granulocytes, and NK cells among all immune cells. Macrophages and granulocytes express both activating and inhibitory CD16, whereas NK cells only express activating CD16.

Because of the recent development of antibody therapies for cancers, monoclonal antibodies such as trastuzumab (Herceptin®, F. Hoffman–La Roche Ltd., Basel, Switzerland), rituximab (Rituxan®, F. Hoffman–La Roche Ltd., Basel, Switzerland), and anti-EGF receptor mAbs, which are effective against cancers, have been used clinically. NK cells, which possess Fc receptors, play a significant role in the mechanism of these therapies [21,22].

Regarding HIV infection, HIV-infected cells bound by anti-gp120 antibodies are destroyed by NK cells [17]. It has also been reported that higher ADCC activities specific for the HIV envelope protein are associated with lower disease stages of HIV infection [23]. Furthermore, it has become possible to destroy HIV-infected cells through ADCC with an IgG-IgA chimera-bound protein with strong binding ability for CD16 [24].

In addition to further development of pharmaceutical antibody products, the development of specific and powerful treatment methods against tumor cells and virus-infected cells based on the ADCC of NK cells, such as the concomitant use of *ex vivo*–expanded NK cells, is expected.

CROSS-REGULATION OF DENDRITIC CELLS AND NK CELLS

Dendritic cells (DCs) are key cells that lead antigen-specific immune reactions as potent antigen-presenting cells. NK cells induce innate immune responses through reciprocal interactions with DCs [25,26]. Furthermore, these interactions initiate T-cell responses [27].

Interleukin (IL)-12 and IL-2, the cytokines produced by mature DCs, induce the production of IFN-γ by NK cells [27,28]. On the other hand, it is considered that activated NK cells mainly produce Th1-type cytokines, such as IFN-γ, TNF-α, or granulocyte-macrophage colony-stimulating factor, induce the maturation and activation of DCs, and are also involved in the shift from innate immunity to adaptive immunity [25,29].

NK cells also produce and secrete large quantities of CC-chemokines such as CC-chemokine ligand 3 (CCL3; generic name, MIP1α), CCL4 (generic name, MIP1β), and CCL5 (generic name, RANTES), which are the ligands of CC-chemokine receptor 5 [30,31]. It is considered that migration of immature DCs, as well as CTL and Th1-type T cells, to tumor cells or lymphatic nodes is induced by these chemokines.

Reciprocal DC-NK cell interactions that normally occur after an inflammatory insult are affected during HIV-1 infection [32]. The amounts of secreted IFN-γ, a potent inducer of DC differentiation, are decreased when NK cells are exposed to autologous mature DCs generated from viremic but not aviremic HIV-1–infected individuals [32]. Although the ability to promote DC maturation is essentially confined to NK cells expressing a $KIR^{neg}/NKG2A^{dull}/NKp30^{pos}$ phenotype [33], freshly purified NK cells from HIV-1 viremic individuals express increased levels of KIRs and downregulate levels of NKG2A and NKp30 [34]. These data suggest that impaired maturation of DCs may occur due to functional impairment of NK cells in patients with poorly controlled viremia.

The functional cross-talk between NK cells and DCs implies a critical role for NK cells in the initiation and regulation of cellular immunity. Therefore, decreased NK cell activity detected *in vivo* may indicate that innate and adaptive immune reactivities against cancers or viral infections are both decreased.

NK Cell Subsets

At least two subsets of NK cells exist in the peripheral blood. The main subsets are $CD3^-CD56^{dim}$ NK cells whose main function is cytotoxic activity and $CD3^-CD56^{bright}$ cells that produce large amounts of Th1-type cytokines such as IFN-γ [35].

NK cells expanded by our recently developed culture method [36,37] were found to possess not only the $CD3^-CD56^{bright}$ phenotype with high cytokine production ability but also high cytotoxic activity (Fig. 1). Because NK cells showing high immune reactivity are not commonly observed in the peripheral blood, the roles of such cells *in vivo* need to be further discussed in the future.

NK Cell Immunotherapy of Human Malignancies

As mentioned above, NK cells are immune cells that show cytotoxic activity from the early stage of the *in vivo* defense mechanism against tumor cells and virus-infected cells without being sensitized to the antigens and play a significant role in association with DCs when adaptive immunity starts to become active. In fact, epidemiologic data indicate that cancer is more likely to develop if NK cell activity is low [38]. In addition, NK cell activity in cancer patients is significantly decreased compared with that in healthy individuals [39,40]. NK cell infiltration is a positive prognostic parameter in several cancer types, including

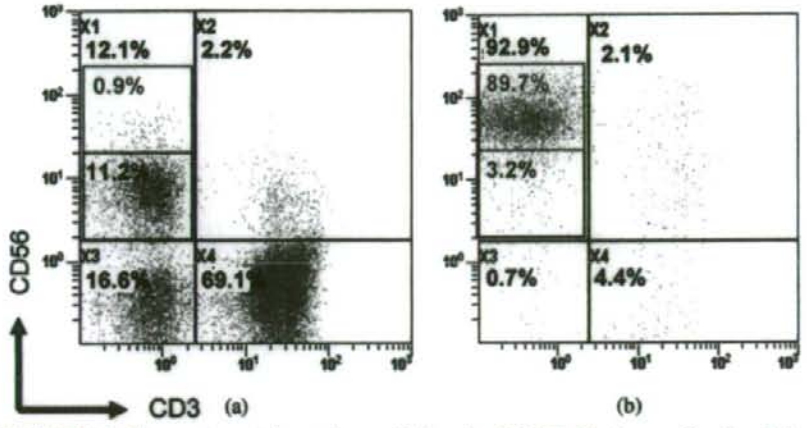

FIGURE 1 Flow cytometric analyses (Cytomics FC500; Beckman Coulter, Miami, FL) reveal the percentages of $CD3^-CD56^{bright}$ NK cells (red squares) and $CD3^-CD56^{dim}$ NK cells (blue squares) in (a) blood and (b) among cells cultured for 14 days.

gastric carcinoma, squamous cell lung carcinoma, and colorectal cancer [41–43]. In addition, we found that human malignant cells diminish and metastasis is inhibited after administration of human NK cells to living mice using the NOG/SCID γc(null) (NOG) mouse model [36,37]. These results suggest a role for NK cells in tumor immunosurveillance and their beneficial effects for many experimentally successful immunotherapy strategies.

Clinical trials using autologous NK cells in metastatic renal cell carcinoma patients resulted in remarkable tumor regressions [44]. A positive treatment effect has also been reported for brain tumors [45]. On the other hand, another report indicated that the efficacy of adoptive transfer of autologous NK cells is limited [46]. The efficacies of NK cell treatments for solid tumors vary among these reports because the activities and numbers of NK cells used differ among the studies. It has also been difficult to culture sufficient numbers of highly active NK cells. Therefore, stable clinical data are not available, and it is necessary to develop a practical NK cell expansion methodology.

In recent reports [36,37], we described a feasible and safe culture method that enables the generation of activated NK cells expanded *ex vivo* by several hundred- to thousand-fold for 2 weeks under good manufacturing practice–conformant conditions (Fig. 2). These cultured $CD3^-CD56^{bright}$ NK cells not only show high cytotoxicity but also express the activation marker CD69 and produce large amounts of IFN-γ

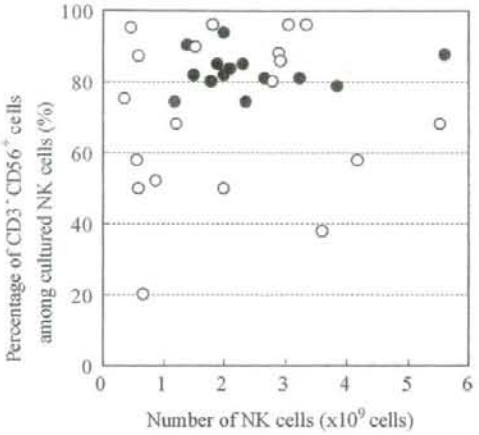

FIGURE 2 The absolute numbers and percentages of NK cells among *ex vivo*–expanded cells from 50-mL blood samples obtained from healthy donors (closed circle) and patients with various stage IV advanced cancers (open circle) after 14 days in culture.

(Fig. 3). The absolute number and cytotoxic activity of these NK cells were 10 times higher than those of cultured lymphokine-activated killer (LAK) cells. Therefore, higher antitumor activity can be expected compared with previous methods. Furthermore, when these NK cells were infused into healthy volunteers, not only NK cells but also T and B cells accumulated in the peripheral blood, and the NK cell activity also increased in the peripheral blood without any adverse effects (Fig. 4). The absolute numbers of $CD3^-CD56^{bright}$ and $CD3^-CD56^{dim}$ NK cells increased several times for a period of 2 weeks after NK cell infusion. The proportion of $CD3^-CD56^{bright}$ NK cell subsets slightly increased in volunteers' NK cells after the infusion of cultured cells. These data suggest that these cells function effectively in tumor immunosurveillance *in vivo*. When we infused autologous NK cells into patients, CT scanning revealed some clinical effects in two cancer patients (Fig. 5). In addition, when the *in vivo* kinetics of infused NK cells were investigated by labeling them with radioactive isotopes, it was confirmed that the cells accumulated in the lungs immediately after infusion and then became distributed to the liver, spleen, and bone marrow. In patients with tumors, infused NK cells were absorbed into the tumors [47]. It is necessary to accumulate a large amount of clinical data for NK cell therapy in order to evaluate NK cell usefulness for cancer and viral immunotherapy.

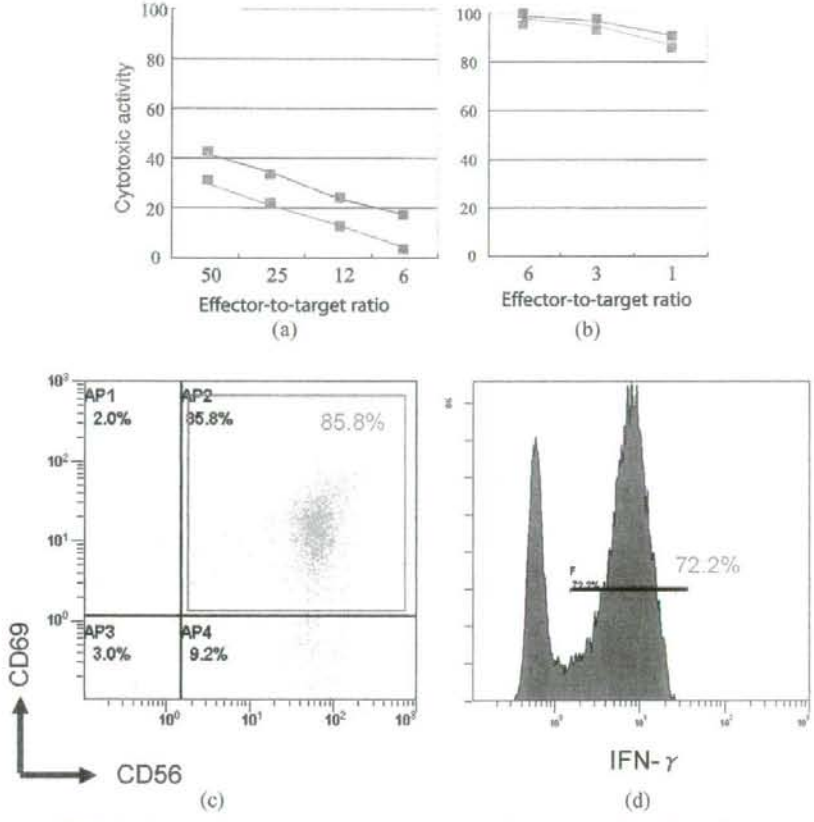

FIGURE 3 Characterization of *ex vivo*–expanded NK cells after 14 days in culture. The spontaneous cytotoxic activities of (a) freshly isolated peripheral blood mononuclear cells from two volunteers and (b) *ex vivo*–expanded NK cells cultured for 14 days were measured against K562 cells at different effector-to-target ratios in a calcein-AM release assay using a TERASCAN VP (Minerva Tech., Tokyo, Japan) as previously described (35). (c) Increased expression levels of CD69, an activation marker for NK cells that is not expressed on NK cells in blood, and (d) induction of intracellular IFN-γ after incubation with 50 μg phorbol 12-myristate 13-acetate, 1 μM ionomycin, and 1 μM monensin for 6 h in *ex vivo*–expanded NK cells were measured by flow cytometry.

LYMPHODEPLETION IN NK CELL THERAPY

Recently, performance of systemic lymphodepleting chemotherapy prior to concomitant therapy of adoptive immunotherapy with tumor-reactive T cells and IL-2 administration was reported to make the

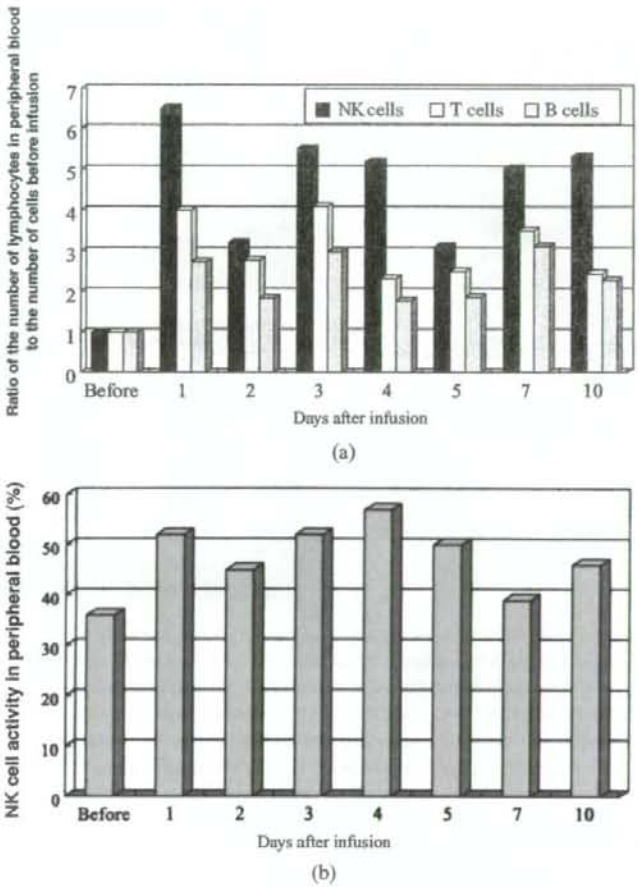

FIGURE 4 Safety and immune responses of autologous activated NK cells. Autologous activated NK cells (2×10^9) were transfused into two healthy donors, and blood was collected at various time points. (a) The changes of the number of lymphocytes in the peripheral blood are shown as the ratio of cells relative to the number of cells before infusion. The number of cells before infusion was set as the baseline value. (b) The NK cell activities in peripheral blood lymphocytes were measured as described in Figure 3.

concomitant therapy more efficient, and this finding is drawing attention [48]. Removal of $CD4^+CD25^+$ regulatory T cells (Treg) that show inhibitory actions in immune responses accounts for the effectiveness of lymphodepletion. In cancer patients, increased Treg inhibit not only the function of tumor-reactive T cells but also the function of NK cells

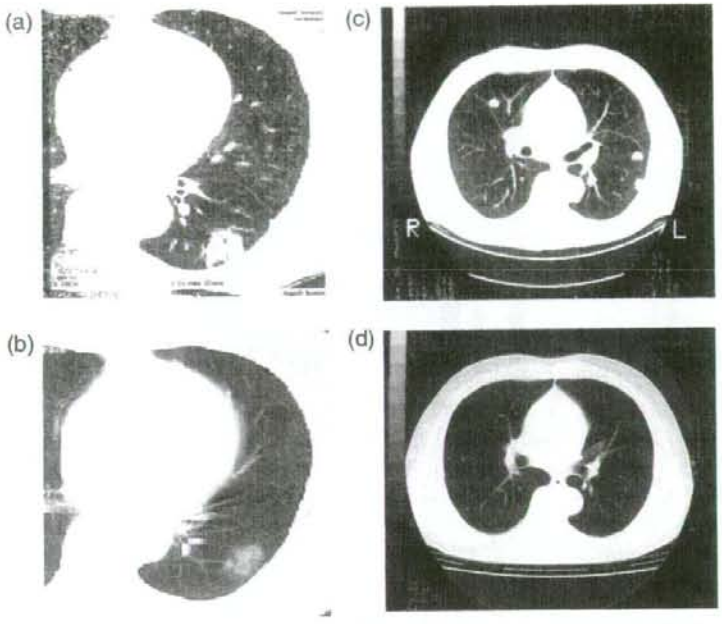

FIGURE 5 Antitumor effects of autologous activated NK cells. (a, b) Images for a 61-year-old female patient with pulmonary adenocarcinoma. (c, d) Images for a 58-year-old male patient with lung metastasis of renal cell carcinoma. Both patients refused standard treatments such as surgical operations, chemotherapy, and radiotherapy. After receiving informed consent from the patients, autologous activated NK cells (2×10^9 cells/injection) were transfused into the patients via 6 injections at 2-week intervals. The effects of the infusions were evaluated clinically by computed tomography scanning before the first cell infusion (a, c) and after the last cell transfusion (b, d).

[49,50]. Current clinical immunosuppressive agents have little effect on NK cell function [51]. We recently reported the possibility of Treg depletion using mild hyperthermia [52]. The results suggest that the effect of NK cell–based adoptive immunotherapy can be intensified by removing Treg or inhibiting their function using an immunosuppressant or hyperthermia, without performing lymphodepletion [51–54].

ALLOGENIC NK CELL THERAPY

Allogenic NK cells, which do not accept inhibitory signals, damage tumor cells more effectively than do autologous NK cells. Acute myeloid leukemia (AML) relapse can be completely controlled by transplants

of KIR-incompatible, T-cell-depleted alloreactive donor NK cells [55]. At the present time, graft-versus-host disease is also controlled in the transplanted patient. Moreover, the remission rates were significantly high when KIR-mismatched alloreactive NK cells were used in studies on the transfer of haploidentical NK cells [56]. Higher sensitivity against KIR-incompatible NK cells is seen *in vitro* in solid tumors such as renal cell carcinoma or melanoma [57]. Thus, effective treatments can be expected by preparing sufficient amounts of highly purified allogenic NK cells.

THE POSSIBILITY OF NK CELL THERAPY AND FURTHER ISSUES

Although NK cells are recognized as cells that widely attack cells with missing-self molecules, such as tumor cells and virus-infected cells, the details of their action mechanisms remained unknown for a long time. However, the details of NK cell function have been clarified over the past few years by the increased number of molecular biological analyses of receptors and immunologic studies of innate immunity, and the significance of these cells has been re-recognized. Based on new findings regarding NK cells, the possibility of cellular immunotherapy using NK cells has become attractive. However, a sufficiently large amount of clinical data for NK cell therapy is not yet available. In addition, the quality and quantity of immune cell cultures differ according to individual institutions, and it is important to make appropriate evaluations of differences in cell quality, including their safety, after adjustment for each institution [58].

To date, treatments for cancers or viral infections have focused on how to remove the tumor cells or virus from the body using inhibitory drugs. However, *in vivo* immunity should be used to the maximum extent in the future. For this purpose, the possibility of cellular immunotherapy using NK cells has been further introduced through the progress in elucidating their molecular biology and involvement in innate immunity as well as the development of a culture method for NK cells.

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Original article

Statin-induced inhibition of HIV-1 release from latently infected U1 cells reveals a critical role for protein prenylation in HIV-1 replication

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Abstract

Latent infection of human immunodeficiency virus type 1 (HIV-1) represents a major hurdle in the treatment of acquired immunodeficiency syndrome (AIDS) patients. Statins were recently reported to suppress acute HIV-1 infection and reduce infectious virion production, but the precise mechanism of inhibition has remained elusive. Here we demonstrate that lypophilic statins suppress HIV-1 virion release from tumor necrosis factor alpha-stimulated latently infected U1 cells through inhibition of protein geranylgeranylation, but not by cholesterol depletion. Indeed, this suppression was reversed by the addition of geranylgeranylpyrophosphate, and a geranylgeranyltransferase-1 inhibitor reduced HIV-1 production. Notably, silencing of the endogenous Rab11a GTPase expression in U1 cells by RNA interference destabilized Gag and reduced virion production both in vitro and in NOD/SCID/γc^{null} mice. Our findings thus suggest that small GTPase proteins play an important role in HIV-1 replication, and therefore could be attractive molecular targets for anti-HIV-1 therapy.

Keywords: Statins; Prenylation; HIV-1; Rab11a; Small GTPases

1. Introduction

Infection with human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS), is characterized clinically by a long asymptomatic period of latency preceding the development of AIDS. Even during this period of latency, the virus is continuously replicating and causing *de novo* infection. Recent studies using combination anti-retroviral therapy have revealed a population of latently infected cells that are refractory to antiviral therapy, which is believed to be a leading cause of the persistence of infection [1]. Although patients treated successfully with the highly active anti-retroviral therapy (HAART) achieved undetectable levels of virus load, viremia recurred in almost every patient when the drug therapy was stopped, because latent virus in reservoir cells is not susceptible to this anti-retroviral therapy or host immune responses [2,3]. Thus, HIV-1 infection remains incurable and new therapeutic approaches need to be developed.

Recent studies have suggested that lypophilic statins have direct anti-HIV effects. del Real et al. showed that lovastatin reduced acute infection by HIV-1 NL4-3.Luc.R.E. pseudotyped with HIV-R5 or X4 envelopes, but not that by the virus pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G) envelope. Lovastatin treatment of HEK 293T producer cells also reduced HIV-1-X4-enveloped infectious virus production, but not that of VSV-G-pseudotyped virus. The

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proposed mechanism was that statins targeted Rho GTPases and affected the actin cytoskeleton re-arrangement necessary for virus entry or budding [4,5]. It was also reported that statins suppressed virion-associated intercellular cell adhesion molecule 1–leukocyte function antigen 1 interactions that are required for viral entry [6]. Audoly et al., using inhibitory toxins, proposed that small GTP-binding proteins are involved in the assembly of HIV-1 Gag in their acute infection model [7]. Quite recently, Nabatov et al. reported that statins disrupt CCR5 and RANTES expression levels in CD4+ T lymphocytes in vitro and preferentially decrease infection of R5 versus X4 HIV-1 [8]. However, the effect of statins in chronically HIV-1-infected cells and its precise mechanism remain to be uncovered.

Statins, which are used to treat hypercholesterolemia, inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in cholesterol biosynthesis in the liver catalyzing the conversion of HMG-CoA to mevalonic acid [9,10]. In addition to inhibiting cholesterol synthesis, statins also block the synthesis of isoprenoid intermediates such as farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGpp). Both FPP and GGpp serve as important lipid attachments for the post-translational modification of variety of proteins, including heterotrimeric G proteins and small GTP-binding proteins such as the Ras, Rho, Rap, and Rab GTPase family proteins [11,12]. This modification, called protein prenylation, is a common mechanism for membrane association of approximately 0.5% of all intracellular proteins. Prenylation consists of the covalent attachment, via thioether linkage, of a C15 (farnesyl) or C20 (geranylgeranyl) isoprenoid group to a C-terminal cysteine residue in the context of a 'prenylation motif'. Farnesyl and geranylgeranyl moieties can bind covalently to several low molecular weight GTPase proteins, and this binding is catalyzed by three prenyltransferases: farnesyltransferase (FTase), geranylgeranyltransferase-1 (GGTase-I) or geranylgeranyltransferase-2 (GGTase-2, also called Rab GGTase). Thus, inhibition of the mevalonate pathway or geranylgeranyltransferases leads to impairment of protein prenylation.

Protein prenylation is critical for intracellular localization and function of small GTPase proteins. In general, modification with FPP is necessary for proper localization of Ras family proteins, whereas GGpp is required for Rho, Rab, and Rap family proteins. Among them, Rab GTPase proteins form the largest family within the Ras-like GTPase superfamily [13,14]. More than 50 Rab proteins have been identified in mammalian cells. Each Rab is believed to be localized to a specific subcellular compartment, reflecting the complexity and variety of trafficking events found in mammalian cells. Rab proteins, unlike other small GTPases, exhibit a variety of prenylation motifs at their C-termini, containing either one or more frequently, two cysteine residues, both of which are modified by geranylgeranyl groups [15]. It was recently reported that siRNA-mediated silencing of Rab9 expression in JC53 HeLa-derived indicator cells inhibited HIV replication, as did silencing expression of other genes that facilitate the late-endosome-to-*trans*-Golgi vesicular transport [16]. Interestingly, acute HIV-1 replication in JC53 cells was also affected, although less profoundly, by silencing expression of Rab11a. It has been well documented that Rab11a is mainly located on pericentriolar recycling endosomes and plays a key role in regulating vesicle trafficking through recycling endosomes to the plasma membrane as well as in exocytosis [17,18].

Here we investigated the effect of statins on virus production in chronically HIV-1-infected promonocytic U1 cells, and showed a critical role for protein prenylation in the late phase of HIV-1 replication.

2. Materials and methods

2.1. Reagents and cells

Simvastatin and lovastatin were purchased from LKT Laboratories, Inc. (MN, USA), and activated by dissolving in ethanol and treatment with 0.1 M NaOH. The pH was then adjusted to 7.0 with HCl. GGTI-298 and FTI-277 were purchased from Calbiochem (Darmstadt, Germany). Anti-Rab11a monoclonal antibody was purchased from BD transduction laboratories (Japan). The serum derived from an HIV-1-infected patient was described previously [19]. Anti-mouse IgG (H&L), anti-human rabbit HRP-linked antibody was obtained from American Qualex manufactures (CA, USA). DMRIE-C reagent for transfection was purchased from Invitrogen (CA, USA). All other reagents including anti-tubulin (T-9026) monoclonal antibody, squalene, GGpp, cycloheximide, TNF-α and phorbol-12-myristate-13 acetate (PMA) were purchased from Sigma (MO, USA). U1 and HEK 293T cells were grown in RPMI 1640 and DMEM, respectively, supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml of penicillin and streptomycin at 37 °C.

2.2. Treatment and stimulation of cells

Cells were treated with or without simvastatin or lovastatin for 2 days and equivalent numbers of viable cells were stimulated with TNF-α or PMA for additional 2 days in the presence or absence of statins, and then intracellular and extracellular Gag (p24 and p55) antigen was quantified. More than 80% of cells were found viable after treatment with 1 μM of simvastatin, and we normalized the levels of Gag protein (p24 and p55) based on the number of viable cells in each sample. The amount of Gag per viable cell was calculated by dividing the Gag value with the number of viable cells. In some of the experiments, GGpp (1 μM), squalene (50 μg/ml) or GGTI-298 (1 μM) was added during the entire course of the experiment.

2.3. HIV-1 Gag quantification

Culture supernatant was collected after centrifugation and subjected to quantification of the HIV-1 Gag (p55 and p24) antigen by automated enzyme-linked immunosorbent assay (ELISA) (Fuji Rebio Inc., Tokyo, Japan). Cell pellets were

washed three times with PBS, re-suspended with the p24 lysis buffer (0.5% Triton X-100 in PBS), put on ice for 30 min, and then the Gag antigen was quantified by using auto-ELISA system. The amount of Gag was normalized by dividing the Gag value with the number of viable cells. The relative amounts of Gag were expressed as percentages of that for cells simply stimulated with TNF-α or PMA (arbitrarily set at 100%). The ratio of Gag amount in culture supernatant to that in cells was calculated by dividing the normalized Gag amount in supernatant with that in cell lysate.

2.4. Western blotting

Cells were lysed in a lysis buffer (20 mM Tris–HCl [pH 8.0], 150 mM NaCl, 1% Triton X-100, 10% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethanesulphonylfluoride (PMSF), 0.1% aprotinin, and 0.1% leupeptin) for preparation of whole-cell extracts. Thirty micrograms aliquots of protein, determined by the Bradford assay, were resolved by SDS–PAGE and detected by standard immunoblotting procedures using the specific primary antibodies.

2.5. Transmission electron microscopy

Cells were fixed with 2.5% glutaraldehyde in PBS for 2 h, washed and fixed overnight at 4 °C in the same buffer and post-fixed with 1% OsO_4 buffered with PBS for 2 h. The cells were then dehydrated in a graded series of ethanol and embedded in Epon 812. Ultrathin (90 nm) sections were cut on an ultracut S microtome (Reichert, Vienna, Austria), double-stained with uranyl acetate and lead citrate, and then examined by transmission electron microscopy (H-7100, Hitachi, Hitachinaka, Japan).

2.6. Lentivirus vectors

Annealed oligonucleotides containing the targeting *rab11a* (5′-GAGCGATATCGAGCTATAA-3′) or *Renilla luciferase* (5′-GTAGCGCGGTGTATTATAC-3′) sequence were first inserted immediately downstream of the H1 promoter of the pSuperRetro vector (Oligoengine), generating pSR-Rab11a-i and pSR-Ctrl-i, respectively. The shRNA expression cassettes were then transferred to a newly constructed lentivirus vector, pCS-puro-PRE, carrying a puromycin resistance gene expressed under the control of the phosphoglycerate kinase (PGK) promoter. Construction details for pCS-puro-PRE will be described elsewhere (Saitoh et al., unpublished). EcoRI–XhoI fragments containing the H1 promoter and targeting sequence from pSR-Rab11a-i or pSR-Ctrl-i were inserted between the EcoRI and XhoI sites of pCS-puro-PRE, generating pCS-puro-Rab11a-i and pCS-puro-Ctrl-i, respectively.

2.7. Transfection and infection

The VSV-G-pseudotyped lentivirus was produced by co-transfection of HEK 293T cells with pCS-puro-Rab11a-i or pCS-puro-Ctrl-i, pHCMV-VSV-G encoding the vesicular stomatitis virus glycoprotein (VSV-G) and a packaging construct pCMVΔR8.2 (a kind gift from ISY Chen, USA), using FuGENE 6 (Roche Diagnostics, IN, USA) according to the manufacturer's instructions. Culture supernatants of 293T cells were collected 48 h post-transfection, filtered through 0.20-μm pore-size filters, supplemented with polybrene (10 μg/ml), and used immediately for infection of U1 cells. Infected cells were selected in the presence of 3 μg/ml of puromycin.

2.8. Animal experiments

NOD/SCID/γc^{null} (NOG) mice were obtained from the Central Institute for Experimental Animals (Kawasaki, Japan). All mice were maintained under specific pathogen-free conditions in the animal center of Tokyo Medical and Dental University (Tokyo, Japan). The Ethical Review Committee of the Institute approved the experimental protocol. NOG mice were inoculated intraperitoneally with approximately 2.5×10^6 Rab11a-depleted or control U1 cells per mouse as described previously [20]. Blood and ascites were examined for HIV-1 p24 amount 2 weeks after cell inoculation.

3. Results

3.1. Statins suppressed HIV-1 release from U1 cells

We used U1 cells that do not constitutively produce or release HIV-1 virions to the culture supernatant. U1 cells are derived from U937 promonocytic cells that survived the cytopathic effect associated with the acute infection by HIV-1 LAI/IIIB. U1 cells contain two integrated copies of proviral HIV-1 DNA and are characterized by low constitutive levels of virus expression that can be up-regulated by several cytokines and phorbol esters. Upon stimulation of U1 cells with PMA or with cytokines such as TNF-α, a dramatic increase in HIV-1 gene expression and robust virion release can be induced. Virions were shown to be released from U1 cells in a manner similar to that for cells of monocytic lineage [21,22]. U1 cells were treated with or without simvastatin for 48 h and then stimulated with TNF-α. We found that treatment of U1 cells with 1 μM of simvastatin, which is within the clinically relevant range, suppressed TNF-α-induced release of p24 to culture supernatant (Fig. 1A). Conversely, intracellular Gag protein (including both p55 and its processed form p24) was increased after the treatment with simvastatin and TNF-α. As a result, the ratio of Gag in culture supernatant to Gag in cell lysate was reduced (Fig. 1B). The release of p24 from cells treated with simvastatin and PMA (Fig. 1A,B) was not profoundly suppressed, but this treatment increased intracellular level of Gag. Essentially similar results were obtained with another lipophilic statin lovastatin (Fig. 1C,D), but not with hydrophilic paravastatin (data not shown), suggesting that lypophilic statins, such as simvastatin and lovastatin, successfully entered cultured cells and worked as HMG-CoA reductase inhibitors.

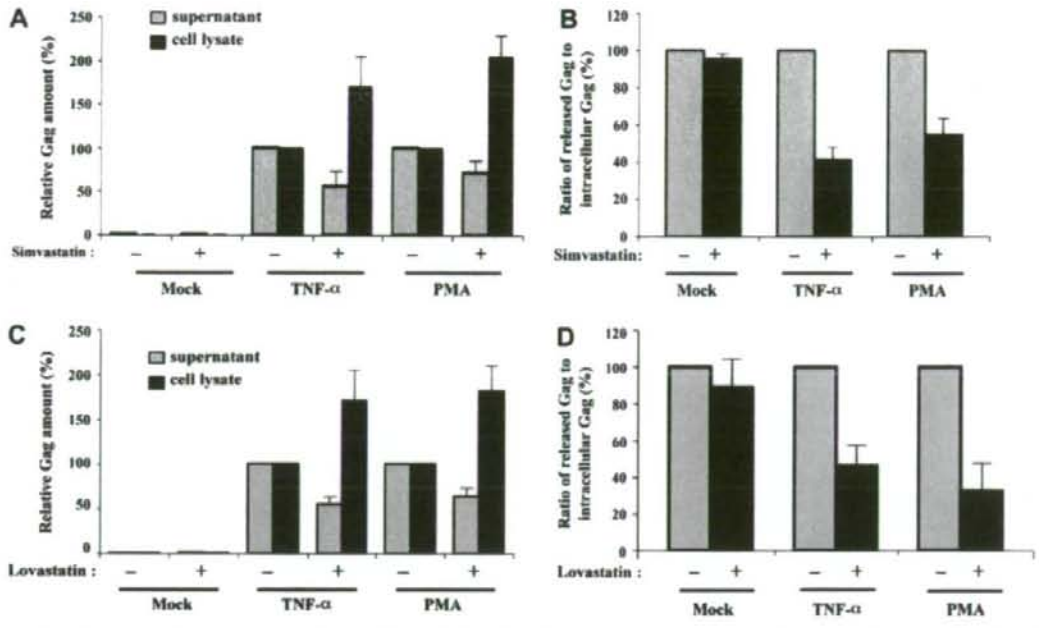

Fig. 1. Statins reduced virus release and increased intracellular Gag in U1 cells. U1 cells were treated or not with simvastatin (A) or lovastatin (C) for 2 days, followed by stimulation with TNF-α (1 ng/ml) or PMA (1 ng/ml) for additional 2 days in the continued presence or absence of simvastatin or lovastatin. Gag (p24 and p55) in culture supernatants (gray bars) and in cell lysates (filled bars) were then quantified. (A) and (C) The relative amounts of Gag in statin-treated (+) cultures are shown in percentage of that of cells (−) stimulated with TNF-α or PMA alone (arbitrarily set at 100%). (B) and (D) Ratios of released Gag to intracellular Gag for statin-treated cells are shown in percentage of the ratio obtained for cells simply stimulated with TNF-α or PMA (arbitrarily set at 100%). Results shown are mean ± SD values of three independent experiments.

3.2. Geranylgeranylation is required for HIV-1 replication in U1 cells

A previous report showed that treatment of 293T cells with lovastatin reduced production of wild type, but not VSV-G-pseudotyped HIV-1, and that this inhibition was reversed by the addition of GGpp [4]. To examine if the reduced p24 release from U1 cells treated with simvastatin resulted from impaired production of geranylgeranyl, we treated U1 cells with simvastatin and TNF-α in the presence of 1 μM GGpp (Fig. 2A). No cytotoxicity was observed after the treatment with GGpp. Addition of GGpp restored the p24 release to the level for control cells stimulated with TNF-α. In contrast, the amount of Gag in cells treated with simvastatin and TNF-α in the presence of GGpp remained higher than that in cells treated with simvastatin and TNF-α. Squalene, one of the metabolites in the cholesterol biosynthesis from FPP, did not interfere with simvastatin-induced inhibition of virion release or intracellular Gag protein accumulation (Fig. 2B). To further investigate the importance of protein prenylation in HIV-1 replication in U1 cells, we tested if geranylgeranyltransferase-1 inhibitor (GGTI) could inhibit virus replication. GGTI was not toxic to U1 cells at 1 μM, whereas farnesyltransferase inhibitor (FTI) was too toxic to be tested in U1 cells (data not shown). GGTI reduced both p24 release and intracellular Gag in TNF-α-stimulated U1 cells (Fig. 2C), suggesting that geranylgeranylation of small GTPase proteins plays a critical role in HIV-1 production.

3.3. Simvastatin enhances intracellular Gag accumulation in U1 cells

We next examined how simvastatin modifies expression of intracellular HIV-1 Gag-related proteins, p55 and its processed form p24. Immunoblotting with anti-HIV-1 Gag antiserum that detected both p55 and p24 revealed that p24 was increased in the presence of simvastatin, while the amount of p55 remained almost unchanged (Fig. 3A). Since the results shown in Fig. 2A suggested the importance of geranylgeranylation, we examined the prenylation status of Rab11a, one of the Rab family small GTPases known to be involved in trafficking of recycling endosomes and exocytosis. As shown in Fig. 3A, treatment with 1 μM simvastatin resulted in almost complete upward shifting of the Rab11a band, indicating accumulation of the non-prenylated form of Rab11a. Besides, GGpp counteracted the simvastatin effect on the prenylation of Rab11a (Fig. 3B). This suggests that simvastatin inhibited the biosynthesis of geranylgeranyl, leading to impaired prenylation of small GTPases involved in intracellular vesicle trafficking. To further gain insight into the effects of simvastatin on HIV-1 replication, we performed transmission electron microscopic (TEM) analysis. Many virus particles were found to be

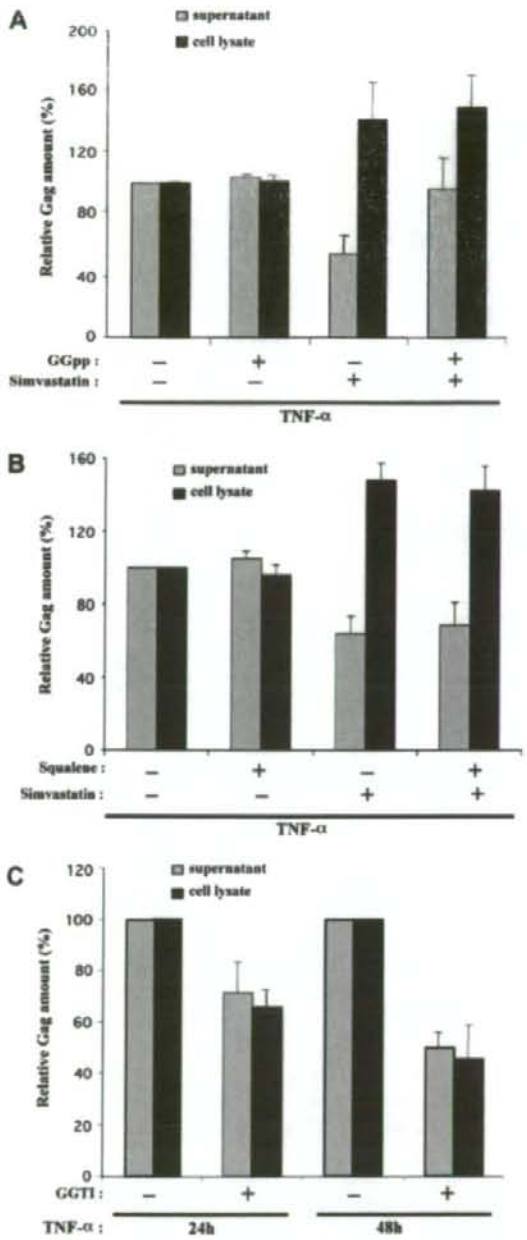

Fig. 2. GGpp restored simvastatin-inhibited virion release, and GGTI suppressed HIV-1 Gag production in U1 cells. U1 cells were cultured for 2 days in the presence (+) or absence (−) of simvastatin, GGpp (A), squalene (B), and GGTI (C). Cells were then stimulated with TNF-α (1 ng/ml) for additional 1 or 2 days, and Gag in supernatants (hatched bars) and cell lysates (filled bars) was quantified. The relative amounts of Gag are shown in percentage of that of cells simply stimulated with TNF-α (arbitrarily set at 100%). Data shown are mean ± SD values of three independent experiments.

released from TNF-α-stimulated U1 cells in the mature form (Fig. 4A,B), but only a few from simvastatin- and TNF-α-treated U1 cells. In contrast, many mature virus particles could be seen in intracellular vesicles of U1 cells treated with simvastatin and TNF-α, whereas it was difficult to find mature virions in vesicles of U1 cells treated with TNF-α alone (Fig. 4C,D). These results suggested impaired release or intracellular trafficking of virions.

3.4. Rab11a mediates HIV-1 replication in U1 cells

In order to further investigate the role of Rab11a in HIV-1 replication in U1 cells, we suppressed the expression of endogenous Rab11a by RNA interference. Immunoblotting analyses (Fig. 5A) demonstrated that the level of Rab11a expression was reduced by ~80–90% in cells expressing Rab11a-specific shRNA (Rab11a-i) compared to cells expressing control shRNA (Ctrl-i). While Rab11a depletion did not affect the growth of U1 cells (data not shown), it reduced the release of p24 as well as intracellular Gag expression induced by TNF-α (Fig. 5B). Immunoblotting analyses revealed that both p24 Gag and p55 Gag are decreased in Rab11a-depleted cells compared to control cells (Fig. 5C). These findings indicate that HIV-1 requires Rab11a for its efficient replication in U1 cells. We next examined if Rab11a depletion affects the stability of Gag, using a protein synthesis inhibitor cycloheximide. As shown in Fig. 5C, the levels of p55 Gag and p24 Gag in Rab11a-depleted cells were generally lower than those in control cells. Importantly, while the expression of p55 Gag remained almost unchanged up to 12 h after CHX treatment in control cells, p55 Gag in Rab11a-depleted cells rapidly decreased with a half-life of ~6 h (Fig. 5D,E). The expression of p24 Gag in Rab11a-depleted cells was lost even more rapidly following cycloheximide treatment, while p24 Gag was only marginally reduced in control cells. These results indicate that Rab11a depletion reduced the stability of Gag, which led to inefficient viral replication in U1 cells.

3.5. Rab11a depletion affects HIV-1 replication in NOG mice

The significant suppression by Rab11a depletion of TNF-α-induced HIV-1 replication in cultured U1 cells prompted us to examine whether depletion of Rab11a in U1 cells can also suppress virus replication in NOG mice. We inoculated Rab11a-depleted or control U1 cells in the peritoneal cavity of immune-deficient NOG mice. Blood and ascites were recovered 2 weeks after inoculation, and then the Gag amounts were determined. Knockdown of Rab11a expression in U1 cells did not apparently influence the growth of cells in mice, but efficiently suppressed HIV-1 replication *in vivo* as revealed by Gag amounts in both serum and ascites (Fig. 6A,B).

4. Discussion

The inhibition of HIV-1 replication by statins was previously reported in acute HIV-1 infection models, and three

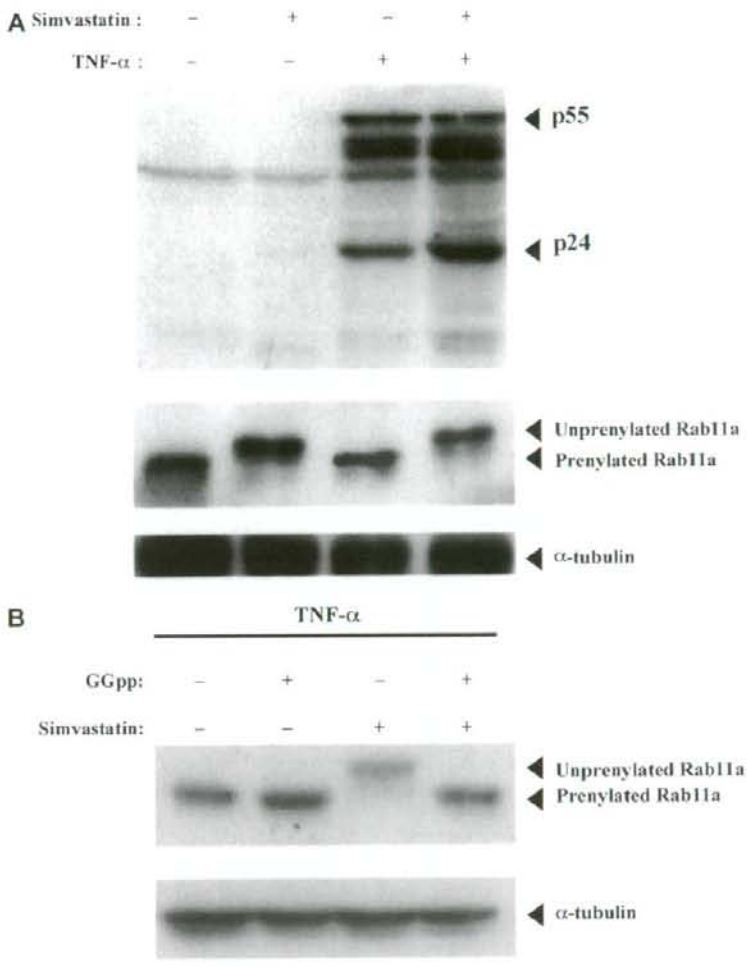

Fig. 3. Simvastatin increased p24 and ablated prenylation of Rab11a in U1 cells. U1 cells were incubated for 2 days with or without simvastatin (A) or GGpp (B), and then stimulated with TNF-α in the continued presence or absence of simvastatin or GGpp for additional 2 days. Whole-cell lysates were prepared and subjected to immunoblotting with serum derived from an HIV-1-infected patient or antibodies to Rab11a or α-tubulin.

different mechanisms were proposed. First, inhibition of HMG-CoA reductase activity resulted in impaired synthesis of GGpp required for prenylation of a small GTPase protein Rho [4]. Second, direct binding of statins to lymphocyte-function-associated antigen 1 (LFA-1) diminished HIV-1 attachment to target cells by preventing the interaction between virion-associated host intercellular adhesion molecule 1 and its natural cell surface ligand LFA-1 [6]. Third, statins disrupted CCR5 and RANTES expression [8]. In this report, we showed statin-induced increase in intracellular Gag and decrease in virus release from chronically HIV-1 infected cells, and defined diminished geranylgeranylation as a principal mechanism of statin-induced inhibition of virus release. The inhibition was associated with nearly a complete loss of prenylation of a small GTPase protein Rab11a, which facilitates vesicle trafficking to the plasma membrane from both the *trans*-Golgi network and recycling endosomes. Indeed, RNA interference-mediated silencing of Rab11a expression also led to a marked reduction in both intracellular and secreted Gag protein. These observations are not limited to TNF-α-induced HIV-1 production in vitro, because the silencing of Rab11a expression also reduced p24 release from U1 cells inoculated in immune-deficient mice.

The effects of simvastatin on HIV-1 replication in U1 cells, increase in intracellular Gag and decrease in virus release, cannot solely be explained by the loss of functional small GTPases involved in vesicle trafficking, because supplementing GGpp in the presence of simvastatin, indeed, restored virus release, but did not normalize the level of intracellular Gag. The increase in intracellular Gag by simvastatin treatment

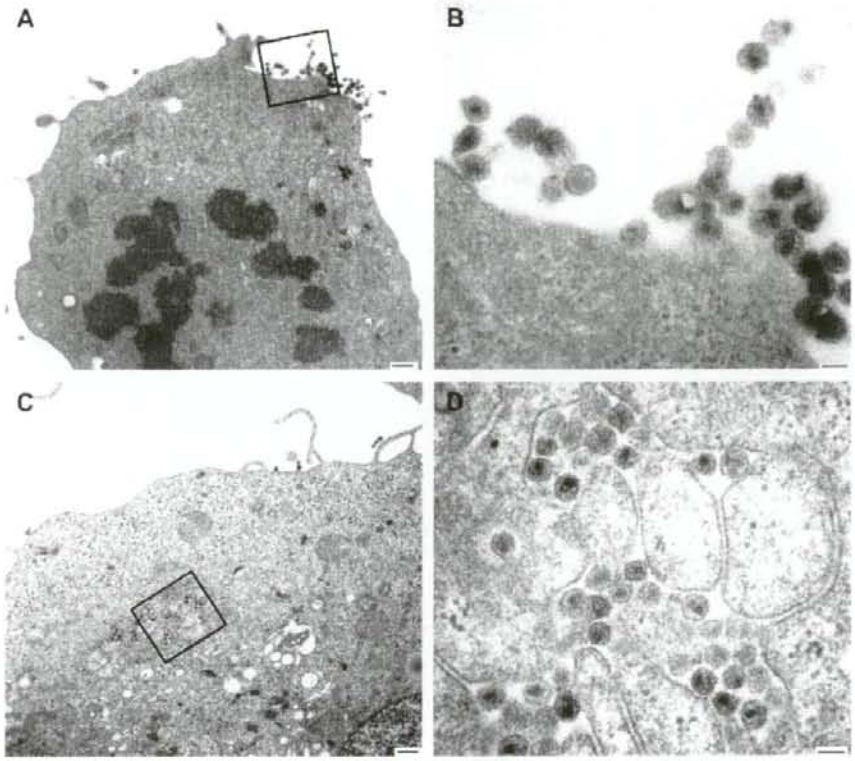

Fig. 4. Transmission electron microscopic (TEM) images of U1 cells. (A) TNF-α-stimulated U1 cell. Many HIV-1 particles are visible at the cell surface. Bar indicates 500 nm (10,000×). (B) Higher magnification of the area indicated by the square in (A). The cone-shaped core structure is evident. Bar indicates 100 nm (60,000×). (C) U1 cell treated with simvastatin and TNF-α. Large vesicles near the Golgi zone contain maturated HIV-1 particles. Bar indicates 500 nm (10,000×). (D) Higher magnification of the area indicated by the square in (C). Bar indicates 100 nm (60,000×).

might simply be a result of accumulation of virions due to impaired virion release, but the results of more specified inhibition by GGTI or gene silencing indicate that loss of prenylation or depletion of Rab11a GTPase reduces both intracellular and extracellular Gag. Thus, simvastatin appears to have yet unknown actions to increase intracellular p24 in U1 cells. In this regard, simvastatin may potentially enhance production of Gag as lovastatin was previously reported to augment HIV-1 LTR-directed transcription in Jurkat cells [4]. The inhibition of virus release by simvastatin would, therefore, be due to loss of prenylation of a yet unidentified protein.

Recent reports support a model of intracellular Gag trafficking common to a variety of cell types in which Gag localizes initially to perinuclear clusters, and then to late endosomes and MVBs and/or MVB-like compartments [23,24]. Both in macrophages and dendritic cells, HIV-1 Gag can be detected in CD63-positive late endosomes and viral exit proceeds through TSG101-dependent budding into the lumen of late endosomes to form multivesicular bodies, followed by the export of viral particles as exosomes [25–28]. However, the transport mechanism of endosomal compartments or MVBs to the cell surface during the course of viral maturation and budding remains to be fully elucidated. Small GTPase proteins have been reported to be involved in vesicle trafficking and actin polymerization. It should be noted that Rab11a is mainly located on pericentriolar recycling endosomes and regulates vesicle trafficking through recycling endosomes to the plasma membrane as well as release of exosomes [29]. In the present report, depletion of Rab11a resulted in an obvious destabilization of Gag p55 and p24, suggesting that Gag failed to traffic through the endosomal compartments or MVBs and could be directed to lysosomal degradation.

Since lipophylic statins cannot be used for HIV-1 infected patients due to its pharmacokinetic interaction with protease inhibitors, inhibiting prenylation of small GTPases involved in Gag trafficking by GGTIs could represent an alternative strategy for effective anti-HIV-1 therapy. GGTI used in this study was previously reported to arrest human tumor cells in G0/G1 and induces $p21^{WAF1/CIP1/SDI1}$ expression in a p53-independent manner and was considered potentially useful in cancer therapy [30]. Perhaps, specific inhibition of individual GTPases involved in HIV-1 replication such as Rab11a would