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Infection of Human Dendritic Cells by a Sindbis Virus Replicon Vector Is Determined by a Single Amino Acid Substitution in the E2 Glycoprotein

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The ability to target antigen-presenting cells with vectors encoding desired antigens holds the promise of potent prophylactic and therapeutic vaccines for infectious diseases and cancer. Toward this goal, we derived variants of the prototype alphavirus, Sindbis virus (SIN), with differential abilities to infect human dendritic cells. Cloning and sequencing of the SIN variant genomes revealed that the genetic determinant for human dendritic cell (DC) tropism mapped to a single amino acid substitution at residue 160 of the envelope glycoprotein E2. Packaging of SIN replicon vectors with the E2 glycoprotein from a DC-tropic variant conferred a similar ability to efficiently infect immature human DC, whereupon those DC were observed to undergo rapid activation and maturation. The SIN replicon particles infected skin-resident mouse DC in vivo, which subsequently migrated to the draining lymph nodes and upregulated cell surface expression of major histocompatibility complex and costimulatory molecules. Furthermore, SIN replicon particles encoding human immunodeficiency virus type 1 $p55^{Gag}$ elicited robust Gag-specific T-cell responses in vitro and in vivo, demonstrating that infected DC maintained their ability to process and present replicon-encoded antigen. Interestingly, human and mouse DC were differentially infected by selected SIN variants, suggesting differences in receptor expression between human and murine DC. Taken together, these data illustrate the tremendous potential of using a directed approach in generating alphavirus vaccine vectors that target and activate antigen-presenting cells, resulting in robust antigen-specific immune responses.

Dendritic cells (DC) are the most potent antigen-presenting cell population and play a major role in the activation of both memory and naïve T cells. Immature DC capture antigen in the periphery and migrate to the draining lymph nodes, where they undergo maturation. Presentation of acquired antigen by mature DC is critical for induction of antigen-specific immune responses (1, 9, 13, 36) and stimulation of protective T-cell responses (3, 10). Transduction of autologous cultured DC ex vivo with gene delivery vectors encoding a desired antigen, followed by adoptive transfer, has been shown to stimulate antigen-specific T-cell responses *in vivo* (45, 46). Unfortunately, the ability to target the DC cell population *in vivo* has been quite limited or has been shown to interfere with DC function or development (5, 17, 20, 23, 32, 39). We rationalized that enhanced delivery of antigen to immature DC may provide an opportunity for improvement of vaccines, particularly for gene-based vaccination approaches.

Toward a goal of improving DC-targeting approaches, we have focused on alphavirus-based vectors. The use of alphavirus vectors for vaccine and gene therapy applications is a rapidly emerging field (15, 42, 44). These RNA-based vectors, known as "replicons" because they retain the replicase functions necessary for RNA self-amplification and high-level expression, can be launched *in vivo* following transfection with plasmid DNA (16, 25) or transduction with replicon-containing particles (30, 35, 37, 48). Infection of cells by alphavirus replicon particles is a receptor-specific event mediated by the viral envelope glycoproteins, E1 and E2, and single amino acid substitutions in these glycoproteins can result in dramatic changes to the biological properties or cell tropism of alphaviruses (8, 31, 32, 34). Packaging of alphavirus RNA replicons into vector particles is accomplished by supplying the envelope glycoproteins in *trans* (35, 42, 44), facilitating the evaluation of specific glycoprotein gene modifications on vector tropism.

Sindbis virus (SIN) is the prototype alphavirus and has a number of attractive features that support its use as a gene-based delivery platform for human clinical applications. SIN is a Biosafety Level II virus, and its replicon-based vectors are propagation-incompetent "suicide" vectors that can be packaged in the absence of detectable replication-competent virus (18, 35, 42, 44, 48), precluding the generation and spread of any infectious virus. In addition, SIN replicon particles can be produced efficiently using stable packaging cell lines (35), an integral component for large-scale development and manufacturing. Alphavirus replicon particles, including those derived from SIN, Semliki Forest virus (SFV), and Venezuelan equine encephalitis virus (VEE), have been shown in numerous preclinical studies to induce robust cellular, humoral, and mucosal immune responses to the vector-encoded antigens (25, 26, 35, 37, 42, 49). Although no direct comparative studies among the various alphavirus replicons have been performed, differences in natural cell tropism are known to exist. For example, the lymphotropic VEE recently was shown to transduce murine DC (32), while SIN and SFV are not lymphotropic. Infection of human DC has never been demonstrated for any of the alphaviruses or their derived vectors.

In this report, we describe the identification of SIN variants

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that are highly efficient for growth in immature human DC. The genetic determinant of human DC tropism was mapped to a single amino acid substitution in glycoprotein E2, and using this information, we demonstrate for the first time the generation of alphavirus replicon particles which can be used to target human DC. Detailed characterization using *in vitro* and *in vivo* systems revealed that the replicon-infected cells maintained their developmental and antigen presentation capabilities, demonstrating the potential utility of the DC-targeted SIN replicons for vaccine applications against infectious and malignant disease.

MATERIALS AND METHODS

DC cultures. Human DC were derived from peripheral blood monocytes purified from the buffy coats of healthy volunteers by using anti-CD14 antibody-coated magnetic beads (Miltenyi, Auburn, Calif.). Immature DC were harvested after 3 to 4 days of culture with interleukin-4 (IL-4) (1,000 U/ml; PeproTech, Rocky Hill, N.J.) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (1,000 U/ml; PeproTech), and >90% of the cells routinely expressed CD1a, as assessed by flourescence-activated cell sorter (FACS) analysis. Maturation of DC was accomplished by the addition of monocyte-conditioned media (MCM) at 30% (vol/vol) for 24 to 48 h (4, 40).

For murine DC, female BALB/c mice 6 to 8 weeks old (Charles River Laboratories, Holister, Calif.) were sacrificed and bone marrow was recovered from femurs and tibiae by flushing with RPMI 1640. Bone marrow was dissociated by pipetting, and the resulting single-cell suspension was cryopreserved. For each experiment, frozen bone marrow cells were rapidly thawed, washed, and cultured in media supplemented with 200 U of murine GM-CSF (PeproTech)/ml. Media and GM-CSF were replenished by demi-depletion on days 3 and 5 of culture. The nonadherent and slightly adherent cells were harvested by gentle pipetting on days 6 or 7 and were 80 to 90% immature DC, as characterized by cell surface staining for $CD11b^+$, $CD11c^+$, $H\text{-}2K^{d+}$, $I\text{-}a^{d\,dim}$, $CD80^{dim}$, and $CD86^{dim}$. In some experiments, DC were further purified by isolation of CD11c-positive cells using anti-CD11c antibody (N418)-coated magnetic beads (Miltenyi). Similar results were obtained using the total DC fraction or $CD11c^+$-selected cells.

Cloning of SIN variants and generation of vector particles. Genomic RNA was extracted from pelleted SIN virions with TRIzol reagent and was used as template for cDNA cloning by reverse transcriptase PCR. Fourteen pairs of oligonucleotide PCR primers based on the published SIN HR strain sequence (43) were used to generate a series of overlapping cDNA clones spanning the SIN genome. PCR products initially were cloned into pRS2 (a pUC19 derivative), and genome sequence data were obtained from three independent clones per fragment. The fragments subsequently were assembled into defective helper packaging constructs and a replicon vector (designated SINCR) essentially as previously described (16, 35). Production and partial purification of vector particles containing the SIN-green fluorescent protein (SIN-GFP) or SIN-*gag* replicons was performed as described previously (35), and vector particles were resuspended in phosphate-buffered saline containing 40 mg of lactose/ml. Endotoxin levels were measured for all samples and were consistently <0.5 EU/ml.

Infection of DC with SIN replicon particles. DC infection with various particle preparations was done in suspension (2×10^5 cells, multiplicity of infection [MOI] = 50, unless otherwise specified), in a total volume of 200 to 500 μl of serum-free media. After a 1-h incubation with continuous rocking, 500 μl of media containing GM-CSF and IL-4 was added. Infection efficiency and cell surface phenotype were assessed 24 h later by FACS, and propidium iodide-negative cells were gated for analysis. Antibodies used for flow cytometry were phycoerythrin (PE) conjugated and purchased from Pharmingen (San Diego, Calif.). For antibody inhibition experiments, SINBV-GFP particles packaged with either DC+ or SFV structural proteins were incubated for 30 min at room temperature with either polyclonal anti-SIN rabbit serum or nonspecific rabbit serum (Sigma, St. Louis, Mo.), both diluted 1:1,000 in phosphate-buffered saline with continuous rocking. DC then were infected with either the SIN particles (MOI, 50 to 100) or SFV particles (MOI, 500) and analyzed by FACS after 24 h.

HIV Gag-specific T-cell hybridoma. The murine T-cell hybridoma 12.2 was generated by fusion of splenocytes from human immunodeficiency virus (HIV) Gag-immunized CB6F1 mice with the BWZ.36 fusion partner, followed by cloning and selection based on IL-2 production in response to antigen-presenting cells loaded with HIV-derived peptides. The 12.2 T-cell hybridoma specifically recognizes the peptide sequence AMQMLKETI (p7g) of the HIV-1 SF2 Gag protein in the context of $H\text{-}2K^d$. The p7g Gag-specific T-cell hybridoma 12.2 was plated at 10^5 cells/well in 96-well, U-bottom microtiter plates. Various numbers of DC were added to wells in volumes of 100 μl, for a total culture volume of 200 μl. As a positive control, DC from each treatment condition were assayed in the presence of 1 ng of a p7g peptide (AMQMLKETI)/ml, as well as in media alone. Negative control wells containing DC or the T-cell hybridoma alone were also included in each experiment and reliably yielded <20 pg/ml of IL-2. Each experimental condition was assayed in duplicate. After coculture at 37°C for 1 day, supernatants were removed and assayed for IL-2 production by enzyme-linked immunosorbent assay according to the manufacturer's instructions (Endogen, Woburn, Mass.). The 12.2 T-cell hybridoma produces IL-2 in a dose-responsive manner upon coculture with p7g peptide-loaded $H\text{-}2K^d$ DC and is unresponsive to DC loaded with another Gag-derived peptide, SQVTNPANI.

DC infection *in vivo*. Female BALB/c mice (5 animals/group; Charles River, Charles River, Mass.) were inoculated intradermally with 2×10^6 to 8×10^6 particles in a 25-μl volume in each ear. For trafficking studies, rhodamine (2%) was applied epicutaneously at the site of injection as previously described (14, 47). After 24 h, the draining lymph nodes were collected, embedded in OCT resin, and snap-frozen, and 5-μm-thick sections were prepared for either immunohistostaining or analysis with a laser scanning cytometer (LSC; Compucyte, Cambridge, Mass.). The LSC scans sections with multiple-wavelength lasers to excite fluorochrome-labeled probes, and the detected fluorescent signals are digitally integrated on a per-cell basis (21). Fluorescence signals are converted into histograms defining individual cells in the entire tissue section, permitting quantitative and spatial analysis at a level of sensitivity superior to that of conventional fluorescence microscopy. For analysis, the GFP signal was gated and rhodamine-positive cells were analyzed in the second channel. Appropriate controls were included to establish gates for positive signals. Confocal microscopy was performed using an LSM 410 machine (Carl Zeiss). For single-cell analysis, draining lymph nodes were gently disaggregated by careful dissection, followed by digestion with collagenase (CLS4, 1.6 mg/ml; Worthington Biochemical, Lakewood, N.J.) and DNase I (0.02 mg/ml; Boehringer Mannheim, Indianapolis, Ind.) at 37°C for 90 min. Positive selection of $CD11c^+$ DC was carried out with N418 magnetic beads and columns according to the manufacturer's instructions (Miltenyi). Cell suspensions were analyzed by FACS using PE-conjugated antibodies from Pharmingen.

***In vivo* immunization studies.** Groups of five CB6F1 mice were immunized with various SIN replicon particle preparations containing a codon-optimized HIV-1 $p55^{gag}$ gene (50) at doses of either 10^5 or 10^3 IU, by subcutaneous and intramuscular routes. The SIN particles were generated using combinations of the SINBV-*gag* or SINCR-*gag* replicons, together with the LP, DC+, or VIGN structural proteins. After 28 days, immunized mice were challenged with 10^7 PFU of recombinant vaccinia virus expressing Gag. Five days postchallenge, the spleens were removed, pooled spleen cell suspensions were prepared, and samples were analyzed by flow cytometry for gamma interferon (IFN-γ) secreting CD8-positive T cells following stimulation with Gag peptides as described previously (50).

RESULTS

Selection of SIN variants in human DC. To derive an alphavirus variant that could target immunostimulatory human DC, we serially propagated SIN in highly enriched precursors of the human myeloid DC subpopulation (38). Immature DC were derived from $CD14^+$ purified human monocytes by culture in GM-CSF and IL-4 (4, 40), and after 3 to 4 days, the cells displayed the characteristic $CD1a^+$ $HLA\text{-}DR^{dim}$ $CD80^{dim}$ $CD14^-$ $CD83^-$ phenotype of immature DC (data not shown). A heterogeneous stock of SIN that had been isolated originally from mosquitoes and passaged minimally in mouse brain and cultured BHK-21 cells was used to sequentially infect immature DC derived from four independent donors, with intermediate plaque purification steps in 293 and BHK-21 cells. This selection strategy resulted in the isolation of two SIN variants with distinct plaque phenotypes in BHK-21 cells. One variant, designated SIN DC+, exhibited a small plaque phenotype and produced virus titers of $>10^8$ PFU/ml in immature human DC. The second variant, designated SIN LP, was a stable large-plaque revertant that consistently appeared at low frequency during plaque purification in BHK-21 cells. Growth of the SIN LP variant was dramatically less efficient in immature human DC, producing virus titers of $<10^5$ PFU/ml.

Amino acid 160 of the E2 glycoprotein is critical for human DC infection. To identify the genetic determinant(s) of SIN that conferred efficient DC infection, we first generated a series of overlapping cDNA clones that encompassed the complete genomes of both SIN variants. Sequence data were compiled from three independent cDNA clones of each region to eliminate any potential PCR artifacts. This sequence analysis revealed that, strikingly, the small-plaque (SIN DC+) and large-plaque (SIN LP) variants differed by only a single amino acid, at position 160 of the envelope glycoprotein E2 (Fig.1). In

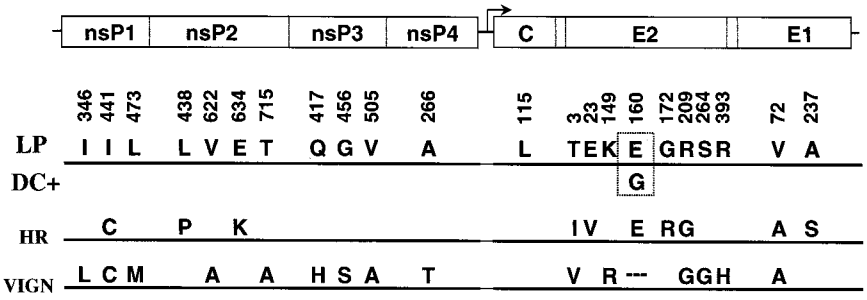

FIG. 1. Sequence analysis of the DC+ and LP strains of SIN. The schematic diagram illustrates gene order and subgenomic promoter for the 11,703-nucleotide genome, but it is not drawn to scale. Codon numbering within each gene is shown only when an amino acid-coding difference exists for at least one of the four strains. Amino acid reference sequences (conventional, single-letter nomenclature) are indicated at each numbered codon for the LP strain, and noncoding nucleotide differences are not shown. The HR and VIGN SIN strains have been described and sequenced previously (16, 43).

the SIN DC+ variant, a glycine residue was encoded at $E2_{160}$, while in the SIN LP variant, glutamic acid was encoded at this residue.

In order to test the hypothesis that a single amino acid substitution at $E2_{160}$ was sufficient for human DC tropism, SIN replicons encoding a GFP reporter (SINBV-GFP) (16) were packaged with either the E2-GLY_{160} or E2-GLU_{160} structural proteins and tested for their ability to infect immature human DC. FACS analysis confirmed that replicons packaged with the E2-GLY_{160} structural proteins efficiently infected DC (Fig. 2A). GFP-expressing cells were positive for the characteristic DC surface marker CD1a, and contaminating precursor $CD14^+$ monocytes were insignificant (<0.1%). In contrast, SINBV-GFP replicons packaged with the LP strain-derived E2-GLU_{160} structural proteins did not infect human DC efficiently (Fig. 2A). These data clearly demonstrated the pivotal role of this single amino acid substitution for DC infection. BHK-21 cells were infected equivalently by the same LP and DC+ SIN-GFP particle preparations (data not shown). Interestingly, a broad distribution of GFP expression was observed in the infected DC population but not in the BHK-21 cells.

We next compared the relative efficiency of structural proteins derived from other SIN strains and also SFV to mediate replicon particle infection of human DC. SINBV-GFP replicons packaged with the structural proteins of our laboratory SIN strain, designated SIN VIGN (16), also infected immature human DC, albeit at a lower, yet reproducible, efficiency than the DC+ SIN particles (Fig. 2B). In comparison, SINBV-GFP replicons packaged with SFV structural proteins infected DC only at a very low efficiency, even when tested at a high MOI. Interestingly, the SIN VIGN strain contains, among numerous other differences, a deleted codon corresponding to $E2_{160}$ (Fig. 1). Although highly adapted by extensive passage in cell lines, the VIGN structural proteins could mediate infection of human DC, presumably as a result of the deleted amino acid at position 160 of E2.

We reasoned that the $E2_{160}$ deletion likely confers some degree of DC tropism to replicon particles packaged with the VIGN structural proteins. To further confirm that the $E2_{160}$ determinant was critical for DC tropism, this codon was deleted in the genetic background of the SIN LP strain. Furthermore, given the relative instability of the DC+ small-plaque phenotype in BHK-21 cells, a deletion of the $E2_{160}$ codon could be genetically stable. As shown in Fig. 2B, $E2_{160}$ codon-deleted particles ($E2\Delta_{160}$) infected human DC with efficiency comparable to that of the DC+ vector particles. Therefore, E2 position 160 is a critical determinant of SIN tropism for immature human DC and is likely integral in determining the structure of E2 that is essential for binding a specific human DC receptor.

Optimized nonstructural proteins enhance replicon efficiency in human DC. The SIN replicon (SINBV) used for the previous studies contained a number of amino acid changes in the nonstructural protein genes compared with other published SIN strains and the variants described here. These differences are likely adaptive and result from extensive cell culture passage of its parental virus, SIN VIGN (Fig. 1). Most of these changes are not present in the nonstructural proteins of the variant viruses (SIN LP and SIN DC+) isolated by passage in primary human DC. To investigate the possible effect of nonstructural protein variation on SIN replicon function in DC, a new vector replicon backbone, designated SINCR, was constructed, based on the genes cloned from the DC+ SIN virus variant (Fig. 1). SINCR replicons expressing GFP were packaged with either the E2-GLY_{160} or E2-GLU_{160} structural proteins and used to infect immature human DC (Fig. 2C). The E2-GLY_{160} structural proteins combined with the new SINCR replicon resulted in an increased number of GFP-positive DC compared to the original SINBV replicon, when both replicons were packaged in the same structural proteins. In addition, more than 50% of the DC scored positive for GFP after infection at an MOI of 80 to 100 with the DC+ SINCR replicon particles, while less than 3% of cells expressed GFP following infection with LP SINCR replicon particles at the same MOI (data not shown). Therefore, the combination of E2-GLY_{160} structural proteins with the optimized SINCR replicon conferred the most efficient infection and expression in human DC.

Immature human DC are preferentially infected by SIN particles and undergo rapid maturation and activation *in vitro*. To characterize the interaction of SIN replicon particles with DC in more detail, we next examined the overall impact of replicon expression on the biology of infected DC. As shown in Fig. 3A, DC+ SIN particles preferentially infected immature DC, in contrast to more differentiated DC (day 7) or activated, mature DC (day 7, posttreatment with MCM), isolated sequentially from developing cultures of human DC in vitro. These data suggest a differential susceptibility to SIN vector infection based on the DC developmental stage. Other cells of the hematopoetic lineage, including primary human T cells, B cells, monocytes, and NK cells, were refractory to infection by DC+ SIN replicon particles, even at a high MOI (>100) (data not shown).

Infection of immature DC was completely inhibited by preincubation of the SIN DC+ particles with a SIN virion-specific neutralizing antiserum (35) (Fig. 3B). In contrast, this serum

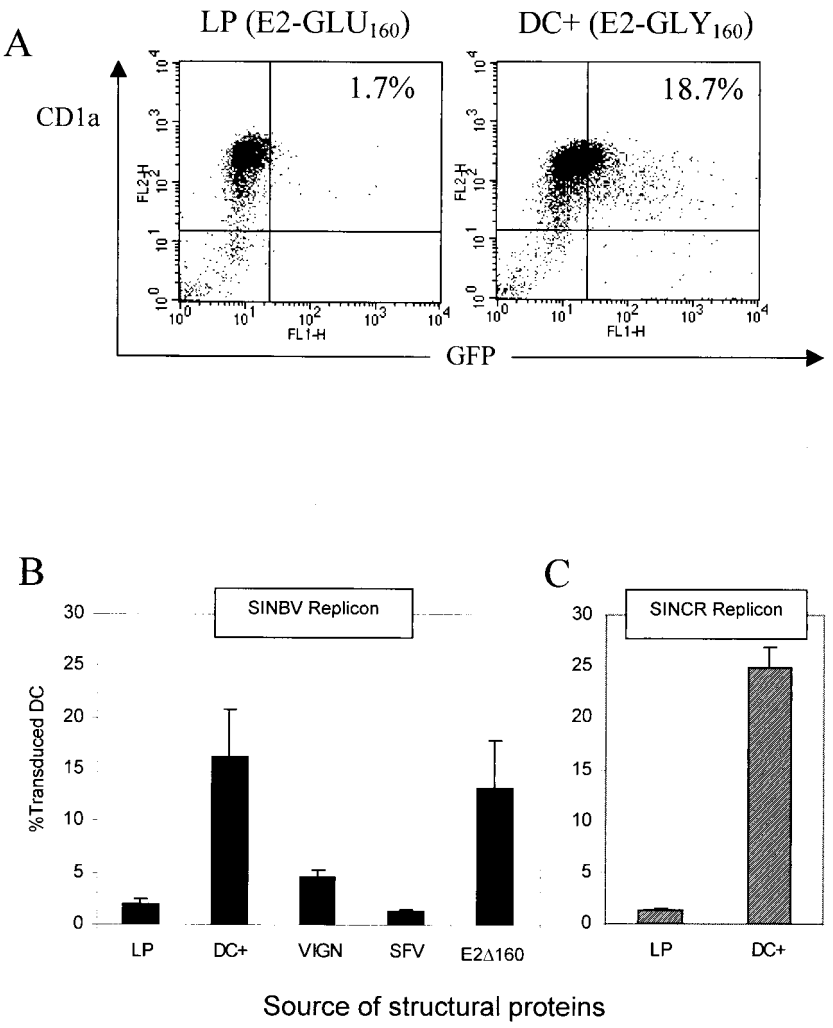

FIG. 2. (A) Infection of immature DC by SIN-GFP replicon particles. Flow cytometric analysis of immature DC 24 h after infection at an MOI of 50 with SINBV-GFP replicons packaged with either LP (E2-GLU_{160}) or DC+ (E2-GLY_{160}) structural proteins. Anti-CD1a-PE antibody was used to identify DC, and only viable cells that excluded propidium iodide were analyzed. Quadrants for analysis were established with a nonspecific isotype-matched control antibody, and mock-infected cells were <0.5% GFP^+. A representative result from at least five experiments with different cell donors is shown. (B) Position $E2_{160}$ is critical for efficient infection of human DC. Infected GFP^+ $CD1a^+$ immature DC were quantified by FACS analysis 24 h after infection at an MOI of 50 with SINBV-GFP vectors packaged in alternative structural proteins. Data (means ± standard error of the mean) were taken from at least four DC donors. (C) In later experiments, the optimized SINCR replicon was used, and data (mean ± standard error of the mean) are from at least four DC donors.

had no effect on infection by replicon particles packaged with SFV structural proteins, which did occur to a limited extent only at a high MOI (≥500) (Fig. 3B). Taken together, these data demonstrated that SIN DC+ infection was mediated by a specific receptor expressed on the surface of immature DC, rather than the result of nonspecific phagocytic uptake.

To evaluate the impact of SIN replicon infection on the immunophenotype of DC, we measured changes in cell surface marker expression by FACS analysis (Fig. 3C). Molecules involved with antigen presentation and DC activation (CD80, CD86, HLA-DR) were rapidly upregulated on infected DC. In addition, surface expression of the DC maturation marker, CD83, was elevated sixfold following infection. These surface modifications closely mirrored the changes induced by MCM and are consistent with virus-induced activation and maturation of immature DC observed after infection with influenza virus (12, 24).

SIN replicon-infected murine DC present expressed antigen to Class I-restricted T cells in vitro and infection is independent of structural proteins. Immature DC capture antigen in the periphery and migrate to secondary lymph organs, where they undergo maturation and activation (3, 10). Mature DC present the processed antigen to naïve T cells, thus eliciting antigen-specific T-cell responses. To demonstrate that SIN replicon-infected DC maintain their ability to process and present antigen, we used replicon particles containing a codon-optimized $p55^{gag}$ gene from HIV-1 (50) in an *in vitro* murine T-cell stimulation assay. IL-2 secretion from an HIV $p55^{gag}$-restricted T-cell hybridoma was measured after incubation with murine $CD11c^+$ DC that had been infected with LP or DC+ SIN replicon particles expressing $p55^{Gag}$. Both the DC+ and LP SIN replicon particles expressing $p55^{Gag}$ were found to elicit Class I-restricted T-cell responses in an MOI-dependent fashion (Fig. 4), demonstrating that immature DC process and present replicon-encoded antigen to T cells after infection. Importantly, infection of DC with replicon particles encoding GFP or UV-inactivated replicon particles encoding $p55^{Gag}$ did not result in T-cell stimulation (data not shown). Thus, antigen

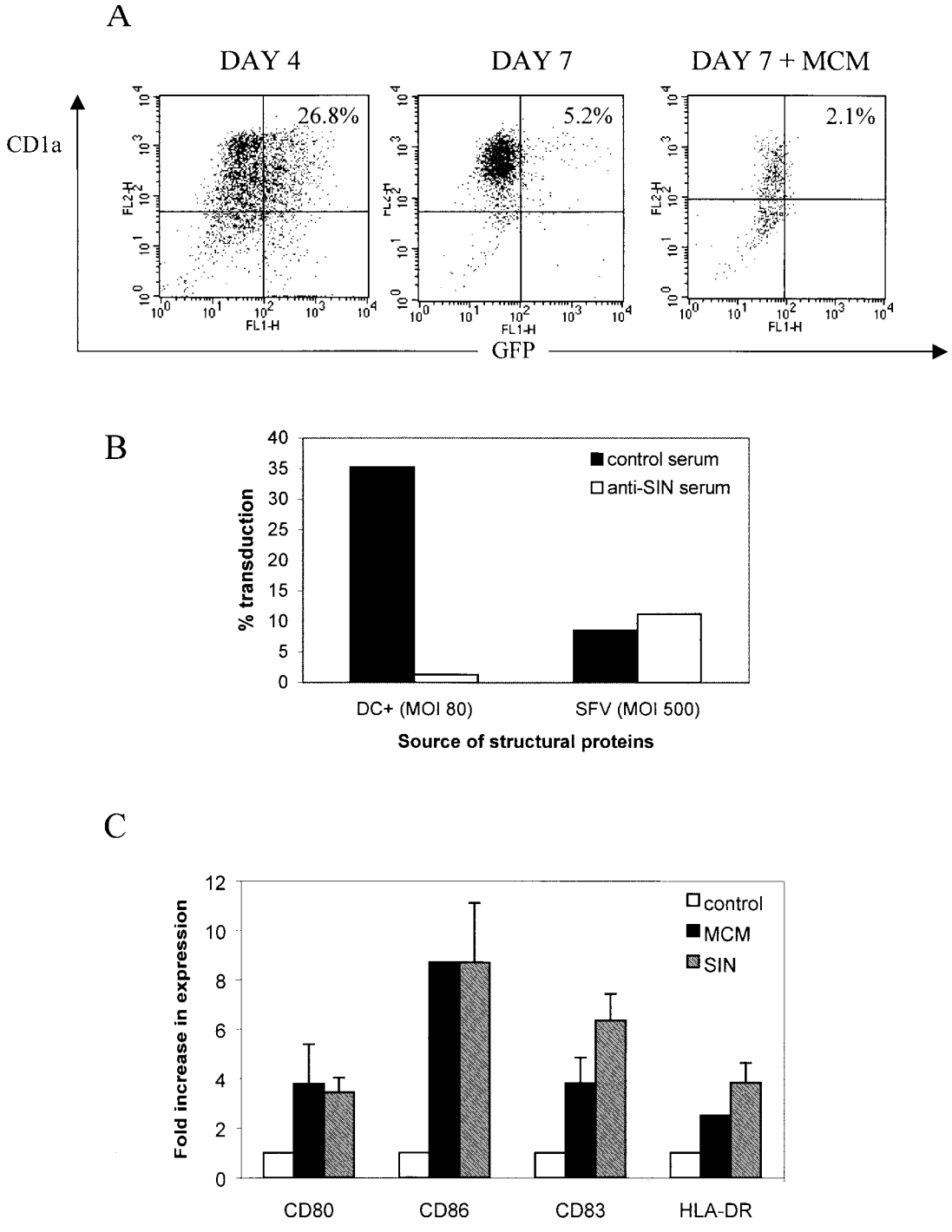

FIG. 3. (A) Differential susceptibility of human DC to SIN replicon particle infection. Developing DC were sequentially isolated from the *in vitro* culture system and infected on different days at an MOI of 80 with either SINCR-GFP or SINBV-GFP replicons packaged with the DC+ structural proteins. FACS analysis was performed after 24 h as previously described, and data are representative of three experiments. (B) Inhibition of DC infection by anti-SIN neutralizing serum. SINBV-GFP replicon particles were preincubated as described in Materials and Methods and were immediately used to infect DC. Data are from a representative experiment with three DC donors. (C) Activation and maturation of DC following SIN replicon infection. Immature DC were infected with DC+ SIN-GFP particles as described and were analyzed by FACS analysis 24 h later. Mean fluorescent intensity (MFI) of GFP-expressing cells was divided by the MFI of mock-infected DC. MFI from DC following MCM treatment over control MFI is included for comparison. Data are means ± standard error of the mean from at least three independent experiments, and similar data were obtained for SINBV and SINCR vectors.

presentation resulted from SIN replicon expression of $p55^{Gag}$ within infected DC rather than nonspecific uptake and presentation of $p55^{Gag}$ virus-like particles that might have been produced when the SIN replicon particles were packaged. Notably, replicons packaged with either LP or DC+ structural proteins stimulated similar T-cell hybridoma responses, suggesting that infection of murine DC, unlike human DC, was independent of the amino acid at $E2_{160}$. To test this hypothesis

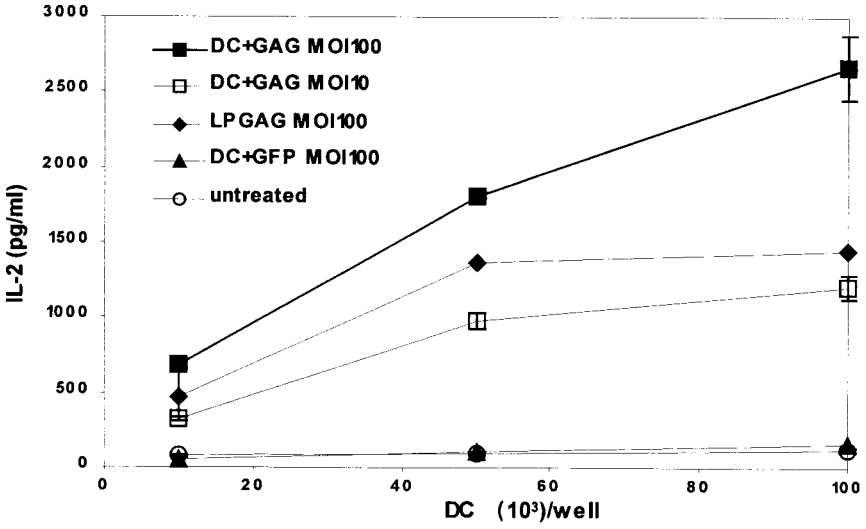

FIG. 4. SIN replicon particles infect murine DC which elicit antigen-dependent T-cell activation. DC derived from BALB/c mice were infected for 2 h with SINCR replicons encoding Gag or GFP, packaged with either LP or DC+ structural proteins. After recovery in medium overnight, CD11c$^+$ DC were collected and cocultured with a MHC-I-restricted, Gag-specific T-cell hybridoma. Supernatants from the cocultures were collected and assayed for IL-2 by enzyme-linked immunosorbent assay. In a parallel experiment, IL-2 production by the T-cell hybridoma in response to peptide-loaded DCs was linear in the range of 100 to 10,000 APCs (data not shown).

directly, SINBV-GFP replicons were packaged with SIN LP, SIN DC+, SIN VIGN, or SFV structural proteins and then tested for their capacity to infect murine DC in vitro. FACS analysis demonstrated that CD11c$^+$ murine DC were infected by replicons packaged with each of the structural proteins equivalently, but that the overall efficiency (4 to 6% at an MOI of 40) was lower than that typically observed for SIN DC+ infection of human DC cells.

Skin-resident DC are infected by SIN replicon particles *in vivo*, leading to activation and migration to draining lymph nodes. While DC+ SIN replicon particles could infect human DC in vitro and both DC+ and LP SIN replicon particles could infect murine DC in vitro, we wished to determine whether these particles could efficiently infect DC in vivo. Immature DC reside in the skin and respond to inflammatory signals and pathogens by modulating chemokine receptors and adhesion molecules that, in turn, initiate rapid migration of DC to the draining lymph nodes. Therefore, we inoculated mice intradermally with SIN replicon particles packaged with a variety of structural proteins and examined the draining lymph nodes 24 h later for DC that expressed GFP. As shown in Fig. 5A, and similar to infection of murine DC in vitro, SINCR-GFP replicons packaged with either LP or DC+ structural proteins efficiently infected murine DC, and comparable numbers of CD11c$^+$ DC expressing GFP were detected in lymph nodes draining the site of infection. Furthermore, a substantial increase in the overall number of CD11c$^+$ DC was observed in draining lymph nodes. In contrast, inoculation with the lab-adapted replicon SINBV-GFP packaged with SIN VIGN structural proteins resulted in fewer infected DC in the nodes.

The increased number of DC in the draining lymph nodes of mice receiving SIN replicon particles (Fig. 5A) suggested that immune activation was occurring in vivo. Therefore, we next examined the immunophenotype of the GFP-positive DC population in the lymph nodes. Similar to the findings for human DC in vitro, SIN replicon-infected DC isolated from nodes of intradermally injected mice expressed variable levels of GFP, but were activated, and expressed elevated levels of surface major MHC histocompatibility complex class II-II) molecules (Fig. 5B) and CD86 (B7-2) (data not shown). The same surface molecules were not upregulated on DC isolated from control animals, reflecting the inactivated state of the secondary immune system.

To confirm that skin-resident DC were infected by SIN replicon particles prior to migration into the draining lymph nodes, rhodamine paint was applied epicutaneously at the site of intradermal injection of replicon particles (13, 47). LSC analysis of lymph node sections 24 h after injection with doses of 10^4 to 10^6 particles revealed that numerous cells were positive for both phagocytic uptake of rhodamine and also GFP expression arising from SIN replicon infection (Fig. 5C). Significantly, more GFP$^+$/rhodamine$^+$ cells were detected in lymph nodes from mice immunized with the DC+ SINCR-GFP replicon particles than with the SINBV-GFP particles packaged with SIN VIGN structural proteins. Confocal microscopy also demonstrated that lymph node cells in parallel-injected mice both were GFP$^+$ and expressed the characteristic interdigitating DC marker, DEC-205 (27) (Fig. 5C, inset).

SIN replicon particles selected in human DC elicit robust T-cell responses in mice. T-cell responses are known to be important for control of many diseases, including HIV (6). We have shown that SIN replicon particles stimulated MHC class I-restricted T cells in vitro independent of structural proteins. Therefore, we wished to determine if a similar activation and antigen presentation profile would be elicited in vivo, using a murine model for HIV-1 vaccination. Mice were immunized with SINCR replicons encoding HIV-1 p55^{Gag} and packaged with the DC+ or LP structural proteins. For comparison, mice were also immunized with the SINBV replicon encoding p55^{Gag} and packaged with the VIGN structural proteins. Robust Gag-specific CD8$^+$ T-cell responses were detected in the spleens from mice (Fig. 6) and demonstrated that SINCR-*gag* replicons packaged with LP and DC+ structural genes elicited similar potent T-cell responses. Gag-specific CD8$^+$ T-cell proliferation was indicative of priming of the MHC-I pathway following replicon expression in DC. In contrast, the levels of Gag-specific T cells were significantly lower in the group that was immunized with the lab-adapted SINBV-*gag* replicon par-

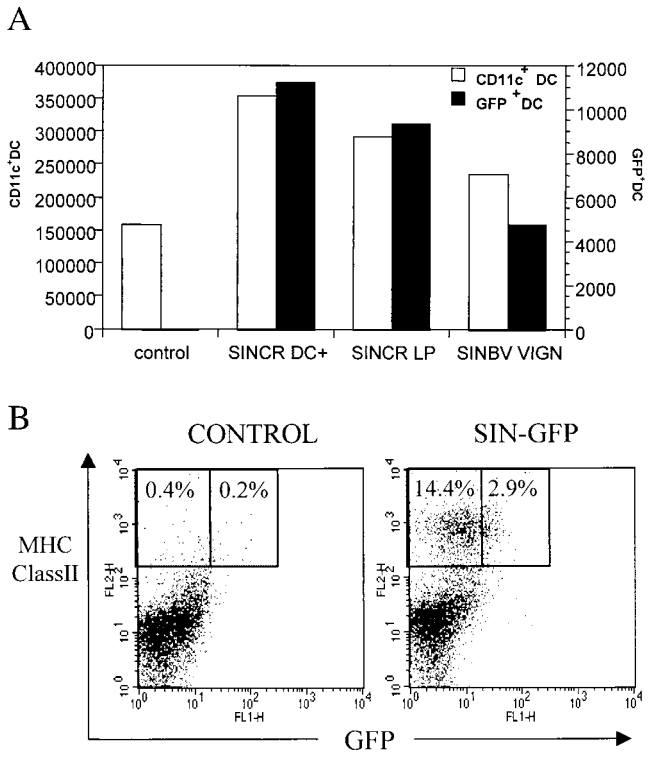

FIG. 5. Infection and activation of DC by SIN vectors *in vivo*. SINCR-GFP replicons packaged with LP or DC+ structural proteins were inoculated in the skin of mice, and draining lymph nodes were analyzed 24 h later. (A) Quantitation of the effect of SIN-GFP inoculation on DC number in the draining lymph nodes. $CD11c^+$ DC (left axis) were prepared by positive selection from single-cell suspensions of draining lymph nodes, and GFP-positive cells were enumerated by FACS analysis (right axis). Data are from groups of five mice and are representative of two experiments. (B) DC in the draining lymph nodes are activated after intradermal inoculation of SINCR replicon particles. $CD11c^+$ DC isolated from the draining lymph nodes following staining with PE-conjugated antibodies recognizing MHC-II (Ia^d) were analyzed by FACS analysis. Quadrants were set using cells from control groups from animals and isotype PE-antibody controls. (C) LSC analysis of lymph nodes following VIGN SINBV-GFP replicon particle or DC+ SINCR-GFP replicon particle injection into rhodamine-painted skin. Double-positive GFP^+/$rhodamine^+$ cells were detected by LSC and fluorescent intensity using gates established with sections from control mice inoculated with buffer alone with and without rhodamine. Fluorescent cells were localized in the lymph node sections, and representative data are shown with each group. (Inset) Confocal microscopic image of lymph node section of a GFP^+/$DEC\text{-}205^+$ DC in draining nodes from mice injected with DC+ SINCR-GFP replicon particles in parallel. Yellow represents the colocalization of GFP (green) and DEC205 signal (red) and is indicative of infected DC.

ticles with VIGN structural proteins. The enhanced efficiency of the SINCR-*gag* particles was most apparent at lower doses (10^3 particles), at which robust $CD8^+$ T-cell proliferation was detected with LP and DC+ replicon particles, but was undetectable with VIGN-based SINBV-*gag* particles (data not shown). These data corroborated the in vivo GFP marking data and presumably reflect enhanced infection and expression.

DISCUSSION

Our results support the exciting potential of alphavirus replicons as gene delivery vehicles for vaccination because we could select SIN variants that not only infect immature DC in vitro and in vivo but also induce DC activation, maturation, and trafficking to lymph nodes. These are requisite steps for antigen presentation to T cells in secondary lymphoid organs

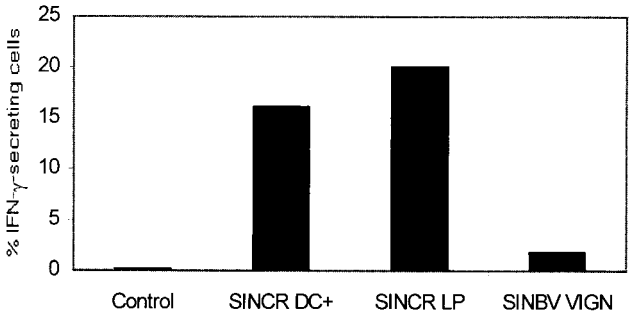

FIG. 6. Induction of $p55^{Gag}$-specific $CD8^+$ T cells by SIN replicon particles. SINCR-$p55^{gag}$ replicons packaged with LP or DC+ structural proteins or SINBV-$p55^{gag}$ replicons packaged with the VIGN structural proteins were injected (10^5 particles per mouse) into CB6F1 mice. Unimmunized mice served as controls. Twenty-eight days after immunization, all mice were challenged with a recombinant vaccinia virus containing *gag-pol* given intraperitoneally at a dose of 10^7 PFU. The numbers of $p55^{Gag}$-specific, IFN-γ-synthesizing $CD8^+$ T cells were determined 5 days later.

and concomitant induction of humoral and cell-mediated immune responses (11, 41). We have shown that robust antigen-specific T-cell responses occur in vivo after a single administration of SIN replicon particles encoding HIV-1 Gag. These hallmarks of DC infection and antigen expression by SIN replicons are in contrast to many widely used viral vaccine vectors, including those derived from adenovirus, vaccinia virus, and herpes simplex virus, which inhibit DC function and development (5, 17, 20, 23, 39).

In order to examine the various aspects of DC development and function after infection with SIN replicon particles, experiments were necessarily performed using a combination of human and murine DC. Mouse models are commonly used to test vaccine delivery approaches, and recently, DC-tropic properties have been identified in mouse-marking studies with another alphavirus, VEE (32). In those studies, infected cells with DC characteristics appeared in lymph nodes draining the site of peripheral infection with VEE replicon particles. However, in contrast to the SIN replicon data from our study, DC infected with VEE were shown to remain immature and inactivated after migration to the lymph nodes, and antigen presentation or immunostimulatory functions were not assessed. In addition, the susceptibility of human DC to infection with VEE replicons was not reported in this study.

To derive a DC-targeted SIN vaccine vector that could be useful for human clinical trials, we elected to utilize primary immature human DC for the selection of variants. The specificity of human DC infection by SIN variants was mapped to a single amino acid residue at position 160 in the E2 glycoprotein. Amino acids 62, 96, and 159 in SIN glycoprotein E2 previously have been defined as a conformational antigenic site (E2c), which is a target for neutralizing antibodies in mice (33). In addition, changes to specific positively charged amino acids at E2 positions 159 and 161 resulted in decreased binding to heparin sulfate and a large-plaque phenotype (8). Single amino acid substitutions at these and other residues in glycoprotein E2 can have profound effects on virus-cell interactions. Our data strongly suggest that the residue at position 160 may play a direct role in determining E2 conformation and binding to a receptor on human DC. Although the identification of a candidate receptor and/or coreceptor for SIN has not been conclusively established (7, 28), it is possible that the DC+ variant glycoproteins mediate binding to a receptor that is differentially expressed in a species-specific fashion on immature human DC. Clearly, usage of a murine DC receptor by the SIN LP variant and also SFV did not translate to efficient human DC infection. This finding illustrates the importance of using relevant cell types to derive alphavirus variants, as murine models may not be predictive of behavior in humans. Preliminary data now suggest that rhesus macaque DC are infected more efficiently by SIN replicons packaged with DC+ structural proteins than with LP structural proteins (data not shown), further emphasizing the importance of testing alphavirus replicon vectors in nonhuman primate models. We have also attempted to determine whether differences in heparin sulfate binding (7, 28) may result from amino acid substitution at $E2_{160}$. Unfortunately, the levels of toxicity for primary human DC encountered in these experiments precluded us from obtaining any conclusive data.

As a gene delivery system for vaccination, SIN replicon particles selected in human DC provide numerous advantages over available systems. In addition to the immunologic features described above, replicon particles can be produced with relative ease in stable packaging cell lines (35) and also have a desirable safety profile (42, 44). The mechanisms underlying the immune response to antigens expressed from SIN replicons, or viral vectors in general, are incompletely understood. It is known that alphaviruses produce large amounts of double-stranded RNA intermediates during cytoplasmic amplification of the RNA replicons (42, 44). Exposure of DC to double-stranded RNA has been shown to induce activation and immunostimulation (12), and this feature of alphavirus vectors may play a role in the overall potency of replicons as vaccine delivery systems. Furthermore, alphavirus-derived vectors ultimately induce apoptosis in the infected cell (22, 29). Our studies indicated that apoptosis did not occur in a timeframe that inhibited DC activation and function. However, the subsequent induction of apoptosis and release of antigen following DC migration to the lymph node may actually enhance alphavirus vector potency via cross-priming of noninfected antigen-presenting cells (2, 19). For gene delivery vectors, the relative contributions of directly infected DC and cross-primed DC to the ensuing immune response is not known. Although gene transfer to DC in vivo is predictive of the degree of T-cell stimulation in mouse models, the equivalent susceptibility of murine DC to infection with LP and DC+ SIN replicon particles permits only qualitative comparison. Further evaluation of the comparative efficacy of these replicon particles will require testing in nonhuman primates, and we will be initiating a rhesus macaque study to address this issue.

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