CD4 Incorporation into HIV-1 Viral Particles Exposes Envelope Epitopes Recognized by CD4-Induced Antibodies

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ABSTRACT CD4 downregulation on infected cells is a highly conserved function of primate lentiviruses. It has been shown to positively impact viral replication by a variety of mechanisms, including enhanced viral release and infectivity, decrease of cell reinfection, and protection from antibody-dependent cellular cytotoxicity (ADCC), which is often mediated by antibodies that require CD4 to change envelope (Env) conformation. Here, we report that incorporation of CD4 into HIV-1 viral particles affects Env conformation resulting in the exposure of occluded epitopes recognized by CD4-induced antibodies. This translates into enhanced neutralization susceptibility by these otherwise nonneutralizing antibodies but is prevented by the HIV-1 Nef accessory protein. Altogether, these findings suggest that another functional consequence of Nef-mediated CD4 downregulation is the protection of viral particles from neutralization by commonly elicited CD4-induced antibodies.

IMPORTANCE It has been well established that Env-CD4 complexes expose epitopes recognized by commonly elicited CD4-induced antibodies at the surface of HIV-1-infected cells, rendering them vulnerable to ADCC responses. Here, we show that CD4 incorporation has a profound impact on Env conformation at the surface of viral particles. Incorporated CD4 exposes CD4-induced epitopes on Env, rendering HIV-1 susceptible to neutralization by otherwise nonneutralizing antibodies.

KEYWORDS HIV-1, Env, CD4, incorporation, virus capture assay, nonneutralizing antibodies, neutralization, cold inactivation, Env conformation, CD4-induced antibodies, HIV-1

Human immunodeficiency virus type 1 (HIV-1) entry, mediated by the trimeric viral envelope glycoproteins (Env), is the first step of the viral replication cycle. The Env trimer is the only virus-specific antigen present on the surface of viral particles; as such, it is the target of neutralizing and nonneutralizing antibodies (nnAbs). Env is a highly dynamic molecule that, upon binding the receptor, CD4, transitions from a “closed” conformation (state 1) to an “open” CD4-bound conformation (state 3). CD4 engagement induces an asymmetric intermediate (state 2) adopted on the pathway to state 3 (1–3). The mature HIV-1 Env trimer is derived by proteolytic cleavage of a trimeric gp160 precursor (4, 5) and is composed of the exterior gp120 and transmembrane gp41 subunits. The gp120 is retained on the trimer via noncovalent interactions with the gp41 ectodomain (6–8). The gp120 glycoprotein is responsible for interactions with CD4 (9, 10). CD4 binding triggers conformational changes in gp120 that promote its...
interaction with one of the chemokine receptors, CCR5 or CXCR4 (11–18). CD4 binding also induces conformational changes within the gp41 ectodomain (19–22). The conformational transition of the gp41 ectodomain into a six-helix bundle composed of the HR1 and HR2 heptad repeat regions results in the fusion of the viral and target cell membranes (23–25).

CD4 downregulation is a highly conserved function of primate lentiviruses (26, 27). It has been shown that HIV-1 uses different mechanisms to downregulate CD4 from the cell surface (reviewed in references 28–30). HIV-1 uses its Nef, Vpu, and Env proteins to decrease CD4 cell surface expression. Nef is expressed early during the replication cycle and downregulates CD4 from the plasma membrane by directing the receptor to lysosomal degradation (28, 31–35). Vpu is expressed late in the replication cycle from a bicistronic mRNA also coding for Env. Vpu interacts with newly synthesized CD4 in the endoplasmic reticulum (ER) and induces its degradation through an endoplasmic-reticulum-associated protein degradation (ERAD) mechanism (36–39). The action of Vpu liberates Env from CD4-dependent retention in the ER (40), allowing trafficking in its unliganded form to the plasma membrane.

CD4 downregulation appears to be important for viral replication at different levels (28–30) and was shown to be important for Env incorporation into viral particles, viral infectivity (41–44), and to avoid reinfection of the cell (26, 45–47). CD4 downregulation also prevents exposure of otherwise occluded CD4-induced (CD4i) epitopes, which are recognized by easily elicited nonneutralizing antibodies (nnAbs) (48). In HIV-1-infected individuals, CD4i antibodies are present in different biological fluids, including sera, breast milk, and cervicovaginal lavages (49–52). Some of these antibodies have been shown to possess potent antibody-dependent cellular cytotoxicity (ADCC) activity against cells expressing Env in its “open” CD4-bound conformation (48, 51, 53–56). This “ADCC-susceptible” conformation was recently identified as a fourth Env conformational state named state 2A (57). This new conformation is asymmetric and was shown to be stabilized by a combination of small CD4 mimetics (CD4mc) and two types of CD4i antibodies, anti-coreceptor binding site (CoRBS) and anti-cluster A antibodies. Alternatively, it could be stabilized through Env-CD4 cis interactions. Accordingly, Nef-mediated CD4 downregulation prevented the spontaneous sampling of this antibody-vulnerable conformation at the surface of infected cells (57). This finding raised the intriguing possibility that another functional consequence of HIV-1-mediated CD4 downregulation is to prevent neutralization by otherwise nonneutralizing CD4i antibodies.

Here, using a combination of virus capture assay (VCA), infection, neutralization, and cold-inactivation assays, we have investigated the functional consequences of CD4 incorporation on Env conformation. We report that CD4 incorporation has a significant impact on Env conformation, stabilizing “open” conformational states and increasing the susceptibility of viral particles to neutralization by commonly elicited CD4i antibodies.

RESULTS

CD4 interaction exposes CD4i epitopes on viral particles. To investigate the impact of CD4 on Env conformation at the surface of viral particles, we adapted a previously described virus capture assay (58, 59). This virus capture assay relies on the binding of HIV-1 virions by anti-Env Abs that are immobilized on enzyme-linked immunosorbent assay (ELISA) plates. The viral particles used in this assay are generated by transfecting HEK293T cells with the pNL4.3 Nef− Luc Env− construct (8, 59–61). This construct is cotransfected with a plasmid encoding HIV-1 Env and a plasmid encoding the G glycoprotein from vesicular stomatitis virus (VSV-G), resulting in a virus capable of a single round of infection. Virus-containing supernatants are added to the antibody-coated plate, and unbound virions are washed away. Retention of virions on the surface of the plate by anti-Env Abs is visualized by the addition of HEK293T cells that do not express CD4. Infection of the HEK293T cells is mediated by VSV-G and measured by luciferase activity 2 days after infection. A scheme of the assay is depicted in Fig. 1A.
VSV-G must be present on the virion in order to allow viral infection and subsequent luciferase expression. If only HIV-1 Env is present and that Env is recognized by the capture antibody, the virions are captured but unable to infect HEK293T cells and, therefore, no signal is obtained (Fig. 1B). Similarly, if only VSV-G is present, the anti-Env Abs are unable to capture the virions and, therefore, no signal is obtained. Only the presence of HIV-1 Env and VSV-G on virions results in a signal when using anti-Env Abs, such as 2G12, which recognizes an exposed glycan-dependent epitope on the gp120 outer domain. Since the epitope recognized by the A32 antibody, which targets the gp120 inner domain, is buried in the closed trimer, it fails to capture the virus (Fig. 1B).

Using this virus capture assay (VCA), we evaluated the impact of CD4 incorporation on Env conformation. Briefly, HEK293T cells were cotransfected with pNL4.3 Nef– /H11002 Env /H11002 together with plasmids expressing wild-type (wt) HIV-1JRFL Env or a mutant Env (D368R) unable to engage CD4, VSV-G, and wild-type human CD4 (hCD4) or a mutant CD4 (F43H) impaired in its ability to engage gp120 (48, 62, 63). Released viral particles were collected 2 days after transfection, as described in Materials and Methods. Ninety-six-well plates were coated with anti-HIV-1 Env monoclonal antibodies recognizing the gp120 outer domain (2G12), the V1V2 glycan trimer apex (PG9), CD4-induced gp120 epitopes (17b, A32, C11), the CD4-binding site (VRC03, b12), CD4i gp41 cluster I (F240, QA255.072), anti-HIV immune globulin (HIVIG) (prepared from pooled plasma of asymptomatic HIV-positive donors), and the anti-CD4 OKT4 Ab, which binds to the D3
CD4 incorporation exposes HIV-1 Env CD4i epitopes. VSV-G-pseudotyped viral particles expressing HIV-1 JRFL Env wild-type (A, C) or an Env variant unable to engage CD4 (D368R) (B, D) were produced together with wild-type human CD4 (hCD4) or a mutant CD4 (F43H) that has decreased affinity for gp120. These viral particles were added to plates coated with antibodies targeting different Env epitopes or the anti-CD4 OKT4 antibody. Free virions were washed away, and HEK293T cells were added to the wells. After 48 h, cells were lysed and luciferase activity was measured. Luciferase signals were normalized to those obtained with the 2G12 antibody. Data shown are the mean ± standard deviation (SD) of at least three independent experiments. Statistical significance was evaluated using a paired t test (*, P < 0.05; **, P < 0.01, ***, P < 0.001).

[Graphs showing luciferase activity ratios for different conditions]
CD4i gp120 epitopes. Supporting this observation, the Env D368R variant failed to expose these epitopes despite the incorporation of CD4, as measured by effective capture by OKT4 (Fig. 2B and D). In the absence of incorporated wild-type CD4, the gp41 CD4i epitopes recognized by the F240 and QA255-072 antibodies were more available than the gp120 CD4i epitopes (Fig. 2C). More viruses were captured by these antibodies when wild-type CD4 was incorporated; this effect was nullified by the F43H change in CD4 (Fig. 2C) or by the D368R change in Env (Fig. 2D). These results indicate that the incorporation of CD4 into HIV-1 viral particles leads to CD4-gp120 interaction and increases exposure of CD4i epitopes on Env.

To extend these results beyond the HIV-1JRFL Env, we performed the VCA using viral particles pseudotyped with the HIV-1Yu2 and HIV-1BG505 Envs and obtained similar results (Fig. 3A and B). CD4 incorporation resulted in a significant increase in the interaction of several CD4i Abs with viral particles. As expected, CD4 competed with

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**FIG 3** Exposure of CD4i epitopes on additional HIV-1 strains by incorporated CD4. VSV-G-pseudotyped viral particles expressing HIV-1Yu2 Env (A) or HIV-1BG505 Env (B) were produced with or without human CD4. These viral particles were added to plates coated with antibodies targeting different Env epitopes or the anti-CD4 OKT4 antibody. Free virions were washed away, and HEK293T cells were added to the wells. After 48 h, cells were lysed and luciferase activity was measured. Luciferase signals were normalized to those obtained with the 2G12 antibody. Data shown are the mean ± SD of at least three independent experiments. Statistical significance was evaluated using an unpaired t test (A) or Wilcoxon paired t test (B) (*, \( P \leq 0.05 \); **, \( P < 0.01 \)).
CD4BS Abs for binding, resulting in decreased interaction of VRC03 and b12 with HIV-1YU2. Opening of Env by CD4 also decreased recognition by PG9, an antibody that preferentially binds the closed state 1 conformation. The gp41 epitopes recognized by the F240 and QA255-072 antibodies were exposed in the presence of CD4 on HIV-1YU2 more than on HIV-1BG505. This may relate to the differential trigger-ability of these Envs by CD4. Altogether, these results confirm that incorporated CD4 alters the conformational landscape of Env to sample more open conformations.

CD4 interaction sensitizes viral particles to cold inactivation. For some HIV-1 Env isolates, prolonged incubation on ice results in functional inactivation. It has been suggested that cold inactivation depends on the ability of the HIV-1 gp120 to sample the CD4-bound conformation and is more efficient for Envs that are prone to undergo conformational changes. Accordingly, viral particles bearing Envs in open conformations are more susceptible to this ligand-free inactivation, which can be modulated by the V1V2 and V3 variable regions of gp120. To evaluate whether incorporated CD4 affects the susceptibility of viral particles to cold inactivation, we incubated them on ice for up to 24 h. Briefly, HIV-1 virions encoding a luciferase reporter (pNL4.3 Nef Env Luc) and bearing wild-type (wt) Env from HIV-1JRFL or HIV-1YU2 were incubated for different amounts of time on ice before being used to infect Cf2Th cells expressing CD4 and CCR5. Luciferase activity was measured 48 h later, as previously described. Env-pseudotyped viral particles produced in the absence of hCD4 were resistant to cold inactivation. CD4 incorporation modestly but significantly enhanced virus susceptibility to cold inactivation. This suggests that Env-CD4 cis interaction changes the conformational landscape of Env, resulting in the stabilization of more open and thus cold-sensitive conformations (Fig. 4).

CD4 incorporation sensitizes viral particles to neutralization by CD4-induced antibodies. As our data indicate that incorporation of CD4 into viral particles affects Env conformation, we evaluated whether CD4 incorporation also affected the susceptibility of viral particles to neutralization by ligands that recognize open conformations.

**FIG 4** Incorporated CD4 sensitizes viral particles to cold inactivation. Viral particles pseudotyped with HIV-1JRFL (A) or HIV-1YU2 (B) were produced by cotransfection with or without human CD4. Viral particles were incubated on ice for different amounts of time. At the indicated time points, aliquots were removed and frozen at −80°C. After completion of the longest incubation, all samples were thawed and infectivity on Cf2Th-CD4/CCR5 cells was measured. Data is representative of results from at least three independent experiments, performed in quadruplicate. Data shown are the mean ± SD of at least three independent experiments. Statistical significance was evaluated using an unpaired t test (*, P < 0.05; **, P < 0.01).
We used plasmids encoding full proviruses of the transmitted/founder infectious molecular clones HIVCH58 and HIVCH77, either wild-type (wt) or deleted in their Nef gene (Nef\(^-\)), to transfect HEK293T cells in the absence of or with different amounts of a plasmid encoding human CD4. By doing so, we generated HIV-1 virion particles enriched in CD4. We used these virions to infect CD4\(^+\)/CCR5\(^+\) TZM-BL cells in the presence of increasing quantities of antibodies. In agreement with previous reports (41–43), we observed that CD4 incorporation decreases viral infectivity (Fig. 5A).

Interestingly, CD4 incorporation significantly reduced infectivity of HIVCH58 wt but not HIVCH77 wt viral particles. Thus, HIVCH77 is intrinsically more resistant to the detrimental effects of CD4 incorporation on viral infectivity. This phenotype was modulated by Nef since nef deletion further impaired viral infectivity of HIVCH58 viral particles but also resulted in a significant dose response decrease in viral infectivity for HIVCH77. Never-
theless, under these conditions, a fraction of the viral particles generated in the presence of CD4 remained infectious, allowing us to evaluate their susceptibility to antibodies with different specificities. As shown in Fig. 5B and C, Nef-defective viral particles produced in the presence of the highest ratio of CD4 were modestly but significantly more susceptible to neutralization by pooled plasma from asymptomatic HIV-1-infected donors (HIVIG). Because this phenotype is reminiscent of the neutralization mediated by nonneutralizing CD4i Abs, such as 17b (anti-CoRBS), 19b (anti-V3), and A32 (anti-cluster A), in the presence of subinhibitory concentrations of CD4mc (71–73), we then tested the susceptibility of viral particles to these antibodies. **Fig. 6** shows that low CD4 incorporation, at a ratio of 0.1, is sufficient to render HIVCH58 Nef/H11002 and HIVCH77 Nef/H11002 viral particles, which bear neutralization-resistant tier 2 Envs, susceptible to neutralization by anti-gp120 Abs 17b and 19b. At this ratio of CD4, HIVCH77 Nef viral particles were also susceptible to A32. Intriguingly, higher expression of CD4 restored baseline sensitivity neutralization of Nef-defective viral particles. This could be explained by the impact of CD4 incorporation on viral infectivity (Fig. 5A). As CD4 incorporation increases, viral infectivity is gradually impaired, thus modifying the nature of the pool of infectious viral particles. At higher levels of CD4, incorporated CD4 abrogates viral infectivity; the remaining infectious viral particles might be those that did not incorporate sufficient CD4 to modulate Env conformation, thus explaining why the neutralization goes to baseline. These results suggest that
there is a fine balance between CD4 incorporation, loss of infectivity, Env-CD4 stoichiometry, its impact on Env conformation, and neutralization by CD4i antibodies. Nevertheless, the protective effect of Nef in this system can apparently be surmounted, as coexpression of higher quantities of CD4, at a ratio of 0.5, was sufficient to sensitize the wild-type HIV-1CH77 to neutralization by these nonneutralizing antibodies. Altogether, these results indicate that CD4 incorporation enhances the susceptibility of viral particles to neutralization by otherwise nonneutralizing CD4i antibodies.

**DISCUSSION**

The presence of receptor molecules on the infected cell surface can present problems for enveloped viruses, leading to viral strategies to minimize potential detrimental
effects on virus replication. For example, sialic acid serves as the receptor for the influenza virus, and is bound by its hemagglutinin (HA) protein. Sialic acid is present on many glycoproteins, but influenza neuraminidase (NA) removes it. If the viral neuraminidase is inactivated, influenza aggregates at the cell surface (76) but also HA conformational changes required for fusion are restricted, leading to premature HA inactivation (77).

HIV-1 also put in place different mechanisms to downregulate its receptor from the cell surface. This function is highly conserved among primate lentiviruses (27) and appears to be important for viral replication in T cells (43, 78). Downregulation of CD4 from the surface of infected cells positively impacts viral pathogenesis by virtue of multiple effects. CD4 downregulation has been shown to enhance viral infectivity by facilitating gp120 incorporation (41–44). CD4 downregulation also prevents superinfection and may facilitate the release of viral particles from the infected cell (26, 45–47). CD4 downregulation may weaken the antiviral immune response by limiting CD4 interaction with the major histocompatibility complex class II, which is involved in T cell activation (79).

Another plausible reason to remove CD4 from the cell surface is to limit Env-CD4 interactions which otherwise expose CD4i epitopes recognized by commonly elicited CD4i ADCC-mediating antibodies (reviewed in references 80, 81). It is well established that Envs from primary HIV-1 isolates intrinsically resist sampling the conformations recognized by CD4i Abs. This resistance is likely due to the stability of state 1 in primary Envs, which rarely make spontaneous transitions to conformations recognized by CD4i Abs (2). Soluble CD4 (sCD4) or CD4mc engagement also drive Env into the more open states 2 and 3, rendering them susceptible to CD4i Abs (1, 48, 51, 54, 73, 82). Interestingly, CD4 incorporation into viral particles was recently shown to stabilize more open Env conformations, including state 2A, which is vulnerable to antibody attack (57).

The asymmetric state 2A conformation is characterized by the exposure of gp120 inner domain cluster A epitopes (57). A32 and C11 are well-characterized anti-cluster A antibodies (8, 83–85). These antibodies failed to capture viral particles bearing different primary Envs unless CD4 was incorporated. Using our VCA, we found that CD4 incorporation into viral particles had a significant impact on the conformational equilibrium of four different primary Envs. Indeed, CD4 incorporation facilitated virus capture by antibodies targeting different CD4i Abs located in the V3, CoRBS, cluster A, and gp41 cluster I regions. Exposure of these epitopes was also accompanied by enhanced neutralization sensitivity to different CD4i Abs, such as 17b, 19b, and A32. It is therefore tempting to speculate that Nef-mediated CD4 downregulation represents a viral mechanism to avoid exposure of vulnerable CD4i epitopes at the surface of viral particles. Importantly, these above-mentioned effects were reduced in the presence of Nef, further demonstrating the crucial role of CD4 downregulation in avoiding immune responses. Altogether, our results suggest that targeting the ability of Nef to downregulate CD4 or strategies aimed at modifying Env conformation to expose CD4i epitopes could have therapeutic utility.

MATERIALS AND METHODS

Cell lines and plasmids. HEK293T human embryonic kidney and Cf2Th canine thymocytes (American Type Culture Collection) were grown at 37°C and 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) containing 5% fetal bovine serum (Sigma), 100 units/ml penicillin, and 100 μg/ml streptomycin (Mediatech, Inc.). Cf2Th cells stably expressing human CD4 and CCR5 (expressor and its F43H variant (70)) were grown in phosphate-buffered saline (PBS) overnight at 4°C. Unbound antibodies were removed by washing
twice the plates with PBS. Plates were subsequently blocked with 3% bovine serum albumin (BSA) in PBS for 1 h at room temperature. After two washes with PBS, 200 μl of virus-containing supernatant was added to the wells. After 4 to 6 h incubation, virions were removed and the wells were washed with PBS 3 times. Viral capture by any given antibody was visualized by adding HEK293T cells (10 × 10^4) in full DMEM medium per well. Forty-eight hours postinfection, cells were lysed by the addition of 30 μl of passive lysis buffer (Promega) and three freeze-thaw cycles. An LB 941 TriStar luminometer (Berthold Technologies) was used to measure the luciferase activity of each well after the addition of 100 μl of luciferin buffer (15 mM MgSO₄, 15 mM KPO₄, pH 7.8, 1 mM ATP, and 1 mM dithiothreitol) and 50 μl of 1 mM o-luciferin potassium salt (Prolume).

**Antibodies.** The following antibodies were used: anti-HIV-1 gp120 MAb recognizing gp120 outer domain (2G12) (NIH AIDS Reagent Program), the V1V2 glycan trimer apex (PG9) (Polymun), CD4-induced gp120 epitopes (17b, A32, C11) (NIH AIDS Reagent Program), the CD4-binding site (VRC03, b12), CD4i gp41 cluster I (F240), QA255-072, anti-HIV-1 Env antigen (HIV-1, HXB2, and HCMV, prepared from pooled plasma of asymptomatic, HIV-positive donors obtained from the NIH AIDS Reagent Program), and the anti-CD4 OKT4 Ab which binds to the D3 domain of CD4 (Invitrogen).

**Virus neutralization.** CH50 and CH77 transmitted/founder infectious molecular clones of HIV-1 were produced by calcium phosphate transfection of HEK293T cells together with an expressor of CD4 wt at a weight ratio of 1 provirus/0.1 CD4 or 1 provirus/0.5 CD4. Two days after transfection, the cell supernatants were harvested. The reverse transcriptase activities of all virus preparations were measured, as described previously (67). Each virus preparation was used immediately and was never frozen. Twenty-four hours before infection, TZM-bl cells were seeded at a density of 5 × 10⁴ cells/well in 96-well plates in a 1:10 weight ratio of cell pellets with tissue culture white plates (Perkin Elmer). Luciferase-expressing viruses (10,000 reverse transcriptase units) were incubated for 1 h at 37°C with serial dilutions of Env ligands in a volume of 200 μl. The recombinant viruses were then incubated in quadruplicate with TZM-bl cells. After a 48-h incubation at 37°C, the medium was removed from each well, and the cells were lysed by the addition of 30 μl of passive lysis buffer (Promega) and three freeze-thaw cycles. After the addition of 100 μl of luciferin buffer (15 mM MgSO₄, 15 mM KPO₄, pH 7.8, 1 mM ATP, and 1 mM dithiothreitol) and 50 μl of 1 mM o-luciferin potassium salt (Prolume), the luciferase activity in each well was measured with an EG&G Berthold microplate luminometer LB 96V.

**Cold-inactivation assay.** To assess the effect of cold on virus infectivity, virus preparations equalized for reverse transcriptase activity were incubated on ice for 0, 8, or 24 h, as described (69). At the end of the incubation, aliquots were removed and transferred to a −80°C freezer until infection. To measure the infectivity of the virus, aliquots were thawed at 37°C just before infection of C2Th-CD4/CCR5 cells in quadruplicate.

**Statistical analyses.** Statistics were analyzed using GraphPad Prism version 6.01 (GraphPad, San Diego, CA, USA). Every data set was tested for statistical normality, and this information was used to apply the appropriate (parametric or nonparametric) statistical test. P values of <0.05 were considered significant; significance values are indicated as follows: *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.

**ACKNOWLEDGMENTS**

We thank Julie Overbaugh for the anti-gp41 QA255-072 antibody and for helpful discussions and Christina Ochsenbauer for the pNL4.3 IRES Bal infectious molecular clones.

This work was supported by CIHR foundation grant no. 352417 to A.F. and by NIH grants R01 AI129769 to M.P. and A.F., R01 AI116274 to M.P., P01 AI150471 to A.F. and J.S., and AI124902 and AI145547 to J.S. A.F. is the recipient of an FRQS postdoctoral fellowship.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript. The authors have no conflicts of interest to report.

The views expressed in this presentation are those of the authors and do not reflect the official policy or position of the Uniformed Services University, U.S. Army, the Department of Defense, or the U.S. Government.

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