Sensitive detection of measles virus infection in the blood and tissues of humanized mouse by one-step quantitative RT-PCR

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INTRODUCTION

Measles, a highly contagious childhood disease caused by the measles virus (MV), affects more than 20 million people each year. MV infection is characterized by a high fever, generalized maculopapular rash, and is often associated with respiratory and neuronal complications (Griffin, 2007). Since the measles virus (MV) infection is a major cause of death among young children under the age of 5 years, especially in countries with weak health infrastructures, and approximately 158,000 measles death occurred in 2011 (http://www.who.int/mediacentre/factsheets/fs286/en/).

The ongoing global vaccination strategy aims to protect small children at high risk. The MV vaccine is safe, effective, and inexpensive. Based on its long and successful vaccination history, several groups have taken advantage of reverse-genetics technology to utilize the live attenuated MV vaccine strain as a viral vector to elicit immune responses against foreign antigens from various pathogens, such as Env or Gag of human immunodeficiency virus (HIV; Lorin et al., 2004; Stebbings et al., 2012), hepatitis B surface (S) antigen (Singh et al., 1999; Reyes-del Valle et al., 2009), fusion protein of respiratory syncytial virus (Sawada et al., 2011), and envelope glycoprotein of West Nile virus (Despres et al., 2005; Brandler et al., 2012). MV is a human-tropic virus that uses CD46, epithelial-cell receptor nectin-4 (PVRL4, see review in Kato et al., 2012) as receptors.

Therefore, as an infectious disease that has been hampered by the lack of a small-animal model, the humanized mouse, a recently developed system in which an immunodeficient mouse is transplanted with human fetal tissues or hematopoietic stem cells, may represent a suitable model. Here, we developed a sensitive one-step quantitative reverse transcription qRT-PCR that simultaneously measures nucleocapsid (N) and human RNAse P mRNA levels. The results can be used to monitor MV infection in a humanized mouse model. Using this method, we elucidated the replication kinetics of MV expressing enhanced green fluorescent protein both in vitro and in humanized mice in parallel with flow-cytometric analysis. Because our qRT-PCR system was sensitive enough to detect MV expression using RNA extracted from a small number of cells, it can be used to monitor MV infection in humanized mice by sequential blood sampling.

Keywords: measles virus infection, humanized mouse, quantitative RT-PCR, EGFP expression, flow cytometry
A large number of studies have described the development of cytometry, the actual frequency of MV-infected cells, as determined by flow system works and demonstrate that the results obtained reflect humanized mice. In this study, we describe how this monitor—blood mononuclear cells (PBMCs) circulating in the blood of PCR) system to monitor MV infection in human peripheral it is important to assess MV infection kinetics in an animal in systemic lymphoid tissues and bone marrow (BM). Because these human MV receptor-expressing mouse models is severely after obtaining the approval of the institutional ethical commit-tee of the National Institute of Infectious Diseases (NIID; No. 350) and written informed consent from each subject. PBMCs were separated by Ficoll–Hypaque density-gradient centrifugation (Lymphoprep, IBL, Gunma, Japan).

To obtain monocyte-derived dendritic cells (MDDCs), mono-cytes were enriched from PBMCs using CD14 microbeads (Mil-tenyi Biotec) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, and antibiotics in the presence of interleukin-4 (IL-4) and granulocyte–macrophage colony-stimulating factor (GM-CSF; both 10 ng/ml, from Pepro-Tech Inc., London, UK) for 1 week. T cells were isolated from CD14-negative PBMCs using the Total T Cell Enrichment Kit (STEMCELL technologies, Vancouver, BC, Canada).

**PREPARATION OF RNA**

Total RNA was extracted from mouse blood, IBM, and spleen of humanized mice, human PBMCs, and Jurkat cells expressing human SLAM (Jurkat/hSLAM) using the RNaseasy Mini Kit (QIA-GEN, Valencia, CA, USA) or the Total RNA Isolation Mini Kit (Agilent Technologies, Santa Clara, CA, USA).

To prepare a standard of MV RNA, the cDNA encoding measles virus nucleocapsid (N) (MV-N: AB052821) was subcloned into the pBluescript II vector, and then MV-N RNA was produced by in vitro RNA transcription using the T7 RiboMAX™ Express Large Scale RNA Production System (Promega, Madison, WI, USA). The RNA product was purified by DNase treatment, fol-lowed by phenol–chloroform extraction and ethanol precipitation, according to the protocol supplied by the manufacturer. The final concentration of RNA was measured using an ND-1000 spectrophotometer (Thermo, Waltham, MA, USA).

**PREPARATION OF STANDARD TEMPLATE DNA**

To prepare a standard template DNA, cDNAs of human CD45 (hCD45: NG_007730) and RNase P (NM_006413) were syn-thesized from total RNA of GEM cells by reverse transcription (RT)-PCR using SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen) and then MV-N RNA was produced by in vitro RNA transcription using the T7 RiboMAX™ Express Large Scale RNA Production System (Promega, Madison, WI, USA). The RNA product was purified by DNase treatment, fol-lowed by phenol–chloroform extraction and ethanol precipitation, according to the protocol supplied by the manufacturer.

**REAL-TIME RT-PCR ASSAY**

To perform real-time qRT-PCR, SuperScript III Platinum One-Step Quantitative RT-PCR system (Invitrogen) was used according to the manufacturer’s instructions. Briefly, each reaction contained 1× reaction mix, ROX reference dye, SuperScript III Platinum TaqMix, 0.2 μM specific primers, and 0.1 μM TaqMan probe. Reactions were performed on an Mx3000P qPCR system (Agilent Technologies). Thermocycling parameters included a RT step at 50°C for 2 min, followed by a DNA polymerase activation step at 95°C for 2 min and 30 PCR cycles (95°C for 20 s, 60°C for 30 s). Threshold cycle (Ct) values were calculated for each reaction; Ct represents the cycle at which a statistically significant increase in the emission intensity of the reporter relative to the passive reference dye is first detected.
For detection of hCD45 mRNA, the following sequences were used: forward primer, 5'-GGA AGT GCT GCA ATG TGT CAT T-3', reverse primer, 5'-CTT GAG CAT AGT ACT ATT ATC TGA TGT CA-3', TaqMan probe, 5'-FAM-ACA ACT AAA AGT TAC TAT CTT CC-3'. For detection of RNase P mRNA: forward primer, 5'-AGA TTT GGA CCT GGC AGC G-3', reverse primer, 5'-GAG CCG CTG TCT CCA CAA GT-3', TaqMan probe, 5'-FAM-TTC TGA CCT GAA GCC TCT GGG CG-BHQ1-3'. For detection of MV-N RNA: forward primer, 5'-GGA TGA CCC TGA GGT TAG CA-3', reverse primer, 5'-GGG AAG GTA AGG CCA CAT TG-3', TaqMan probe, 5'-FAM-AGG CTG TTA GAG GAT GTT GTC CAG AGT GAC CAG-BHQ1-3' (Hummel et al., 2006).

**GENERATION OF HUMANIZED MICE**

Humanized NOD/SCID/JAK3null mice were established as described previously (Terahara et al., 2013). In brief, NOD mice were transplanted with human HSCs (0.5-1 × 10^6) enriched from human umbilical cord blood cells into the livers of irradiated (1 Gy) newborn mice within 2 days after birth. All mice were maintained under specific pathogen-free conditions in the animal facility at NIID and were treated in accordance with the guidelines issued by the Institutional Animal Care and Committee of NIID.

Human umbilical cord blood was donated by the Tokyo Cord Blood Bank (Tokyo, Japan) after obtaining informed consent. The use of human umbilical cord blood cells was approved by the Institutional Ethical Committees of NIID and the Tokyo Cord Blood Bank. Human HSCs were isolated using the CD343 Microbeads Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity was approximately 90% as assessed by flow cytometry.

**PREPARATION AND INFECTION OF MV**

Recombinant wild-type MV (IC323: AB016162) expressing EGFP (IC323-EGFP; Hashimoto et al., 2002) and a recombinant vaccine (IC323-EGFP; Hashimoto et al., 2002) were purchased from BioLegend Inc. (San Diego, CA, USA). Jurkat/hSLAM cells were infected with various doses of MV (multiplicity of infection (MOI) = 0.25, 0.5, 0.01) by incubation at 37°C for 1 h, washed twice with phosphate buffered saline (PBS), and seeded on 24-well plates. Cells were harvested immediately after washing (time 0) or 6, 12, 18, or 24 h later. The harvested cells were either lysed for mRNA extraction or analyzed by flow cytometry.

Humanized NOD/SCID/JAK3null mice were challenged intravenously (i.v.) with different doses [200, 2000, 10,000, or 20,000 plaque-forming units (pfu)] of AIK-C-EGFP. Peripheral blood was obtained from MV-infected hNOJ mice at 3, 5, 7, 10, 14, and 21 days post-infection (p.i.). In some experiments, MV-infected hNOJ mice were sacrificed at day 7 p.i. At the time of sacrifice, peripheral blood, BM, spleen, and mesenteric lymph nodes (MLNs) were harvested, and red blood cells were lysed in ACK buffer (0.15 M NaCl, 1 mM KHCO3, and 0.1 mM EDTA-2Na; pH 7.2-7.4).

For detection of hCD45 mRNA, the following sequences were used: forward primer, 5'-GGA AGT GCT GCA ATG TGT CAT T-3', reverse primer, 5'-CTT GAG CAT AGT ACT ATT ATC TGA TGT CA-3', TaqMan probe, 5'-FAM-ACA ACT AAA AGT TAC TAT CTT CC-3'. For detection of RNase P mRNA: forward primer, 5'-AGA TTT GGA CCT GGC AGC G-3', reverse primer, 5'-GAG CCG CTG TCT CCA CAA GT-3', TaqMan probe, 5'-FAM-TTC TGA CCT GAA GCC TCT GGG CG-BHQ1-3' (Hummel et al., 2006).
FIGURE 1 | Selection of an endogenous control for the analysis of MV-infected human PBMCs. (A) RNA was extracted from spleen cells of NSG and non-humanized NOJ, and one-step qRT-PCR was performed using primer and probe sets designed against the human-specific hCD45 and RNaseP mRNAs. To calculate copy numbers of these genes, the PCR products of human CD45 and RNase P were subcloned into plasmids and used as standard DNAs. (B) Human PBMCs from five donors were fractionated into CD14+ monocytes and T cells. RNA from these cell populations was extracted, and the expression levels of hCD45 and RNase P were analyzed by qRT-PCR. The graph depicts the expression levels in these fractionated cells relative to the levels in PBMCs defined as 1. Statistical differences in hCD45 and RNase P expression among these cell populations were evaluated by non-parametric one-way ANOVA test (*P < 0.05).

infection in Jurkat/hSLAM cells can be clearly visualized by flow cytometry. We infected Jurkat/hSLAM cells with a wild-type MV encoding EGFP (IC323-EGFP) at MOI of 0.01, 0.05, and 0.25. Cells were washed and harvested at 6, 12, 18, or 24 h after MV infection. A subset of the cells in each sample was analyzed by flow cytometry, and the remainder of the sample was used for RNA extraction. The mRNA levels of MV-N and RNase P were determined by qRT-PCR, and the level of MV-N mRNA relative to RNase P RNA was calculated. Representative results of three experiments are shown in Figure 2A (flow cytometry) and Figure 2B (qRT-PCR). Because of the rapid and strong cytopathic effect by MV at the highest MOI (0.25), we omitted the flow cytometry data corresponding to that condition. At MOI 0.01, a similar frequency of GFP+ cells was detectable at 12 and 18 h p.i., whereas at MOI 0.05, the GFP+ cell frequency was already high at 12 h p.i. Note that the level of hSLAM was not down-modulated by MV infection. Over the time course, relative MV-N expression level at all three MOIs increased in parallel over two orders of magnitude, indicating that these two methods yield comparable results (as shown in Figure 2C) and are useful for monitoring the replication kinetics of MV infection in vitro.

PARALLEL INCREASE OF MV-INFECTED CELL FREQUENCY AND MV-N RNA LEVELS IN VIVO
We then applied these detection systems in vivo in MV-infected hNOJ mice. hNOJ mice were infected with an MV vaccine strain expressing EGFP (AIK-C-EGFP) at 2000 pfu, and the animals were sacrificed 7 days later. Blood PBMCs and BM cells were washed with PBS, and a subset of the cells in each sample were stained with anti-hCD45 mAb. Representative results of flow-cytometric analysis of BM cells from three mice are shown in Figure 3A. The percentages of GFP+ cells in mice 127-1, 127-4, and 127-5 were low (0.002%), high (0.35%), and intermediate (0.028%), respectively. The number of human PBMCs obtained from mouse blood was not sufficient to determine GFP+
FIGURE 2 | Time course of MV infection in vitro. Jurkat/hSLAM cells were infected with wild-type MV IC323-EGFP at MOI of 0.01, 0.05, and 0.25, washed, and harvested at the indicated time points. (A) Cells were stained with PE-conjugated anti-hSLAM mAb, fixed with 2% formalin/PBS, and GFP expression was analyzed. (B) RNA was extracted from cells, and expression levels of MV-N and RNase P were analyzed by one-step qRT-PCR. The copy numbers of MV-N and RNase P were determined, and the ratio of MV-N copies to RNase P copies is depicted on the vertical axis. (C) Correlation between the percentage of GFP⁺ Jurkat/hSLAM cells and the time course of MV-N expression. Spearman's rank correlation coefficient was used for statistical analysis.

cell frequencies by flow cytometry. Next, we extracted RNA from PBMCs and BM cells and analyzed MV-N expression by qRT-PCR, as described in the previous section. MV-N expression paralleled the GFP⁺ frequencies in BM (Figure 3B). Notably, a high level of MV-N expression was also detected in PBMCs of mouse 127-4, suggesting that the level of MV-N expression per single hematopoietic cell is similar between blood and BM. We plotted the GFP⁺ frequency and MV-N expression level in BM cells of eight mice. As shown in Figure 3C, these values were well correlated (R = 0.9286). Taken together, these data indicate that MV infection in vivo is detectable in BM by both flow cytometry and MV-N RNA qRT-PCR analysis, but only MV-N RNA qRT-PCR is
FIGURE 3 | Analysis of MV infection in vivo. Three hNOJ mice (127-1, -4, and -5) were infected intravenously with 2,000 pfu of the MV vaccine strain, AIK-C-EGFP. Mice were sacrificed at day 7 post-infection, and blood and bone marrow cells (BM) were obtained. (A) BM cells were stained with PB-anti-human CD45 mAb, fixed with 2% formalin/PBS, and GFP expression was analyzed. (B) PBMCs from blood and BM cells were lysed, and RNA was prepared. The expression of MV-N and RNase P was analyzed as described in the legend for Figure 2B. (C) Correlation between the percentage of GFP+ cells among hCD45+ cells in BM and the level of MV-N expression in MV-infected hNOJ mice, at day 7 (n = 4) or day 10 (n = 4) p.i. Spearman’s rank correlation coefficient was used for statistical analysis.

sensitive enough to detect PBMC-associated MV infection in the blood.

KINETICS OF MV GROWTH CAN BE MONITORED IN THE BLOOD OF hNOJ MOUSE

Finally, we measured MV growth kinetics in vivo by qRT-PCR analysis using sequential blood samples obtained from MV-infected hNOJ mice; it was not feasible to perform these measurements by flow cytometry because of the paucity of human PBMCs in the blood. Two or three hNOJ mice in each group were infected intravenously with 200, 2000, or 20,000 pfu AIK-C-EGFP and followed up to 21 days p.i. The level of PBMC-associated MV RNA in individual mice is shown in Figure 4A. We noticed two peaks of MV replication, the first at around day 3 p.i., and the second at day 10 p.i., irrespective of the initial inoculum. Two mice infected with 20,000 pfu MV exhibited a high level of MV replication that peaked at day 10 p.i. One mouse infected with 2,000 pfu exhibited a high level of MV replication at day 3 p.i., followed by a small peak at day 10 p.i. For some mice, we counted the number of human cells per 50 μl of blood used for RNA extraction. The data are shown in Figure 4B. We were able to detect MV replication in individual mice containing less than 2,000 cells, indicating that the qRT-PCR system is sensitive enough to detect low numbers of MV-infected human cells.

Although MV replication was not obvious in three mice infected with the smallest dose (200 pfu), one of these animals exhibited an increase in MV RNA expression at day 21 p.i. (gray circle). We sacrificed this particular mouse and used flow cytometry to analyze GFP expression in its blood, spleen, MLN, and BM. As shown in Figure 4C, GFP+ cells were present in spleen (0.308%) and all the other tissues, albeit at a lower frequency, indicating that MV infection can occur even at a low dose (200 pfu) and spread slowly in the systemic lymphoid tissues of hNOJ.

It may be necessary to acquire at least 30,000 events to be sure of having >10,000 cells for flow cytometry analysis. This is because of the substantial amount of sample loss that occurs in this system. The flow cytometry data presented in Figure 4C were obtained by analyzing ~0.4 ml blood from a sacrificed mouse. However, even under these conditions, the proportion of MV-infected cells detected was only 0.006%; indeed, the cells are barely visible on the plot. Therefore, it appears that flow cytometry is not a suitable method for the sequential monitoring of infected (GFP+) cells. Thus, the qRT-PCR system we have developed here allowed us to monitor systemic MV replication using a small volume of blood from humanized mice.

DISCUSSION

Based on a highly sensitive MV-N RNA detection method previously developed by Hummel et al. (2006), which could detect one copy of synthetic MV RNA/reaction, we developed a novel one-step real-time qRT-PCR system for the purpose of monitoring MV replication in the blood of MV-infected humanized mice.
Because MV replication usually occurs in association with cells (Griffin, 2007), it is necessary to evaluate the endogenous RNA expression level of human PBMCs that co-exist with mouse blood cells. To this end, we designed human-specific primer/probe sets for the CD45 and RNase P mRNAs. When we analyzed the detection efficiencies of these two primer/probe sets using distinct cell types present in human PBMCs, we found that RNase P expression was less dependent than CD45 expression on cell type. Using this qRT-PCR system with RNase P as an internal control, we can reliably detect MV replication with high sensitivity in humanized mice in vivo. When MV expressing GFP was used for infections in vitro or in vivo, the level of MV-N RNA was closely correlated with the frequencies of GFP+ MV-infected cells determined by flow cytometry.

Our qRT-PCR system allowed us to follow MV replication in vivo using a small amount of blood, with no need to sacrifice mice at each time point. Although flow-cytometric analysis provides valuable information, such as the proportions of various cell types and the surface phenotypes of MV-infected cells, the small number of human cells circulating in the mouse blood may not be sufficient for precise estimation of MV-infected cells by flow cytometry. By contrast, our qRT-PCR system was able to detect MV-N RNA in fewer than 2,000 human PBMCs (Figure 4B). This is an important technological advantage considering that individual humanized mice exhibit variable levels of human cell engraftment, i.e., chimerism (Terahara et al., 2013); moreover, there may exist donor-to-donor variation in susceptibility to MV infection. Thus, it should be possible to select humanized mice with a degree of MV infection appropriate for the purpose of a given experiment.

In this study, MV was inoculated through the tail vein, and infected cells were distributed to systemic lymphoid tissues as well as BMs, where human hematopoietic cells localize in humanized mice (Draggini et al., 2008). MV may also be distributed to other organs, such as lung and intestinal tissue, as demonstrated in the case of HIV infection using the BLT mouse (Sun et al., 2007). To our surprise, by monitoring MV replication in PBMCs of humanized mice, we noticed two peaks of MV replication, at around 3 and 10 days p.i., in some mice. This pattern of MV replication did not depend on the initial dose of MV inoculum. We do not know why MV replication showed two peaks in many animals. However, it was recently reported in a monkey model that MV RNA persists in PBMCs for more than 1 month after primary infection, and declined in three phases (Lin et al., 2012). The authors of that study hypothesized that both T cells, including regulatory T cells (Treg), and antibody responses contributed to the dynamics of MV replication in vivo. Although hNOJ mice are reported to show poor immune responses, the role of regulatory T cells should be considered. This is because these cells regulate HIV-1 infection in humanized mice (Jiang et al., 2008). Alternatively, it may be that the intravenous injection of MV rapidly kills the target cells (probably those showing an activated phenotype) within 3 days. The low number of MV-infected cells then gradually transmits the virus to the human cells that are replenished from the BM stem cell pool. Further investigations are required to clarify this issue.

The humanized mouse model is expected to be a useful tool for studying virus infection (Akkina, 2013). Although the human immune system is not fully reconstructed by the transplantation of human HSCs alone, we believe that further improvements are possible, which will allow us to utilize this mouse model to not only evaluate vaccine and drug efficacy but also to increase our understanding of the pathogenesis of MV infection. The described novel method of monitoring MV-infected human cells in the blood will
We thank Prof. Yusuke Yanagi for providing IC323-EGFP, and Ms. Kahori Okano for her excellent technical assistance. We also thank the Tokyo Cord Blood Bank for donating human umbilical cord blood. This work was supported in part by a grant from the Ministry of Health, Labor, and Welfare of Japan, and in part by a grant from the Ministry of Education, Science, Sports, and Culture of Japan. Shota Ikeno is supported by the Global COE Program (Practical Chemical Wisdom) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

ACKNOWLEDGMENTS

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