure 1A**), or by a receptor expressing both SAα2,3 and SAα2,6 (**Figure 1B**). In these two cases, engagement of avian IAV to a putative receptor containing SAα2,3 would result in activation of signaling pathways leading to increased cytokine production and inflammation. However, human IAV detection would result in delayed induction of cytokines. At that stage of infection, IAV proteins involved in innate immune evasion (such as NS1 or PB1-F2) could antagonize innate immunity, resulting in lower expression of pro-inflammatory cytokines.

On the other hand, another study by a different group found that recombinant HA proteins from an H5N1 and a 2009 H1N1 virus were able to induce DC activation of murine DCs in a MyD88/TLR4 dependent manner (Liu et al., 2010), indicating a possible role for this Toll-like receptor (TLR) in recognition of IAV HA. In this work, no clear differences were observed between H5 and H1 HAs, but it is important to note that the cytokine profile induced in murine DCs could differ of that observed in human DCs, probably due to different sialylation patterns in those two systems.

Interestingly, Eierhoff et al. (2010) recently discovered that IAV interact with epidermal growth factor receptor (EGFR) in a SA-dependent manner. They clearly showed that IAV attachment to this receptor tyrosine kinase (RTK) family member promotes its activation, resulting in increased IAV uptake. Furthermore, they observed recruitment of the regulatory subunit p85 of PI3K to the EGFR in response to viral attachment, indicating activation of downstream signaling pathways, which are necessary for endocytosis triggering. These results are of great relevance, indicating that the interaction of the viral particles with the cell surface via RTK might have important impact not only in viral uptake, as the authors demonstrated, but its activation could play a role also in the innate immune response to IAV, since it is known that the EGFR pathway is involved in the production of IL-8 by respiratory epithelial cells, resulting in neutrophils recruitment in airway inflammatory diseases (Subauste and Proud, 2001; Nakanaga et al., 2007). Moreover, as EGFR activation was observed to be SA-dependent, further studies to elucidate if the same signaling pathways are trigged by viruses with different SAα2,3 or SAα2,6 receptor specificity would be of great interest.

IAV INTERACTION WITH CELL SURFACE CARBOHYDRATE RECOGNIZING RECEPTORS AND THE INNATE IMMUNE RESPONSE

Influenza A viruses are recognized by different PRRs in host cells, leading to the initiation of the innate immune response aimed to defeat the virus. The most extensively studied receptors for IAV

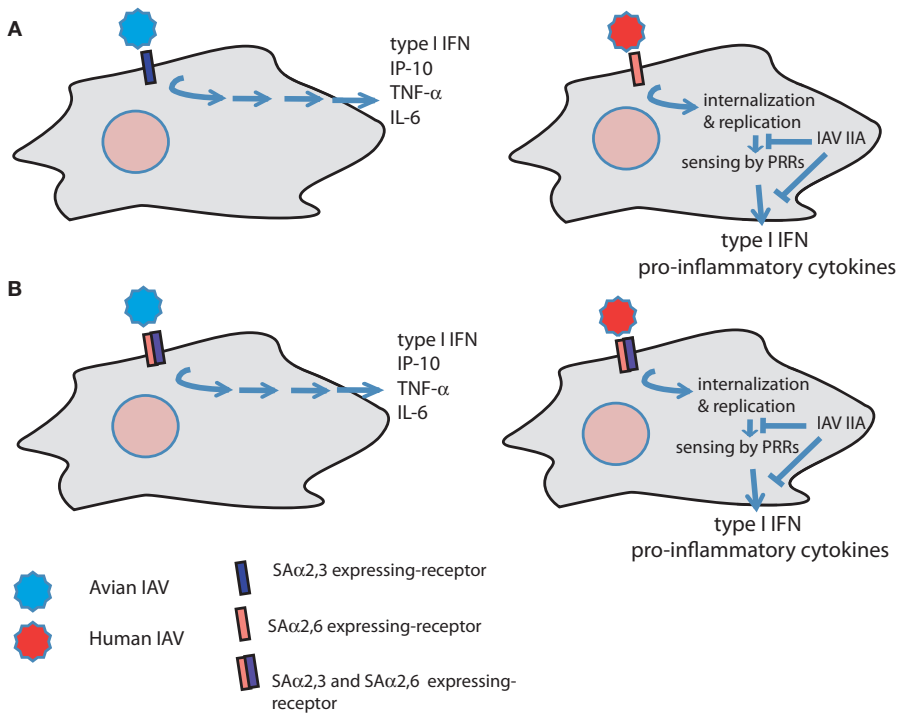

FIGURE 1 | Model for recognition of human and avian IAV by human immune cells. (A) IAV are recognized by separate receptors that are differently sialylated (SAα2,3 and SAα2,6). **(B)** IAV are sensed by an unknown SAα2,3 and SAα2,6 containing receptor that activates production of cytokines differently upon interaction with avian or human IAV. IIA, innate immunity antagonists.

are nucleic acid recognizing receptors, such as RIG-I-like receptors (RLRs) and TLRs (Garcia-Sastre, 2011), and more recently, nucleotide oligomerization domain receptors or Nod-like receptors (NLRs) have been shown to have also an important role in IAV infection (Allen et al., 2009; Ichinohe et al., 2009). However, although not much attention has been paid so far to the IAV glycoproteins as pathogen associated molecular patterns (PAMPs) there is increasing evidence for the importance of their interaction with the host cell and the initiation of signaling pathways related with either internalization/endocytosis and cytokine production (**Table 1**).

Several studies showed that interactions of the HA of the IAV with C-type lectins receptors (CLRs) are important for IAV infectivity and for induction of type I IFN production in immune cells, since these are reduced upon pre-incubation with mannans and/or blocking antibodies (Miller and Anders, 2003; Londrigan et al., 2011; Seeds et al., 2011). CLRs are important PRRs in the immune response to pathogens, since they mediate attachment of the pathogens to DCs and macrophages. Attachment events facilitate phagocytosis, and also induce the activation of several signaling pathways that modulate immune responses to that pathogen (Geijtenbeek and Gringhuis, 2009).

Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) is one of the best-characterized CLRs, which is expressed in immature DCs and recognizes and binds to high mannose containing glycoproteins, commonly found in several pathogens, including viruses. H5N1 pseudotyped viruses were demonstrated to efficiently infect THP-1/DC-SIGN expressing cells, and the infectivity was reduced using DC-SIGN blocking antibodies (Wang et al., 2008). Another interesting observation in this manuscript was that DC-SIGN dependent entry was observed to be also dependent on SA. In contrast, another study focused in the role of DC-SIGN and L-SIGN in IAV entry, using SA-deficient cells and the virus strain BJx109 (H3N2), indicated that the entry mediated by these receptors was SA-independent (Londrigan et al., 2011). These discrepancies are likely to be due to differences on experimental designs as, for instance, the avian H5 and the H3 from BJx109 present affinity for differently galactose-linked SA (SAα2,3 and SAα2,6, respectively). SIGN-R1 is one of the human DC-SIGN homologous proteins in mouse, and it is expressed in some subsets of macrophages and DCs in the spleen and the lymph node (Kang et al., 2003; Gonzalez et al., 2010; Lyszkiewicz et al., 2011). Similarly to human DC-SIGN, SIGN-R1 has also been reported to interact with IAV. Gonzalez et al. (2010) observed that a SIGN-R1-specific blocking antibody reduces IAV PR8 binding to lymph node DCs both *in vitro* and *in vivo* (using multiphoton intra-vital microscopy), and the viral uptake via this receptor was essential for local humoral immunity.

Additional C-type lectins that IAV have been shown to interact with are the murine macrophage galactose-type lectin (MGL) and macrophage mannose receptor (MMR). These interactions were observed to be SA-independent, and correlated with the different HA glycosylation levels of a few IAV strains (Reading et al., 2000; Upham et al., 2010). On the other hand, the mannose binding lectin (MBL), which is a soluble PRR belonging to the collectins family, is known to have anti-IAV activity, since it inhibits hemagglutination, resulting in viral neutralization, opsonizes viral particles allowing for better recognition and ingestion by phagocytic cells, and also activates the lectin complement pathway (Kawai et al., 2007; Chang et al., 2010; Gonzalez et al., 2010).

CONCLUDING REMARKS

The IAV interaction with surface cellular receptors is important for the pathogenesis and the immune responses generated in the host. The receptor specificity of IAV is determinant for allowing entry in the cell, and differential SA specificity accounts for host and cell tropism. Several specific cell receptors have been identified to interact with IAV in a SA-dependent or independent way. Further studies will allow for a better understanding of the outcome of these interactions and identification of new receptors, which could be important for the immune responses necessary for virus clearance or exaggerated responses that result in acute infections

Table 1 | Summary of cell surface or soluble host molecules reported to interact with IAV.

Cell receptor/ interacting molecule	Host studied/cell types	IAV subtype	Remarks	Reference
DC-SIGN	B-THP-1, B-THP-1/DC-SIGN (human), MDDCs (human)	H5N1 pseudotyped virus particles	SA-dependent interaction	Wang et al. (2008)
	Lec2 CHO cells (hamster)	H3N2 and highly glycosylated H1N1	SA-independent interaction	Londrigan et al. (2011)
L-SIGN	Lec2 CHO cells	H3N2 and highly glycosylated H1N1	SA-independent interaction	Londrigan et al. (2011)
SIGN-R1	Mouse model-*in vivo*/DCs	PR8 (H1N1)	Involved in humoral immunity	Gonzalez et al. (2010)
MMR	J774E (mouse)	H3N2, PR8	SA-independent interaction	Reading et al. (2000), Upham et al. (2010)
MGL	J774E	H3N2, PR8	SA-independent interaction	Upham et al. (2010)
EGFR c-Met receptor	A549 (human)	H7N7 (avian), PR8	SA-dependent interaction	Eierhoff et al. (2010)
MBL (opsonization)	Mouse model-*in vivo*/ macrophages	PR8 (H1N1)	Dispensable for humoral immunity	Gonzalez et al. (2010)
	MDCK cells (canine)	H3N2		Kawai et al. (2007)
	Mouse model-*in vivo*	H3N2		Chang et al. (2010)

and complications in high risk populations, as well as in infection by HPAIV. Recent data from several groups, including ours, strongly suggest that these receptors, when present in immune cells, might account for specific pro-inflammatory responses or facilitation of virus entry into cells. These findings may shed new light into the observed exacerbated immune responses in humans infected with viruses with different host tropism, such as HPAIV H5N1. Further studies need to be performed to analyze if IAV with different SA binding affinity indeed bind different receptors in antigen presenting cells or if the different binding by those HAs induces distinct signaling cascades in those cells.

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Norovirus recognition sites on histo-blood group antigens

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Norovirus (NoV) is the major causative agent of acute viral gastroenteritis worldwide. Based on genetic analyses, human NoV strains have been classified into at least three genogroups: genogroup I (GI), GII, and GIV, which contain at least 15, 18, and 1 genotypes, respectively (Kageyama et al., 2004). Notably, these NoV genotypes are morphologically similar to one another but differ antigenically (Kapikian, 1996; Estes et al., 1997; Hansman et al., 2006). Since no cell culture system has been developed yet, molecular analyses to elucidate the mechanisms underlying infection or productive replication have been stalled until recently. The primary NoV replication site in humans is unknown, but intestinal biopsy from volunteer who became ill following oral administration of NoV exhibited histopathologic lesions, in which the villi of the proximal small intestine were blunted (Agus et al., 1973). More than 30 years ago, the initial NoV challenge studies conducted in volunteers found that a subset of individuals was repeatedly susceptible to NoV infection, whereas a second subset was repeatedly resistant to infection (Parrino et al., 1977). A genetic factor, possibly a receptor, was hypothesized to affect the susceptibility of an individual to NoV infection. Recently, a mechanism that explains the susceptibility or resistance to NoV infection has been identified; namely, NoV attaches to histo-blood group antigens (HBGAs) on potential host cells in the gut (Lindesmith et al., 2003).

HBGAs are structurally related oligosaccharides that include ABH antigens and Lewis (Le) antigens (**Figure 1**). Polymorphisms in the ABH antigens are induced by variations in the core structure. Type 1 chain ABH antigens are widely expressed in endodermally derived tissues such as the lining epithelia and the glandular epithelia (Oriol et al., 1986). Meanwhile, type 2 chain ABH antigens are found primarily in ecto- or meso-dermally derived tissues, including skin and erythrocytes (Hakomori, 1981; Dabelsteen et al., 1982; Oriol et al., 1986). In the human gastroduodenal junction, type 1 structures are found exclusively at the level of the surface epithelia, whereas type 2 structures are preferentially found at the glandular level (Mollicone et al., 1985).

Details of the NoV recognition of HBGAs were clarified with *in vitro* experiments, enzyme-linked immunosorbent assays (ELISAs), and surface plasmon resonance (SPR) experiments using virus-like particles (VLPs), as well as crystallization studies using P domains. When the NoV VP1 gene, which encodes a capsid protein, is expressed in insect cells, capsid proteins of approximately 58 kDa are generated and self-assembled into VLPs (Xi et al., 1990; Jiang et al., 1993). Though artificial, these VLPs are morphologically and antigenically similar to those of the respective native virions (Jiang et al., 1992; Green et al., 1993; Prasad et al., 1994, 1999). VP1 has two major domains: a shell (S) domain, which forms the core of the icosahedral shell (Bertolotti-Ciarlet et al., 2002), and a protruding (P) domain, which forms arches extending from the shell and is responsible for host interactions (Tan et al., 2004, 2011; Tan and Jiang, 2005a,b, 2007). The association and dissociation kinetics for NoV VLPs binding to HBGAs were measured with SPR experiments (Choi et al., 2008; Shirato et al., 2008), which allowed us to visualize

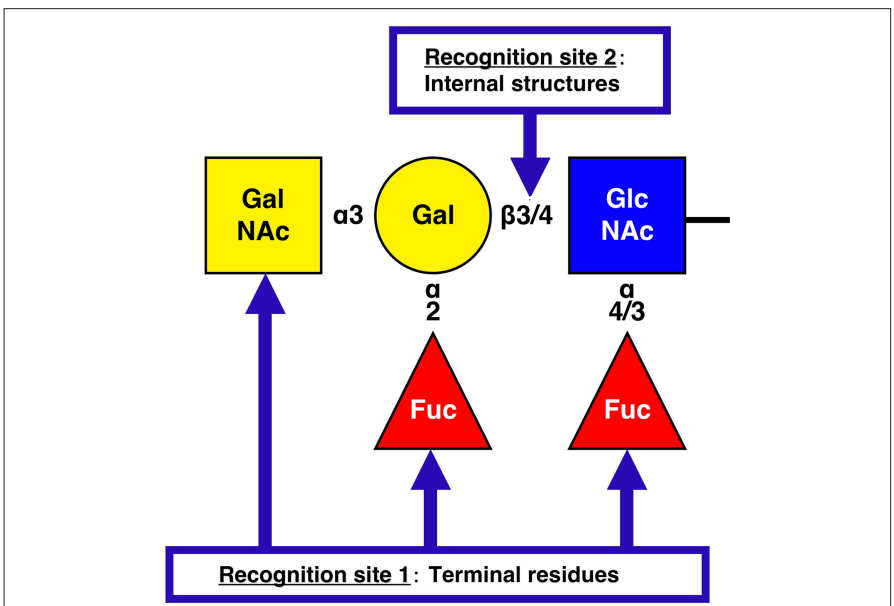

FIGURE 1 | NoV recognition sites on histo-blood group antigens. The structure represents a pentasaccharide as the final product (ALe^b or BLe^b: in the case of BLe^b, the terminal residue is Gal.) of human HBGA synthesis. H types 1 (Fucα1-2Galβ1-3GlcNAcβ1-) and 2 (Fucα1-2Galβ1-4GlcNAcβ1-) are the terminal moieties expressed in histo-blood group type O individuals. However, in A, B, and AB individuals the H antigens are further modified by enzymes that transfer *N*-acetylgalactosamine (GalNAc, type A), Gal (type B), or either carbohydrate (type AB) to the terminal Gal residue of an H antigen in an α3 linkage. For NoV recognition site 1 on HBGAs, the majority of GI NoVs interact with the A [GalNAcα1-3(Fucα1-2)Galβ1-] and Le^a [Galβ1-3(Fucα1-4)GlcNAcβ1-] antigens, whereas GII NoVs exhibit more diverse HBGA-binding patterns, such as for the B [Galα1-3(Fucα1-2)Galβ1-] antigen. For recognition site 2, NoV VLPs are able to distinguish between the type 1 (Galβ1-3GlcNAcβ1-) and type 2 (Galβ1-4GlcNAcβ1-) core structures of HBGAs and bind more tightly to the type 1 carbohydrates than to the type 2 carbohydrates.

Abbreviations: Fuc, fucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; Glc, glucose; GlcNAc, *N*-acetylglucosamine; HBGA, histo-blood group antigen; NoV, norovirus; VP, virion protein.

each binding step in about a 120–1200 s reaction time, whereas ELISA allowed us to visualize the last step in a total reaction time of about 8 h. Recent crystallographic studies have revealed that the P domain dimers form a structure similar to that of the corresponding region of the VP1 protein, indicating that the P domain proteins are valid alternatives for HBGA-binding studies (Choi et al., 2008). Moreover, the P domain proteins have an advantage over VLPs in structural studies, because they are easily purified from extracts of transformed *E. coli* cells. The crystallization studies have identified not only the recognition sites on HBGAs by NoV but also the putative binding sites on the NoV capsid protein. These *in vitro* studies clarified that the binding properties of human NoV to HBGAs were variable and that the terminal residues and internal structures were important in the NoV–HBGA interactions (Harrington et al., 2002, 2004; Huang et al., 2003, 2005; Shirato-Horikoshi et al., 2007; Shirato et al., 2008).

To date, the interactions between carbohydrates and 29 NoV VLPs, including 10 GI and 19 GII VLPs, have been reported. **Figure 1** illustrates the recognition sites on HBGAs by NoV. For recognition site 1, several binding patterns have been identified according to the interaction with the H (α2Fuc), A (αGalNAc), B (αGal), and Le (α4Fuc) epitopes (Huang et al., 2005; Shirato et al., 2008). The binding of NoVs to these terminal residues is genogroup dependent. The majority of GI NoVs interact with the A and Le^a antigens (Hutson et al., 2003; Shirato et al., 2008), whereas GII NoVs exhibit more diverse HBGA-binding patterns, such as in the binding of the B antigen (Harrington et al., 2004; Huang et al., 2005; Cao et al., 2007; Shirato et al., 2008). Indeed, strains of the same genogroup tend to have identical amino acids on the putative binding sites. Moreover, structural studies revealed that the carbohydrate-binding site in the P domain is distinctly different between the GI and GII genogroups both in terms of its location and its structural characteristics (Cao et al., 2007; Choi et al., 2008), despite the VP1 protein sharing a similar S and P domain organization and polypeptide fold between these two genogroups. However, one group has argued that the specificity for HBGA is strain-dependent across GI and GII (Huang et al., 2005). These authors concluded that NoV–HBGA-binding patterns could be classified into two groups, an A/B-binding group and a Le-binding group, and that no correlation was present between the binding patterns and the genogroup. However, a single amino acid change in the P domain was found to result in a change in the pattern of HBGA binding (Tan et al., 2003), so that reaching conclusions is difficult. For recognition site 2, both GI and GII NoV VLPs are able to distinguish the difference between type 1 and type 2 carbohydrates. In SPR experiments, GI and GII NoV VLPs more efficiently bound to type 2 than type 1 for the H, A and B antigens (Shirato et al., 2008). Moreover, the dissociation of GII NoV VLP from the B type 1 antigen was slower than that from the B type 2 antigen (Shirato et al., 2008). ELISA experiments also showed that the NoV VLPs were able to distinguish the difference between the core structures of HBGAs. The GI VLP has been reported to bind synthetic H carbohydrates in the following order of strength: H type 1 trisaccharides, H type 2 trisaccharides, and H disaccharides (Hutson et al., 2003). The binding of GII NoV VLP to B type 1 has been reported to be stronger than that to B type 2 (Shirato et al., 2008). In addition, immunohistochemical analysis has revealed that the binding of GI to the gastroduodenal junction correlated with the presence of H type 1 antigen but not to H type 2 antigen (Marionneau et al., 2002). These studies indicate that the NoV VLPs are able to distinguish between type 1 and type 2 core structures of HBGAs and to bind more tightly to the type 1 carbohydrates than to the type 2 carbohydrates.

Avian and equine influenza viruses are known to preferentially bind to the terminal sialic acid α2–3Gal (SAα2–3Gal) linkage, whereas human influenza viruses preferentially bind to the SA α2–6Gal linkage (Rogers and Paulson, 1983; Rogers et al., 1983; Connor et al., 1994), affording a major impact on the host specificity of these influenza viruses. A similar relationship may exist between NoV carbohydrate recognition and the tissue specificity of this virus, since the binding of NoV to the gastroduodenal junction has been reported to correlate with the presence of the H type 1 but not the H type 2 antigen, as described above (Marionneau et al., 2002). In conclusion, HBGAs are important factors in determining the host specificity of NoV, although it remains unclear whether they function as the primary receptor or enhance NoV infectivity and/or attachment to a common cellular receptor. Since NoV comprises a number of antigenically diverse groups, identification of the common NoV-binding epitopes on host cells, if any, should be useful in the development of possible antiviral agents.

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