Ebola viruses constitute a public health threat, particularly in Central and Western Africa. Host cell factors required for spread of ebolaviruses may serve as targets for antiviral intervention. Lectins, TAM receptor tyrosine kinases (Tyro3, Axl, Mer), T cell immunoglobulin and mucin domain (TIM) proteins, integrins, and Niemann-Pick C1 (NPC1) have been reported to promote entry of ebolaviruses into certain cellular systems. However, the factors used by ebolaviruses to invade macrophages, major viral targets, are poorly defined. Here, we show that mannose-specific lectins, TIM-1 and Axl augment entry into certain cell lines but do not contribute to Ebola virus (EBOV)-glycoprotein (GP)-driven transduction of macrophages. In contrast, expression of Mer, integrin αV, and NPC1 was required for efficient GP-mediated transduction and EBOV infection of macrophages. These results define cellular factors hijacked by EBOV for entry into macrophages and, considering that Mer and integrin αV promote phagocytosis of apoptotic cells, support the concept that EBOV relies on apoptotic mimicry to invade target cells.

Keywords: Ebolavirus; macrophage; entry; Mer; NPC-1.
to glycans on GP can enhance GP-driven entry [12]. In addition, certain integrins were found to promote GP-mediated entry, and evidence of GP binding to these factors has been obtained [13]. Furthermore, TAM receptor tyrosine kinases (Tyro3, Axl, Mer) were shown to promote GP-mediated entry [14], possibly by stimulating viral uptake via macropinocytosis [15]. Direct interactions between TAM receptors and GP have not been detected, to our knowledge. Two members of the T cell immunoglobulin and mucin domain (TIM) protein family, TIM-1 and TIM-4, were also shown to promote viral entry, and binding of GP to TIM-1 has been demonstrated [16]. However, binding to GP might not account for TIM-1-mediated augmentation of GP-driven entry. Thus, TIM proteins directly [17] and TAM receptors indirectly [18] bind to phosphatidylserine (PtdSer) on apoptotic cells, and binding to PtdSer on the virion surface was shown to be important for augmentation of GP-driven entry [15, 19–23]. Finally, NPC1 has been identified as an endosomal binding partner for GP [7–9], as outlined above. However, many of the above-mentioned studies focused on the analysis of cell lines, whereas the cellular factors required for entry of ebolaviruses into macrophages, the major viral target cells, are poorly defined and were thus in the focus of the present study.

**MATERIAL AND METHODS**

**Cell Culture**

Cell lines were maintained in Dulbecco’s modified Eagle medium or Roswell Park Memorial Institute 1640 medium, supplemented with 5%–10% fetal bovine serum and antibiotics. THP-1 monocytes were differentiated into macrophages by exposure to phorbol-12-myristate-13-acetate (Sigma). Primary human monocyte-derived macrophages (MDMs) were cultured in monocyte differentiation medium: X-VIVO 10 (Lonza) supplemented with 1% human fibrin-depleted plasma and antibiotics. All cells were grown in a humidified atmosphere at 37°C and 5% carbon dioxide.

**Generation of MDMs**

First, MDMs were prepared from thrombocyte-depleted blood from healthy human donors by means of Ficoll density gradient centrifugation. Subsequently, cells were cultured in monocyte adhesion medium (Roswell Park Memorial Institute 1640 medium with 7.5% human fibrin-depleted plasma and antibiotics), nonadherent cells were removed by washing. The next day, cells were detached, reseeded in monocyte differentiation medium, and cultivated for 7 days. Differentiation into MDMs was controlled by analyzing CD14 expression.

**Plasmids and Antibodies**

Expression plasmids for viral GPs [24, 25], lectins [24, 26], C-type lectin domain family 5 member A (CLEC5A) [27], NPC1 [7], TIM-3 [28], angiotensin-converting enzyme 2 [24], and folate receptor α [29] were described elsewhere. The plasmid encoding Axl was purchased (ImageGenes). Expression plasmids for human TIM-1, TIM-4, and scavenger receptor A (SR-A) were generated by reverse-transcriptase polymerase chain reaction (RT-PCR) and cloning of products into plasmid pCAGGS. The integrity of all PCR-amplified sequences was confirmed by automated sequence analysis. Antibodies against entry factors were purchased from R&D Systems.

**GP-Binding Assay**

293T cells, transfected to express entry factors, were incubated with concentrated cellular supernatants containing comparable amounts (as determined by Western blot analysis) of EBOV-GP1 and severe acute respiratory syndrome coronavirus spike protein, subunit S1 (SARS-S1) fused to the Fc portion of human immunoglobulin, as described elsewhere [24]. After washing, cells were stained with fluorescein isothiocyanate-labeled secondary antibody (Dianova) and analyzed by means of fluorescence-activated cell sorting (FACS).

**Analysis of Small Interfering RNA-Mediated Knock-down of Mer and Scavenger Receptor A Expression**

For Mer expression, THP-1 cells were differentiated into macrophages and small interfering RNA (siRNA) transfected, as described below, and then incubated for 24 hours followed by FACS analysis of Mer expression. For SR-A expression, MDMs were siRNA transfected as described below and lysed in radioimmunoprecipitation assay buffer. Proteins were then separated by sodium dodecyl sulfate gel electrophoresis and blotted onto nitrocellulose membranes (Hartenstein), and SR-A expression was detected with a SR-A–specific antibody and horseradish peroxidase–coupled secondary antibody (Jackson Research). Signal intensities were quantified using the ImageJ software program version 1.47 (National Institutes of Health).

**RT-PCR Analysis**

For detection of TIM-1, Axl, and NPC1 transcripts with quantitative RT-PCR, RNA was extracted from cell lines and MDMs and analyzed using an ABI 7500 FAST real-time PCR system (Applied Biosystems). TaqMan gene expression assays (Life Technologies catalog No. 4448892; assays Hs03054852_g1 [TIM-1], Hs01064444_m1 [Axl], Hs00264835_m1 [NPC1] and Hs99999908_m1 [β-glucuronidase]). The mean cycle threshold (Ct) value for each individual assay was calculated from triplicate measurements and mean Ct values calculated for TIM-1, Axl, and NPC1 were normalized by subtraction from the Ct values obtained for the housekeeping reference β-glucuronidase. Template-free complementary DNA reactions were analyzed in parallel, and no specific signal was detected in any of these experiments.
Transduction Experiments
Lentiviral vectors pseudotyped with heterologous viral GPs (pseudotypes) were generated as described elsewhere [11] and normalized for comparable infectivity for 293T cells. For transduction experiments, target cell lines were seeded in 96-well plates and incubated for 8 hours with vector preparations. The MDMs were spinoculated at 2000 rpm and 21°C for 2 hours and then incubated at 37°C for 6 hours. Subsequently, the medium was replaced by fresh culture medium. Transduction efficiency was determined by quantifying luciferase activities in cell lysates 72 hours after transduction using a commercial kit (Promega). To assess the impact of siRNA knock-down on transduction, the target cells were Lipofectamine 2000 (Invitrogen) transfected with 5 pmol siRNAs (Santa Cruz) at 24 hours before transduction. To determine whether U18666A (Biomol), tannic acid, and mannan (both Sigma-Aldrich) modulated transduction efficiencies, the inhibitors were incubated with target cells for 30–60 minutes before addition of pseudotypes.

Infection Experiments With Ebola Virus
All experiments with replication competent Ebola virus (EBOV, formerly Zaire ebolavirus), strain Mayinga, were performed in the BSL-4 facility of the Institute of Virology at the Philipps-University Marburg. First, MDMs seeded in 8-well chamber slides (ibidi) were transfected with siRNA in duplicates and infected with EBOV at a multiplicity of infection of 10. Inoculum was replaced by fresh culture medium at 1 hour after infection and infection was stopped after 16 hours by treatment of cells with medium containing 4% paraformaldehyde (PFA). After virus inactivation by PFA, cells were permeabilized and stained for nucleoprotein, and nuclei were stained with 4′,6-diamidino-2-phenylindole. To determine infection efficiency, 100 pictures of each well were taken with an automated microscope, and the number of nucleoprotein-positive cells relative to the total number of cells was determined in every tenth photomicrographs.

RESULTS
Directed Expression of Lectins and TIM-1 Increases EBOV-GP–Driven Entry Into Susceptible Cells
TIM proteins and TAM receptor tyrosine kinases are expressed on certain EBOV target cells and were previously implicated in host cell entry of these viruses [10, 14, 16]. To confirm and extend these observations, we compared the ability of lectins, TIM proteins, and the TAM family member Axl to augment EBOV-GP–driven entry into 293T cells, which can be efficiently transduced and are highly susceptible to transduction mediated by ebolavirus GPs [11]. For transduction, we opted for pseudotypes bearing the GP of the highly pathogenic EBOV and an EBOV-GP variant lacking the mucin domain (EBOV-GPΔmuc), which was reported to yield higher vector titers than the wild-type (WT) protein [30].

Expression of the C-type lectins DC-SIGN, DC-SIGN-Related, asialglycoprotein receptor 1 (ASGPR-1), and liver and lymph node sinusoidal endothelial cell C-type lectin; TIM, T cell immunoglobulin and mucin domain; VSV-G, vesicular stomatitis virus glycoprotein.
node sinusoidal endothelial cell C-type lectin (LSECtin) increased EBOV-GP– and EBOV-GPΔmuc–mediated transduction up to 37-fold but had no effect on entry mediated by vesicular stomatitis virus glycoprotein (VSV-G) (Figure 1A), as expected [10, 11, 24, 26]. An increase in transduction efficiency was also observed on expression of TIM-1, whereas expression of TIM-4 and Axl had no effect on transduction mediated by EBOV-GP but slightly enhanced transduction driven by EBOV-GPΔmuc (Figure 1B). Finally, expression of folate receptor α (which does not augment GP-driven entry [29, 31]), TIM-3, and CLEC5A (a lectin that interacts with dengue virus [32]), did not modulate transduction under all conditions tested. These results demonstrate that expression of certain lectins and TIM-1 on a susceptible cell line can increase EBOV-GP–driven entry, supporting a role for these proteins in host cell entry of EBOVs.

**DC-SIGN but not TIM-1 and Axl Bind to the GP1 Subunit of the EBOV-GP**

We next tested whether the entry factors examined in our study interact directly with the GP1 subunit of the EBOV-GP. The expression of all entry factors tested was readily detectable on transfected cells (Figure 2A), and binding of severe acute respiratory syndrome coronavirus spike protein, subunit S1, fused to the Fc portion of human immunoglobulin to DC-SIGN– and angiotensin-converting enzyme 2–expressing cells was markedly above the background signal (Figure 2B), as expected [33]. The EBOV-GP subunit was also able to bind to DC-SIGN, in keeping with published results [10, 11, 33], but binding to the other proteins tested was within the background range, at least under the conditions used (Figure 2B). Thus, augmentation of GP-driven host cell entry by lectins but not by TIM-1 and Axl might rely on efficient binding to the GP1 subunit, in keeping with the concept that the latter factors augment EBOV-GP–driven entry by binding to PtdSer in the viral envelope [20, 23].

**Endogenous TIM-1 and Axl Promote EBOV-GP-Driven Entry Into a Subset of Susceptible Cell Lines**

DC-SIGN is only expressed on a narrow spectrum of target cells of EBOV infection, including dendritic cells and certain tissue macrophages. Therefore, we focused our subsequent analyses on TIM-1 and Axl, which are broadly expressed on cell lines, as confirmed by FACS analysis (Figure 3A), and primary cells. However, a comparison between endogenous TIM-1 and Axl expression on cell lines and susceptibility to EBOV-GP and EBOV-GPΔmuc–mediated transduction revealed no correlation (Figure 3B). Similarly, the effect of siRNA-mediated knock-down of Axl and TIM-1 expression (Figure 3C) on transduction efficiency was dependent on cell type (Figure 3D). Thus, augmentation of EBOV-GP–mediated transduction by Axl and TIM-1 is restricted to certain cell lines, a finding that matches previous results [15, 16].

**NPC1 Expression is Universally Required for EBOV-GP-Driven Transduction**

The NPC1 protein, an intracellular EBOV entry factor, is ubiquitously expressed. We asked whether NPC1, in contrast to Axl and TIM-1, is universally required for EBOV-GP–mediated transduction. Indeed, knock-down of NPC1 expression (Figure 4A) and treatment of target cells with U18666A, an inhibitor that induces
a NPC1 knockout phenotype in treated cells [34], efficiently and specifically reduced EBOV-GP– and EBOV-GPΔmuc–driven transduction (Figure 4B). Moreover, we found that NPC1 messenger RNA (mRNA) was readily detectable in C8166 T
lymphocytes and Raji B cells (Figure 4C), which were resistant to EBOV-GP–driven transduction (Figure 4D), indicating that lack of NPC1 does not account for the well-documented resistance of lymphocytes to EBOV-GP–mediated entry [35].

**NPC1 but Not TIM-1, Axl or Mannose-Specific Lectins Promote EBOV-GP–Mediated Transduction of Macrophages**

Macrophages are targeted early and throughout EBOV infection [36, 37]. Therefore, we next asked whether Axl, TIM-1, and NPC1 contribute to GP-driven transduction of MDMs. Quantitative RT-PCR analysis revealed that MDMs express only low amounts of Axl and TIM-1 compared with the cell lines HeLa and Huh7 (Figure 5A). In keeping with this finding, siRNA-mediated knock-down of Axl and TIM-1 expression had no appreciable effect on EBOV-GP–driven transduction of MDMs (Figure 5B). In contrast, knock-down of NPC1 significantly reduced MDM transduction mediated by EBOV-GP but not VSV-G. Similarly, transduction of MDMs by EBOV-GP–but not VSV-G–bearing pseudotypes was reduced efficiently and in a concentration-dependent manner by U18666A (Figure 5C), indicating that NPC1 might be required for EBOV infection of macrophages. Finally, the mannose-polymer mannan, which inhibits ligand binding to DC-SIGN and related lectins, did not inhibit GP-driven transduction of MDMs, although it markedly reduced transduction of 293T cells expressing DC-SIGN (Figure 5D), indicating that mannose-specific lectins do not promote GP-dependent entry into macrophages.
Evidence That Integrin αV, SR-A and Mer Expression is Required for EBOV-GP–Mediated Transduction of Macrophages

The results obtained so far indicate that, of the previously identified EBOV entry factors, only NPC1 is essential for efficient GP-mediated transduction of MDMs. Notably, 2 factors required for optimal transduction of cell lines, Axl and TIM-1, are intimately involved in the recognition of apoptotic cells [17, 18]. Therefore, we asked whether EBOV-GP might exploit other cellular factors with a comparable activity for macrophage entry. We focused our analysis on integrin αV, CD14, CD36, LOX-1, SR-A, and Mer, all known to be expressed in macrophages and to play a role in the detection of apoptotic cells [38]. Moreover, integrin αV and Mer were previously implicated in EBOV-GP–mediated entry [13, 14]. Transfection of siRNAs directed against integrin αV, SR-A, and Mer expression significantly reduced EBOV-GP– but not VSV-G–mediated transduction of MDMs (Figure 6A, left).
Moreover, a combination of siRNAs against Mer, SR-A and NPC1 reduced transduction more efficiently than the single siRNAs (left) or combinations of siRNAs (right) and transduced with the indicated pseudotypes. Luciferase activities in cell lysates were determined 72 hours after transduction. Transduction of cells transfected with control siRNA (control) was set at 100%. Results on the left represent the mean of 3 (Mer), 6 (SR-A), and up to 15 experiments (NPC1, CD14, CD36, and integrin αV); results on the right, the mean of 3 independent experiments; error bars indicate standard errors of the mean. B, THP-1 cells induced with phorbol-12-myristate-13-acetate were transfected with the indicated siRNAs, and expression of Mer (white bars) was analyzed with flow cytometry. MDMs were transfected with the indicated siRNAs, and SR-A expression (black bars) was analyzed by means of Western blotting. The signals measured were quantified with ImageJ software. Expression of Mer and SR-A in cells transfected with control siRNA was set at 100%. Results represent the mean of 4 (Mer expression) to 6 (SR-A expression) independent experiments.

**Figure 6.** Transfection of macrophages with small interfering RNAs (siRNAs) against Niemann-Pick C1 (NPC1), Mer, and scavenger receptor A (SR-A) reduces Ebola virus (EBOV) glycoprotein (GP)–mediated entry. A, Monocyte-derived macrophages (MDMs) were transfected with the indicated single siRNAs (left) or combinations of siRNAs (right) and transduced with the indicated pseudotypes. Luciferase activities in cell lysates were determined 72 hours after transduction. Transduction of cells transfected with control siRNA (control) was set at 100%. Results on the left represent the mean of 3 (Mer), 6 (SR-A), and up to 15 experiments (NPC1, CD14, CD36, and integrin αV); results on the right, the mean of 3 independent experiments; error bars indicate standard errors of the mean. B, THP-1 cells induced with phorbol-12-myristate-13-acetate were transfected with the indicated siRNAs, and expression of Mer (white bars) was analyzed with flow cytometry. MDMs were transfected with the indicated siRNAs, and SR-A expression (black bars) was analyzed by means of Western blotting. The signals measured were quantified with ImageJ software. Expression of Mer and SR-A in cells transfected with control siRNA was set at 100%. Results represent the mean of 4 (Mer expression) to 6 (SR-A expression) independent experiments. C, HeLa cells, which do not express endogenous Mer or SR-A, were transfected with the respective siRNAs and transduced with the indicated pseudotypes. Luciferase activities in cell lysates were analyzed 72 hours after transduction. Results represent the mean of 4 independent experiment performed with triplicate samples. The transduction of cells transfected with control siRNA was set at 100%. D, MDMs were incubated with the indicated concentrations of tannic acid transduced with the indicated pseudotypes, and transduction efficiency was determined as described above. Transduction of cells incubated with solvent (water) was set at 100%. Results represent the mean of 3 separate experiments performed with triplicate samples. Statistical significance was calculated using the 2-tailed Student t test. *P < .05; †P < .01; ‡P < .001. Abbreviation: VSV-G, vesicular stomatitis virus glycoprotein.
depend on parallel expression of Mer, a scenario in keeping with published findings [39].

To assess the specificity of entry modulation by siRNAs, we investigated HeLa cells, which are negative for SR-A [40] and Mer [41]. NPC1-specific siRNA markedly reduced EBOV-GP–but not VSV-G–driven entry (Figure 6C), as expected. In contrast, siRNA against Mer had no significant effect on EBOV-GP–mediated transduction and slightly augmented transduction driven by VSV-G. Finally, siRNA directed against SR-A reduced EBOV-GP–but not VSV-G–driven transduction by 45% (Figure 6C), suggesting off-target effects. To further investigate the role of SR-A, we asked whether tannic acid, which blocks ligand binding to SR-A [42], inhibits transduction of MDMs. Indeed, tannic acid reduced EBOV-GP–but not VSV-G–mediated transduction in a robust and concentration dependent manner (Figure 6D). Collectively, these results are in keeping with a role of SR-A and Mer in EBOV-GP–dependent macrophage transduction, although off-target effects of the SR-A siRNA cannot be excluded.

**Interference With Mer, SR-A, and NPC1 Expression Reduces EBOV Infection of Macrophages**

It is possible that GP-pseudotyped retroviral particles might not mirror all facets of EBOV entry into host cells. Therefore, we assessed whether the siRNAs directed against Mer and SR-A reduced MDM infection by replication-competent EBOV. We also tested siRNA against integrin αV and NPC1. Transfection of single siRNAs slightly but reproducibly decreased infection efficiency, and a marked drop in the infection rate was observed upon parallel knock-down of Mer, SR-A and NPC1 (Figure 7), indicating that the expression of NPC1, together with several factors involved in the phagocytosis of apoptotic cells, is required for efficient EBOV infection of macrophages.

**DISCUSSION**

The present study confirms that TIM-1 and Axl can promote EBOV-GP–driven entry into certain cell lines and shows that these proteins do not augment entry into macrophages. In contrast, NPC1 was required for entry into macrophages and all cell lines tested. Finally, evidence was obtained that also Mer, integrin αV, and possibly SR-A promote EBOV infection of macrophages.

The comparative analysis of EBOV entry factors revealed that lectins are most efficient at increasing GP-driven entry into already-susceptible 293T cells. However, mannose-specific lectins did not promote GP-dependent transduction of MDMs and have little impact on EBOV infection of monocyte-derived dendritic cells [12]. These in vitro results argue against a major contribution of mannose-specific lectins to EBOV entry into macrophages and dendritic cells in vivo. Nevertheless, one should keep in mind that lectins such as human macrophage C-type lectin specific for galactose and N-acetylgalactosamine, LSECtin, and ASGPR-1, which recognize carbohydrates other than mannose and augment EBOV-GP–driven infection in cell culture [24, 26, 43], might affect viral cell tropism in vivo.

TAM receptor tyrosine kinases and TIM proteins promote entry of ebolaviruses [14, 16] and other enveloped viruses [19, 20, 23, 44] by binding to PtdSer in the viral envelope. Upon expression in 293T cells, TIM-1, but no other TIM proteins or Axl, increased EBOV-GP WT–mediated entry. Augmentation of GP-driven entry upon TIM-1, Axl, and TIM-4 expression was either more pronounced or exclusively observed for EBOV-GPαmuc compared to EBOV-GP WT, suggesting that the presence of the mucinlike domain might modulate augmentation of GP-driven transduction by PtdSer-binding proteins. The inability of TIM-3 to augment entry is due to inadequate presentation of its PtdSer-binding domain [45]. In contrast, it is currently unclear why TIM-1 augments entry driven by EBOV-GP but not VSV-G (and a few other viral GPs), a finding also reported by others [19], and several hypotheses have been proposed [19, 23].

Our finding that endogenous expression of TIM-1 and Axl augments GP-driven entry into a subset of susceptible cell lines matches previous reports [16, 22] and raised the questions whether these factors contribute to GP-mediated entry into macrophages. Quantitative RT-PCR revealed that less Axl and TIM-1 mRNA is produced in MDMs compared with susceptible cell
lines, and siRNA-knock-down showed that expression of these factors is dispensable for GP-driven entry into MDMs. In contrast, Mer but not Axl expression was required for efficient GP-driven transduction and EBOV infection of MDMs. Similarly, Mer but not Axl or Tyro3 was found to be essential for efficient phagocytosis of apoptotic cells by macrophages [46], indicating that Mer is the TAM receptor kinase active in macrophages.

Several previous reports indicate a key role of NPC1 in host cell entry of ebolaviruses [7–9]. Our finding that NPC1 was required for GP-mediated transduction of all target cells tested and was essential for efficient EBOV infection of macrophages suggests that NPC1 may be universally required for host cell infection by ebolaviruses. In this regard, it should be noted that the lymphocytic cell lines examined here expressed robust levels of NPC1 mRNA, suggesting that lack of NPC1 expression does not account for the resistance of lymphocytes to GP-driven entry.

The relatively modest effect of Mer knock-down on GP-driven entry into MDMs and EBOV infection of MDMs suggested that other factors might contribute to these processes, and we speculated that they may also recognize apoptotic cells. Indeed, we found that siRNAs against integrin αV and SR-A reduced GP-driven entry and EBOV infection of MDMs. Integrins αVβ3 and αVβ5 interact with milk fat globule-EGF factor 8, which in turn recognizes PtdSer [47], suggesting that these factors might promote GP-driven entry in a fashion similar to TAM receptor kinases and their PtdSer-binding ligands growth arrest-specific protein 6 or protein S [44]. Such a scenario could be reconciled with a previous study reporting that recombiant αβ1 integrin or antibodies against β1 integrin interfere with GP-driven entry [13]. However, subsequent work indicated that expression of αβ1 integrin is required for full activity of cathepsin L, which processes GP for binding to NPC1 [13, 48]. Integrin αV expression might thus promote macrophage entry of ebolaviruses via several mechanisms.

SR-A is expressed in macrophages and dendritic cells and contributes to the macrophage uptake of a broad spectrum of ligands, including apoptotic cells [49]. EBOV-GP1 did not bind to cells expressing SR-A and directed expression of SR-A did not augment GP-driven transduction (not shown) but treatment of MDMs with SR-A–specific siRNA consistently reduced EBOV-GP– but not VSV-G–driven entry as well as EBOV infection. Furthermore, GP-driven transduction of MDMs was also inhibited by tannic acid and fucoidan (not shown), SR-A ligands [42, 50], arguing for a role of SR-A in entry of EBOVs into macrophages. On the other hand, siRNA against SR-A also reduced GP-driven entry into SR-A–negative HeLa cells, and alveolar macrophages isolated from SR-A knockout and WT mice were equally susceptible to GP-driven transduction (not shown). Further studies are therefore required to fully define the potential role of SR-A in macrophage entry of ebolaviruses. Collectively, our results support the concept that ebolaviruses use apoptotic mimicry for infection of cell lines and macrophages and suggest that the set of factors exploited by the viruses for this purpose may be cell type dependent.

Notes

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