Supporting Information

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SI Text

Subjects. MPV-immune adults contracted MPV during the 2003 outbreak in Wisconsin (1). VV-immune adults from Oregon were vaccinated with DryVax at 1–16 months before sample collection and VV-naïve subjects were recruited from Wisconsin. Each subject provided informed written consent, signed research authorization forms complying with the U.S. Health Insurance Portability and Accountability Act (HIPAA), filled out a medical history questionnaire, and provided a 50-to 100-ml blood sample that was processed at OHSU. PBMCs were cryopreserved in aliquots and stored in liquid nitrogen. The Institutional Review Board of Oregon Health and Science University approved all clinical studies.

Viruses and Cell Lines. VV (Western Reserve strain), CPV (Brighton strain), and MPV (Zaire strain) were grown on BSC-40 cells. Virus lysates were freeze-thawed three times and sonicated or dounced, debris was pelleted at $820 \times g$, and virus supernatants were purified by centrifugation at $55{,}000 \times g$ for 80 min at 4°C through a 36% sucrose cushion. The virus pellet was disrupted by sonication or using a Duall homogenizer, and purified by layering on a 25–40% sucrose gradient followed by centrifugation at $33{,}000 \times g$ for 40 min. The band containing intracellular mature virus (IMV) was extracted, centrifuged $33{,}000 \times g$ for 40 min through 1 mM Tris (pH 8.0), resuspended in Tris, titered on Vero cells, and stored at −80°C.

EBV-transformed, $CD20^+$ LCL lines were prepared from three subjects and grown in RPMI medium 1640 containing 15% FBS, 2 mM L-glutamine, 1 mM Na pyruvate, 0.1 mM nonessential amino acids, 10 mM Hepes and antibiotics. UV-inactivation of purified VV and MPV was performed by exposure to UV light in a laminar flow hood for 40 min and plaque assays demonstrated that virus titers had dropped below limits of detection (<100 PFU/ml). EBV-LCL were infected with MPV or VV at an MOI of 1 for 15 h before being washed twice and mixed with autologous PBMC in the presence of Brefeldin A to determine the effect on virus-specific T cell responses.

***In Vitro* Stimulation Conditions.** PBMC were cultured in medium alone (uninfected) or infected with purified VV or MPV at an optimized MOI of 0.3 or as indicated in the text. After 12 h of culture, Brefeldin A (ICN) was added at a final concentration of 2 μg/ml for an additional 6 h. In some experiments, Arabinoside C (40 μg/ml) was added at 1 h after infection and maintained throughout the culture period. Instead of infecting PBMC directly with virus, in some experiments PBMC were incubated for 6 h with autologous LCL (uninfected, VV-infected, MPV-infected or VV-infected+MPV-infected) at a ratio of 1:1 in the presence of Brefeldin A. To measure T cell responses independent from antigen processing/presentation, PBMC were cultured with medium (uninfected), VV (MOI of 0.3), MPV (MOI of 0.3), for 12 h and then stimulated with an optimized concentration of soluble (0.04 μg/ml) or plate-bound (0.15 μg/ml, 100 μl per well) anti-CD3 (clone HIT3a, NA/LE; PharMingen) for 6 h in the presence of Brefeldin A. Optimized concentrations of this soluble or plate-bound anti-CD3 antibody induced similar levels of T cell activation (data not shown). To determine whether MPV produced a secreted factor that could inhibit T cell responses, PBMC were incubated with supernatants from autologous LCL (uninfected, or infected with VV or MPV at MOI of 1 for 15 h) for 25 min before addition of soluble anti-CD3 (0.04 μg/ml) and Brefeldin A and incubated for an additional 6 h.

Intracellular Cytokine Staining (ICCS). ICCS was performed as described in refs. 1 and 2. Briefly, PBMC, were cultured at 37°C with 5% CO_2 in RPMI medium 1640 containing 5% heat-inactivated FBS (HyClone), 20 mM Hepes, 2 mM L-glutamine and antibiotics under different *in vitro* stimulation conditions (described above). Cells were stained overnight at 4°C with antibodies specific for CD8β (clone 2ST8.5H7; Beckman Coulter) and CD4 (clone L200; PharMingen). Cells were fixed, permeabilized and stained intracellularly using antibodies to IFNγ (clone 4S.B3; PharMingen) and TNFα (clone Mab11; PharMingen). Samples were either acquired on a FACScalibur flow cytometer (BD Biosciences), using CellQuest (BD Biosciences), or on an LSRII flow cytometer (BD Biosciences), using FACS-Diva software (BD Biosciences). Data were analyzed using FlowJo software with a live cell gate based on forward-scatter and side-scatter characteristics. The number of $IFN\gamma^+TNF\alpha^+$ T cells was quantitated after first gating on live $CD4^+CD8^-$ or $CD4^-CD8^+$ cells and subtracting the number of cytokine-positive events from uninfected/unstimulated cultures.

Flow Cytometry Staining for Virus-Infected Cells in Addition to MHC Class I and Class II expression. Cells were either uninfected or infected with VV or MPV for 16 h before being washed and surface stained with antibodies against CD14 (clone RM052, Beckman Coulter), MHC class I (HLA-A, -B, and -C, clone W6/32; Biolegend) and MHC class II (HLA-DR, clone G46–6; PharMingen). The cells were then fixed, permeabilized and stained intracellularly for OPV antigens using biotinylated polyclonal rabbit anti-VV (ViroStat) followed by addition of Streptavidin-Pacific blue. This pan-OPV antibody allows detection of CPV, MPV, and VV. Samples were acquired on a FACScalibur flow cytometer (BD Biosciences), using CellQuest (BD Biosciences), or on an LSRII instrument (BD Biosciences), using FACS-Diva software (BD Biosciences). Data were analyzed using FlowJo software.

Pulse–Chase. HeLa cells were uninfected or infected with VV, CPV, or MPV (MOI of 3), starved for 1 h starting at 4 h after infection (3 h after adsorption for 1 h) and metabolically labeled for 20 min with [^{35}S]cysteine-[^{35}S]methionine (GE Healthcare) (100 μCi per 10^6 cells). In some experiments, MG132 (Boston Biochem) was added at 50 μM at the starvation stage and maintained throughout the experiment. After labeling, the cells were washed twice with PBS, and samples were harvested immediately or chased for 30 or 60 min in regular medium supplemented with excess unlabeled cysteine and methionine as indicated. Cells were lysed in PBS containing 1% Nonidet P-40 and 3% H_2O_2. Lysate was transferred to tubes and an additional 3% H_2O_2 was added and incubated for 30 min to inactivate residual virus. The cell lysate was precleared with protein A/G-agarose beads for 2 h followed by incubation with anti-MHC class I antibody, W6/32 or K455, for 2 h. For analysis of CD44 maturation, lysates were incubated with anti-CD44 antibody (Santa Cruz Biotechnology) for 2 h. Immune complexes were captured by incubation for 2 h with protein A/G-agarose. The precipitate was washed three times with 0.1% Nonidet P-40 and MHC class I was eluted by boiling in 0.2% SDS, 5 mM DTT, and 40 mM sodium phosphate buffer (pH 6.5). Samples were incubated overnight at 37°C with 25U/μl of Endo H (Roche), separated by 10% SDS/PAGE, and detected by autoradiography.

1. Hammarlund E, *et al.* (2005) Multiple diagnostic techniques identify previously vaccinated individuals with protective immunity against monkeypox *Nat Med* 11:1005–1011.

2. Hammarlund E, *et al.* (2003) Duration of antiviral immunity after smallpox vaccination. *Nature Medicine* 9:1131–1137.

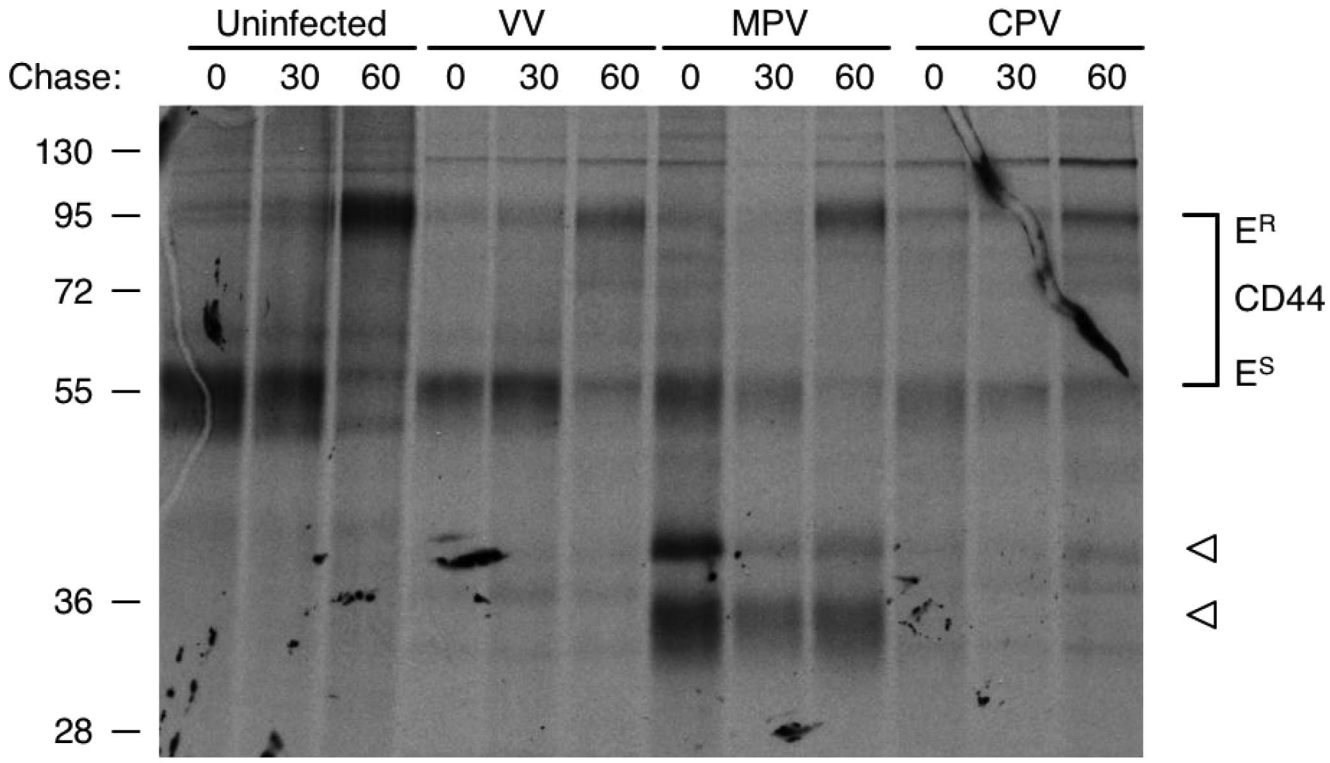

Fig. S1. OPV infection does not inhibit maturation of CD44. CD44 transport through the ER was measured in OPV-infected cells. HeLa cells were uninfected, or infected with VV, MPV, or CPV for 5 h (starved for the last 1 h) and pulse-labeled for 20 min. The labels were chased for 0, 30, or 60 min as indicated. More than 90% of HeLa cells were infected as indicated by staining with anti-OPV antibody (data not shown). Cell lysates were immunoprecipitated with anti-CD44 antibody, and the precipitated material was treated with EndoH and separated by SDS/PAGE. These results demonstrate that infection with any of these viruses does not result in a general interference with glycoprotein trafficking. The open arrowheads indicate the presence of two low molecular weight proteins that were immunoprecipitated with anti-CD44 antibodies from MPV-infected cells. Not that low molecular weight proteins of the same size are also found in the immunoprecipitations of MHC class I (Fig. 2*B*) indicating that these proteins are likely virus-derived proteins binding to the antibodies but not to the immunoprecipitated antigen. E^R, EndoH-resistant band; E^S, EndoH-sensitive band.