# Vaccinia Virus WR induces rapid surface expression of a host molecule detected by the antibody 4C7 that is distinct from CLEC2D

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**RUNNING TITLE:** Externalization of host molecule by WR

**SUBJECT SECTION:** Virology

**SPECIFIED FIELD:** Animal DNA virus

**ABSTRACT**

In this study we examined the effect of active infection with vaccinia virus Western Reserve (VACV WR) on expression of CLEC2D, a ligand of the human natural killer (NK) cell inhibitory receptor NKR-P1. As predicted, VACV infection leads to a loss of CLEC2D mRNA in 221 cells, a B cell lymphoma line. Surprisingly, VACV infection of 221 cells causes a dramatic increase in cell surface staining for one CLEC2D-specific antibody, 4C7. No change occurs with other antibodies specific for CLEC2D and there is no indication that NK cells with NKR-P1A are inhibited, suggesting 4C7 detects a non-CLEC2D molecule following infection. The rapid increase in 4C7 signal requires virus attachment and is disrupted by UV-treatment, but does not depend on new transcription or translation of either cellular or viral proteins. 4C7 does react with the intracellular compartments suggesting the molecule that is detected at the surface following infection is derived from an intracellular store. The phenomenon extends beyond lymphoid cells and is observed in the non-human primate cell line Cos-7, but not with myxoma, a poxvirus belonging to a distinct family. To our knowledge, this is the first report of VACV or any poxvirus leading to the rapid externalization of a host molecule. Among the VACV strains tested, the phenomenon was restricted to VACV WR and IHD-W suggesting virulence as opposed to replication related function.

**KEYWORDS:** CLEC2D, vaccinia virus, LLT1, poxvirus

**ABBREVIATIONS:** AraC, cystosine β-D-arabinofuranoside; CHX, cycloheximide; CLEC2D, C-type lectin domain family 2; DCs, dendritic cells; DMEM, Dulbecco's modified Eagle's medium; DMSO, Dimethyl Sulfoxide; EBV, Epstein Barr virus; EGFP, enhanced green fluorescent protein; EGCG, Epigallocatechin-3-gallate; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; HBV, Hepatitis B virus; HI, heat inactivated; HIV; human immunodeficiency virus; IFA; immunofluorescence assay; MHC I, major histocompatibility complex class I; MOI, multiplicity of infection; MYX, myxoma virus; NK, natural killer, NKR-P1A/B, NK receptor protein-1A/B; PBMC, peripheral blood mononuclear cells; RSV, respiratory syncytial virus; PFA, paraformaldehyde; TK-, thymidine kinase negative; TLR, toll-like receptor; VACV, vaccinia virus; WR, Western Reserve.

## INTRODUCTION

Poxviruses generally limit production of host cellular proteins providing a mechanism for NK cells to detect infection through loss of cell surface proteins that engage NK cell inhibitory receptors (1). In this context, molecules encoded within the natural killer gene complex (NKC) are of particular interest due to evidence that the NKC is involved in NK-mediated host resistance to poxviruses in rodents and humans (2, 3). The NKC consists of several families of receptors that belong to the superfamily of C-type lectin-related receptors including NKG2, NKR-P1 and Ly49. The Ly49 and NKR-P1 families are targeted by rodent cytomegaloviruses to evade the NK cell response (4, 5) and rat cytomegalovirus encodes a decoy NKR-P1 ligand that is also a C-type lectin related protein (5). Poxviruses also encode C-type lectin related molecules with structural similarity to proteins encoded within the NKC such as A33 and A40 (6-8). In the analysis by Wilcock et al. in 1999 (6), the best similarity score was for A40 with human NKG2A. However, the relationship of these poxvirus proteins to other more recently described C-type lectin proteins in humans or mice, and particularly the NKR-P1 receptor pathway, have not been well studied to date. We have previously shown that the mouse NKR-P1B ligand (Clr-b) is rapidly lost upon infection rendering the infected cells more sensitive to NKR-P1B-positive NK cell-mediated cytotoxicity (9). In this study we focus on the influence of vaccinia virus (VACV) infection on the human NKR-P1 ligand known as LLT1, which is isoform 1 of CLEC2D (herein referred to as CLEC2D-1).

There are several isoforms of CLEC2D derived by alternative splicing. There are two secreted forms (CLEC2D-5 and -6), a nonsense RNA decay candidate (CLEC2D-3), and

three isoforms that have transmembrane domains (CLEC2D-1, -2 and -4) (10). CLEC2D-1 is found on the cell surface and interacts with the human NKR-P1A receptor found on NK cells, but the function of CLEC2D-2 and -4 are unknown and the proteins are reported to remain inside the endoplasmic reticulum as immature glycoforms (10). CLEC2D-1 is expressed on activated cells of hematopoietic origin and its primary function is believed to protect activated professional antigen presenting cells from NK cell attack (11). CLEC2D-1 is expressed on Toll-like receptor (TLR) activated plasmacytoid dendritic cells (DCs), monocyte derived DCs, activated B, T and NK cells, and DCs exposed to heat inactivated viruses (11, 12). CLEC2D-1 is also increased on B cells in peripheral blood mononuclear cell cultures infected with Epstein Barr virus (EBV), human immunodeficiency virus (HIV) and Hepatitis B virus (HBV) (12, 13). Lung epithelial cells infected with respiratory syncytial virus (RSV) also increase CLEC2D-1 transcription and protein expression (14). The increase during RSV infection occurs primarily in the uninfected cells of the culture that are likely responding to cytokines or through TLR stimulation (14). CLEC2D-1 is also found constitutively on glioblastoma cells and cell lines such as Raji, NK-92, and YTS (12, 15-17), suggesting it may be involved in tumor evasion of NK cells. It is worth noting here that there are important differences in the expression patterns CLEC2D-1 and its counterpart in mice Clr-b. Clr-b is expressed by all cell types and therefore can be involved in protecting any cell type from NK cell attack while serving as a "missing-self" system similar to MHC-I that can be exploited by viruses (18). In contrast, as described above, CLEC2D-1 has a rather restricted expression and is found mainly following infection, immune activation or transformation.

In this study, we asked whether active infection with VACV would reduce CLEC2D expression in tumor cells that constitutively express CLEC2D-1, or if alternatively, CLEC2D would be induced by VACV as occurs with RSV, EBV and HBV infection (12-14). Our results suggest CLEC2D is down regulated during infection and negates CLEC2D-1 mediated protection of vaccinia-infected antigen presenting cells from NK cells. Surprisingly, we find that one strain of VACV leads to the externalization of a molecule detected by the anti-CLEC2D antibody 4C7. The molecule that is externalized is not any known isoform of CLEC2D and apparently arises from an intracellular store. While the identity of the molecule remains unknown, it may be a protein related to the CLEC2D family given the reactivity with the 4C7 antibody.

## MATERIALS AND METHODS

**Cells.** 721.221 (221; gift from Dr. Eric Long, NIH) were cultured in Iscove's modified Dulbecco's media with 10% heat inactivated (HI) fetal bovine serum (FBS; Gibco, Carlsbad, CA), 2 mM L-glutamine (Gibco), 10,000 units/mL penicillin and 10 mg/mL streptomycin (Gibco). NIH 3T3 cells from the ATCC (Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% HI calf serum. Cos-7 cells (ATCC), 293T cells (ATCC) and thymidine kinase negative (TK-) H143B cells (ATCC) were cultured in DMEM supplemented with 10% HI FBS, 2 mM L-glutamine, 10,000 units/mL penicillin and 10 mg/mL streptomycin. Blood samples were drawn from healthy individuals with written consent and all procedures were performed as approved by the Health Research Ethics Board at the University of Alberta. NK cells were isolated from peripheral blood mononuclear cells (PBMCs) by negative selection using the negative selection human NK cell enrichment kit (Stem Cell, Vancouver, BC) as per the manufacturer's instructions. Isolated NK cells were cultured with irradiated 221 cells in Iscove's medium supplemented with 10% HI male AB human serum (Invitrogen, Carlsbad, CA), 500 mM gentamicin (Gibco) and 200 U/mL rhIL-2 (Tecin, Nutley, NJ) and fed every two to three days. Primary NK cells were used between eight and twenty days following isolation.

**Antibodies.** Antibodies used for primary detection were: anti-CLEC2D, clone 4C7 (MO1; mouse IgG1, κ, Abnova, Walnut, CA), anti-human IgG1, κ (MOPC-21; Sigma-Aldrich, Oakville, ON), anti-CLEC2D, clone 2E11 (MO3; mouse IgG2a, κ, Abnova), anti-human OCIL/CLEC2D APC, clone M402659 (IgG1, R&D Systems, Burlington, ON), polyclonal

goat anti-human OCIL/CLEC2D (IgG, R&D Systems), anti-human-CLEC2A (KACL, IgG1, R&D Systems), anti-human CD161 (clone B199.2, GeneTex, Irvine, CA), mouse IgG2b (GeneTex), anti-human CD161 Alexa Fluor 647 (BioLegend, San Diego, CA), mouse IgG1, κ, Alexa Fluor 647 (BioLegend), anti-human IFN-γ PE-Cy7, clone 4S.B3 (eBioscience, San Diego, CA), mouse IgG1, κ PE-Cy7 (eBioscience), mouse anti-human CD107a PE-Cy5 (BD Pharmingen, Mississauga, ON), and mouse IgG1 κ PE-Cy5 (eBioscience). Antibodies used for secondary detection were: goat anti-mouse IgG APC (Cedarlane Laboratories, Burlington, ON), and Alexa Fluor 647-R-phycoerythrin goat anti-mouse IgG1, (Molecular Probes, Carlsbad, CA).

**Transfection.** MSCV2.2 plasmid containing YFP-LLT1 was kindly provided by Dr. Fiona Culley (Imperial College, London). 293T cells were transfected with 2 μg of YFP-LLT1 plasmid using Lipofectamine (Invitrogen) as per the manufacturer's protocol. Cells were collected forty-eight hours post-transfection and prepared for FACS staining.

**Virus Generation and Infection**. The recombinant VACV WR expressing EGFP has been previously described (19). The EGFP-VACV strain Copenhagen was provided by Dr. Michele Barry (University of Alberta). Recombinant EGFP-expressing VACV IHD-W was generated using the plasmid pSC66 as described previously (19). The ΔM127L-mCherry myxoma virus strain Lausanne was generously provided by Dr. D. Evans (University of Alberta) and purified as previously described (20). All VACV stocks were prepared as previously described (19) and titered on TK-H143B cells. VACV WR was UV inactivated as previously described and verified to be at <0 PFU/mL by titering on TK-H143B cells

(21). Unless otherwise stated, cells were infected at a multiplicity of infection (MOI) of 10 in serum-free medium.

**Flow Cytometry.** Following infection, cells were collected by centrifugation and washed one time with chilled PBS supplemented with 5% FBS and 1 mM ethylenediaminetetraacetic acid (EDTA, Invitrogen). Primary antibodies were added for thirty minutes at 4°C. Cells were then washed and stained with secondary antibody for thirty minutes at 4°C. Cells were also stained with the Molecular Probes LIVE/DEAD Fixable Dead Cell Stain Kit–violet (Invitrogen) according to the protocol supplied by the manufacturer. For intracellular staining, cells were washed one time with chilled PBS/1 mM EDTA and permeabilized using the Cytofix/Cytoperm$^{TM}$ Fixation/Permeabilization solution kit (BD Biosciences) as per the manufacturer's instructions. This was followed by staining as described above. Anti-human IFN-γ PE-Cy7 was used at 1.25 μg/ml and 4C7 at 5 μg/ml. All samples were fixed with 4% paraformaldyhyde (PFA, Fisher Scientific). Cell data was acquired on an FACS Canto II (BD Biosciences) and analyzed using FlowJo Software (version 7.2.5; Tree Star, Ashland, OR). Cells were gated relative to debris by their forward and side scatter and the live/dead stain.

**Analysis of mRNA.** RNA was isolated from $2.5 \times 10^6$ 221 cells using the RNeasy kit (Qiagen, Toronto, ON). Samples were treated on the columns with DNAse to remove any genomic or viral DNA. RT-PCR reactions were performed with 1 μg RNA using the One Step RT PCR Kit (Qiagen) as per the manufacturer's directions. Full length "CLEC2D" Isoform 1 was amplified with the primers 5'-GAA TTC CGG CAA AAT GCA TG-3' and 5'-AAT TAC TCA TTC TCG GGT AT-3'. β-actin was amplified with the primers 5′-AAG

ACC TGT ACG CCA ACA-3′ and 5′-TCC ACA CGG AGG ACT TGC -3′. The resulting RT-PCR products were analyzed on an agarose gel stained with ethidium bromide or SYBR safe.

**Immunofluorescence Assay (IFA).** A filter pad with a circle removed from the center was placed on a microscope slide and put into the cytospin loading cassette. PBS was placed in the circle and the cassette was centrifuged at 70 x g for one minute at 4$^{o}$C to wet the filter pad. A suspension with approximately 5 x $10^4$ cells (221) were fixed in 4% PFA and were placed in the circle and centrifuged at 70 x g for three minutes at 4$^{o}$C. The filter paper was removed and the microscope slide was air-dried. Cells were washed three times with PBS and incubated with MOPC-21 or 4C7 primary antibody at 5 μg/mL for one hour at room temperature. Subsequently, cells were washed three times with PBS and then incubated with the secondary antibody goat anti-mouse IgG APC at 0.9 μg/mL for one hour at room temperature in the dark. Cells were washed three times with PBS and then were mounted and nuclei stained in ProLong gold antifade reagent with DAPI (Invitrogen) and the cover slip was placed and sealed with nail polish. Samples were imaged using a WAVE FX spinning disc confocal microscope (Quorum Technologies) with a 60x oil lens and analyzed using Fiji ImageJ software for Windows (Version 1.48s, National Institutes of Health, USA).

**Inhibitors.** Cells were treated prior to infection with 50 μg/mL cytosine β-D-arabinofuranoside (AraC; Sigma-Aldrich) for five minutes at 37°C or 40 μM of cycloheximide (CHX; MP Biomedicals, Solon, OH) for one hour at 37°C and the inhibitors were maintained for the duration of the experiment. Epigallocatechin-3-gallate (EGCG)

(Sigma-Aldrich) was kindly provided by Dr. L Schang (University of Alberta). For EGCG treatments, virus was treated for ten minutes at 37°C with serum free Iscove's media containing EGCG at 40 μM or equivalent amounts of dimethyl sulfoxide (DMSO) alone. This was followed by treatment on ice for a further twelve minutes. The virus was added to chilled 221 cells in complete media and immediately centrifuged at room temperature and resuspended in PBS for FACS staining.

**Data Analysis.** The statistical significance between conditions was calculated using a Student *t* test, considered significant at a 95% confidence limit, and noted as *$p < 0.01$, **$p < 0.001$, and ***$p < 0.0001$.

## RESULTS

### *Poxvirus proteins with similarity to CLEC2D*

The alignment of NKG2A and CLEC2D-1 with VACV A40 reveals similarity between A40 and CLED2D-1 over a large span of the extracellular domain. There is comparable similarity with NKG2A, which is the molecule previously reported as the best fit with A40 (6). In a search for additional putative decoys that might in bind NKR-P1 receptors, we considered the C-type lectin related proteins encoded by the various sequenced poxviruses using the poxvirus.org database. First, we found that a cowpox protein encoded by GER91-010 bears substantial similarity with Clr-b in the same region (Figure 1). Most strikingly, we found a deerpox protein, DPV-W848_83-142, with a high degree of similarity to the human CLEC2D-1. There is a very high degree of similarity in the area between residues 170-200 that encompasses the ligand-binding region of CLEC2D-1 with identity for 5 of 6 known contact residues indicated in red (Figure 1) (22, 23). While the function of the cowpox and deerpox sequences is not known, their relationship to the NKR-P1 ligands suggests that poxviruses might target the NKR-P1 system to manipulate the immune response in some hosts similar to cytomegaloviruses.

### *VACV infection increases 4C7 reactivity with 221 cells*

To determine the effects of VACV infection on CLEC2D expression, we infected 721.221 (221) cells with EGFP-VACV WR at an MOI of 10 and analyzed the cells by flow cytometry with the CLEC2D antibody 4C7. We used 221 cells to because they have low but detectable levels of CLEC2D-1/LLT1 expression on the surface of the cells as shown in Figure 2a. We chose 4C7 because it detects all three isoforms of CLEC2D that are

expressed by 221 cells (10). At twelve hours post infection cell surface staining with the antibody 4C7 was increased on infected cells in comparison to 4C7 staining on uninfected cells (Figure 2a and b). When cells were treated with UV inactivated EGFP-VACV WR, there was no change in 4C7 staining over uninfected cells (Figure 2c). The increase in 4C7 staining suggested CLEC2D is upregulated upon infection and the upregulation requires active virus.

**Dose dependent increase of 4C7 signal coincides with destruction of CLEC2D mRNA**

A possible explanation for the increase in 4C7 signal is that CLEC2D is transcriptionally induced following infection, which occurs for a select set of host transcripts early after infection (24). To examine the effect of virus dose and duration of infection on CLEC2D expression, 221 cells were infected at an MOI of 1, 5, and 10 and analyzed at two, four and eight hours post infection. EGFP expression was observed at all time points and MOIs, and was well correlated with the dose and time of infection (Figure 3a). After just two hours of infection, upregulation of 4C7 staining was evident for all MOIs and the increase was proportional to the MOI (Figure 3b). At the lowest MOI, the peak expression was reached by two hours and it remained consistent for the duration of the infection (Figure 3b). At an MOI of 5, maximal 4C7 staining was observed at four hours post infection and by eight hours it was reduced to less than that of the two-hour time point (Figure 3b). At an MOI of 10, the maximal upregulation of CLEC2D was observed at two hours post infection (Figure 3b). These results suggest that the degree of 4C7 staining is sensitive to the dose and duration of the infection and suggests that an event that occurs early in the infection is responsible for the upregulation of CLEC2D. In addition, the

reduction in 4C7 staining at later time points suggests the protein is then lost from the cell surface over time.

To investigate whether CLEC2D is induced at the transcriptional level by VACV infection we examined the effect of VACV infection on CLEC2D mRNA at various times post-infection. The amount of CLEC2D transcript was relatively stable in uninfected cells over the course of the infection as shown by a representative analysis in Figure 3c. We did not see any evidence of increased CLEC2D mRNA early following infection at any dose of virus. In fact, the amount of CLEC2D mRNA decreased by four hours post-infection, the point at which VACV causes destruction of most host derived transcripts (25). CLEC2D was undetectable at all MOIs by eight hours post infection. The loss of mRNA was more pronounced with higher MOIs. These results suggest that CLEC2D mRNA is not induced even at early time points post infection and suggests a pre-existing pool of CLEC2D is mobilized upon infection.

The surprising finding that 4C7 staining increased while the mRNA for CLEC2D decreased led us to question if in fact 4C7 was detecting CLEC2D. The 4C7 antibody also can detect a CLEC2D-related protein called KACL/PILAR (10). To test if the 4C7 antibody was detecting the KACL protein, we stained cells after a four-hour infection with EGFP-VACV WR at an MOI 5 with a KACL specific antibody called anti-human CLEC2A. No increase in KACL was detected suggesting infection is not increasing KACL (data not shown). We also addressed the possibility that 4C7 was cross-reacting with a viral protein by examining whether 4C7 stained the EGFP-VACV WR infected mouse cell line, NIH 3T3. NIH 3T3 cells were infected with EGFP-VACV WR for four hours at an MOI of

5 and stained with 4C7 antibody. There was no increase in 4C7 staining in the $EGFP^+$ NIH 3T3 cells (Figure 3d) indicating that the 4C7 antibody is not detecting a viral protein or contaminants from the virus preparation.

***Upon infection 4C7 detects a molecule that is distinct from CLEC2D (and KACL)***

The rapid, yet transient expression of pre-existing CLEC2D on the cell surface could be a mechanism by which the virus protects the infected cell from NK cell-mediated cytotoxicity. To address this possibility, we tested whether NK cells are inhibited at early times post infection when the 4C7 signal is maximal by using cultured primary NK cells from several individuals that express the CLEC2D receptor NKR-P1A (Supporting Information Figure 1). 221 cells were infected with EGFP-VACV WR at an MOI of 10 for maximal induction, and were then co-cultured with primary NK cells for three hours in the presence or absence of an antibody to block the function of the NKR-P1A receptor (anti-CD161) (11). The death of the target cells was assessed with a live/dead dye (Supporting Information Figure 1a). Activation of the NK cells was assessed by degranulation using CD107a and IFN-γ induction (Supporting Information Figure 1b and c). Curiously, NKR-P1A staining was increased on all the NK cells following contact with target cells and which precluded resolving effects on $NKR\text{-}P1A^+$ cells only (Supporting Information Figure 1d). While the presence of the NKR-P1A blocking antibody did produce a very mild increase in 221 cell death and NK cell degranulation for a couple of donors, infection of the target cells did not enhance this effect (Supporting Information Figure 1b and 1c). The infection clearly suppressed production of IFN-γ by the NK cells, but again, this was not influenced by NKR-P1A as determined by addition of a blocking antibody (Supporting

Information Figure 1c) (11). Therefore, in spite of the ability of the relatively short infection time to influence the interaction with NK cells, it did not prevent NK cell killing of the tumor target cell and therefore does not appear to be a result of an increase in NKR-P1A mediated signalling.

A possible explanation for an increase in 4C7 signal without an increase of NKR-P1A-mediated inhibition is that the molecule being detected by the 4C7 antibody is not isoform 1 of CLEC2D. 221 cells express transcripts corresponding to two additional alternatively spliced forms of CLEC2D that are normally retained inside cells and do not bind NKR-P1A (10). Consistent with an intracellular pool of CLEC2D, there is a sizeable increase in the 4C7 signal for permeabilized cells in comparison to surface staining with the same antibody (Figure 4a). Moreover, in a single experiment performed, the amount of 4C7 staining in permeabilized cells did not change up to twelve hours of infection, suggesting that the total pool of protein is maintained (data not shown). To investigate the subcellular localization of the 4C7-reactive molecule, uninfected 221 cells were fixed, permeabilized, and CLEC2D was detected using the 4C7 antibody. There was some diffuse 4C7 signal consistent with the localization of CLEC2D isoform 2 and 4 to the ER (10), but we also noted a stronger vesicular pattern of staining (Figure 4b). Therefore, to address the possibility that intracellular stores of CLEC2D other than CLEC2D-1 might be moving to the cells surface after infection, we used three other commercially available antibodies that bind CLEC2D: 402659, which reacts only with isoform 1 (10), a goat anti-CLEC2D which recognizes isoforms 1, 2 and 4 based upon the peptide sequence it was made against, and the monoclonal antibody 2E11, which has not been characterized in terms of isoform

specificity. As shown in Figure 4c, all of these antibodies worked in our hands to detect cell surface CLEC2D-1 with similar sensitivity using 293T cells transfected with an YFP-tagged construct of CLEC2D isoform 1 (LLT1-YFP) (14). Staining of uninfected 221 cells with these antibodies is very low, suggesting 221 could express an additional molecule that 4C7 detects. The staining with the three antibodies did not substantially change following a two-hour EGFP-VACV WR infection at an MOI of 5, although there was a slight, but not significant, increase with 2E11 (Figure 4d and e). Due to the fact that the other three CLEC2D antibodies show no increase in CLEC2D staining, our results suggest that 4C7 was detecting a non-CLEC2D molecule in 221 cells that increased on the cell surface with infection. While we do not know the identity of the molecule 4C7 was detecting, the ability of VACV to induce this event is intriguing and warrants further investigation.

***Virus binding, but not protein synthesis, is required to increase the 4C7 signal at the cell surface***

To characterize the steps of virus infection required for the 4C7 signal increase we examined early infection events using different inhibitors. To determine if protein synthesis was required to produce the enhanced 4C7 signal, we infected 221 cells with EGFP-VACV WR at an MOI of 5 for four hours in the presence of cycloheximide. Cycloheximide decreased the EGFP signal, although some residual EGFP expression was observed in the cycloheximide treated cells (Figure 5a). The residual EGFP signal is possibly due to EGFP protein carried in the viral preparation. Cycloheximide treatment had no effect on the 4C7 signal in the absence of infection and did not interfere with the VACV-induced upregulation but rather it increased the 4C7 signal (Figure 5a and b). The

lack of inhibition by cycloheximide is consistent with mobilization of a preformed store. In addition, the lack of effect of cycloheximide also indicated that a step very early in infection was sufficient to expose the 4C7 reactive molecule on the cell surface prior to synthesis of viral proteins.

For completeness, we also performed infections in the presence of cystosine β-D-arabinofuranoside (AraC), which prevents replication of the viral genome, as well as late gene expression. Also, we previously found that it protects the viability of lymphocytes during infection with VACV (19). The cells were pre-treated with AraC for five minutes followed by infection with EGFP-VACV WR at a MOI of 10 for twelve hours. The treatment drastically reduces EGFP expression because EGFP is under the control of a synthetic early/late promoter in the recombinant virus (Figure 5c). Moreover, AraC dramatically improved the viability of 221 cells at the late time points of infection as revealed by the live/dead staining used for flow analysis (data not shown). To our surprise, AraC treatment reduced the extent of 4C7 staining at twelve hours suggesting either dying cells or a late gene product was required for the maintaining high levels of 4C7 staining at these time points (Figure 5c and d). Therefore, to determine when the increase in 4C7 staining occurs, we examined earlier time points post-infection in the presence and absence of AraC. We found increases in 4C7 staining as early as two hours post-infection (Figure 5e). At two, four, and eight hours post-infection AraC had no effect on the increase in 4C7 staining (Figure 5e). The effect of AraC on EGFP expression is only obvious at after eight hours of infection when late gene expression is expected (Figure 5e). These results suggest that an event early after infection results in an increase in 4C7 staining and perhaps the

expression of a late gene or the induction of cell death is necessary to sustain the effect for twelve hours. One possibility is that the product of a late gene is incorporated into the virion and acts from within the infected cells to induce the 4C7-reactive molecule when it is released into a newly infected cell.

To investigate how quickly upon fusion of the virus to the cell the 4C7 signal increases, we examined very early time points post-infection. 221 cells were infected with EGFP-VACV WR at an MOI of 5 for five minutes at room temperature, immediately washed with warm media and the cells were pelleted and stained. Surprisingly, with this short exposure to the virus, cells had a low but significant and highly reproducible increase in 4C7 staining (Figure 6a and b). The rapid detection with 4C7 is consistent with a degranulation process that is triggered by virus binding or shortly after fusion into the cells. We also noted that at this early time point there was very low but detectable EGFP expression in the infected cells (Figure 6a). This is similar to when the cells were incubated in the presence of cycloheximide, in line with the idea that some EGFP is carried in our virus preparations (Figure 5a). To test if virus binding was sufficient to induce the 4C7 signal, we allowed virus to adhere to the cells on ice for five minutes and then cells were pelleted and stained. Surprisingly, virus binding alone was sufficient to increase the 4C7 signal to levels similar to that of those cells infected for five minutes at room temperature (Figure 6b). To directly test if binding of the virus to the cells was required for the very early induction, we used epigallocatechingallate (EGCG), a green tea catechin that inhibits VACV binding to heparan sulfate or to sialic acid and prevents attachment (26-28). For this experiment, the virus was pre-treated with the inhibitor for 10 minutes at 37°C,

followed by 12 minutes on ice, and was then used to infect cells for 5 minutes at an MOI of 5. There was no EGFP signal in infected cells (Figure 6c). Uninfected cells had no change in 4C7 staining compared with DMSO treatment alone (Figure 6d and e). EGCG alone caused a small but statistically significant increase in surface 4C7 expression over DMSO treated uninfected cells (Figure 6d and e). The 4C7 level on cells exposed to EGCG-treated virus was less than the increase with DMSO-treated virus and was similar to the EGCG alone control (Figure 6d and e). How EGCG alone increased the 4C7 signal is not clear, but may be due to EGCG perturbing the cell membrane. None-the-less, the lower induction of 4C7 in the presence of EGCG-treated virus is consistent with virus binding being required for the increase. Together the results suggest the triggering of a cellular pathway, such as exocytosis, by virus binding at the cell surface or just after internalization is necessary for an increase in surface 4C7 staining.

***The extent of 4C7-signal varies with strains of VACV***

We next asked whether the phenomenon of increased 4C7 was restricted to VACV or was a more generalized effect with other types of poxviruses. We used mCherry tagged myxoma virus (MYX), a distantly related *Leporipoxvirus* that causes myxomatosis in rabbits. 221 cells were highly infectable by MYX as indicated by the high mCherry expression (Figure 7a). However, MYX infection did not result in an increase in 4C7 reactive molecule (Figure 7a and b), indicating the phenomenon does not occur with all poxviruses.

To determine if different strains of VACV induced the same effect we obtained strain Copenhagen that expresses EGFP, as well as generated a recombinant virus expressing EGFP in strain IHD-W. However, 221 cells were quite refractory to infection with the other strains of VACV at an MOI of 10 as indicated by EGFP expression (Figure 8a) and exposure to higher MOIs did not substantially increase infection (data not shown). The small degree of upregulation of 4C7 detected with these other strains was confined to the few cells that were infected (Figure 8b). Therefore, while these experiments suggest that infection is required to cause the increase in 4C7 staining, they did not adequately address the question of whether the other strains of VACV are capable of inducing a change in 4C7 staining. To address this question we used Cos-7 cells, which are permissive to infection by VACV. Cos-7 cells were infected with the various strains of VACV for two hours at an MOI of 5. All VACV strains tested were able to induce high EGFP expression in Cos-7 cells (Figure 8c). EGFP-IHD-W induced the 4C7 signal to a similar degree as WR, whereas Copenhagen infection resulted in very little upregulation of 4C7 (Figure 8d and e). These results indicate that not all strains of VACV produce the phenomenon and opens the possibility that the process that results in the change in 4C7 staining has a biological function that provides an advantage to the virus.

## DISCUSSION

In this study we set out to investigate the modulation of CLEC2D within an infected cell. Consistent with VACV effect on Clr-b in mouse cells (9) and with what VACV does to most other cellular proteins, CLEC2D mRNA is rapidly lost following infection with EGFP-VACV WR. The loss of CLEC2D mRNA appears to be in contrast to what happens in culture with other viruses that induce CLEC2D transcription through inflammatory cytokine production. The reported upregulation occurs when not all the cells in the culture are infected (12-14) whereas even at the lowest MOIs we tested, the vast majority of 221 cells are infected. Therefore, during *in vivo* infection, cells directly infected by the virus, including antigen presenting cells, are unlikely to increase CLEC2D-1, but uninfected cells may still increase CLEC2D-1 by responding to cytokines. Given that CLEC2D-1 is suppressed in infected cells, it is possible that similar to viruses such as rat cytomegalovirus (5), poxviruses might possess the means to evade the NK response by using a decoy to engage NKR-P1A. For instance, we have shown that molecules such as A40 have some degree of similarity with CLEC2D-1. A40 is a protein of unknown function that is expressed at the cell surface early after infection and may influence VACV pathology in mice (6, 8). Using 221 cells as model cell type because they have low but detectable levels of CLEC2D-1/LLT1 expression, we tested if infection by VACV WR had an impact on the response of NKR-P1A$^+$ NK cells. The response through NKR-P1A on cultured NK cells did not change after infection with 221 cells. Neither did measures of degranulation or the extent of death of the 221 cells as a result of NK cell cytotoxicity (as indicated by the NKR-P1A ligand of CLEC2D-1, which was not increased during infection). However, humans

are not the natural host of VACV, so even if VACV encoded an NKR-P1 decoy protein, it would not be expected to engage human NKRP1A. In the course of the functional experiments with primary NK cells, we made a few curious observations. First, the surface expression of NKR-P1A increased on the vast majority of the NK cells independent of the infection status of the target. Second, VACV suppressed interferon-γ production in NK cells stimulated by 221 cells (Supporting Information Figure 1). The differential effect on interferon-γ production and degranulation may be reflective of changes in stimulatory ligands, limits on the duration of the contact cytokine, or effector proteins released from the infected cells that target cytokine production (29). It may be of interest in the future to determine why there is a dichotomy between cytokine induction and degranulation as the information might be useful for the design of VACV-based vaccines or oncolytic vectors.

In the course of characterizing the effects of active EGFP-VACV WR infection of CLEC2D-1, we uncovered an unexpected upregulation of a molecule detected by the anti-OCIL/CLEC2D antibody, 4C7. We ruled out cell surface upregulation of other proteins known to be detected by 4C7, but not recognized by NKR-P1A such as the typically intracellular isoforms of CLEC2D and KACL. Therefore, the fact that only the 4C7-signal is increased strongly suggests a distinct molecule is being detected by this monoclonal antibody after infection. This commercially available monoclonal antibody was raised against a full-length recombinant CLEC2D molecule (see Materials and Methods). According to the manufacturer, the antibody reacts by Western blot with lysates from transfected 293T cells but not control 293T cells. The Western results suggests that 293T cells do not express the additional protein(s) detected by 4C7 in 221 and other cell types we

tested, although we have had variable results with intracellular staining of 293T (data not shown). The 4C7 antibody has been reported to recognize the three isoforms of CLEC2D that have transmembrane domains, and the CLEC2 family member CLEC2A, also known as KACL (10) by cell surface flow cytometry. Given that 4C7 recognizes all CLEC2D isoforms as well as KACL, it may bind a motif that is present in related proteins. On the other hand, 4C7 may be cross-reacting on a completely unrelated protein. Our efforts to date to identify the molecule bound by 4C7 in 221 cells have not been successful. We cannot detect a protein following infection by immuno-precipitation with surface-bound 4C7 and the long half-life of the molecule detected by 4C7 in the presence of cycloheximide (no loss over four hours) suggest that there are difficulties for methods of screening such as siRNA. However, we do not believe we are detecting a molecule unique to 221 cells as we did find a similar phenomenon in several other leukemic lines such as Jurkat and NK-92 (data not shown), and Cos-7 cells (Figure 8d and e) suggesting a molecule that responds similarly to VACV infection is expressed in other cell types and is as well conserved in the monkey, but not mouse.

How then does the 4C7-reactive molecule appear at the cell surface and what in the virus triggers the process? Our results suggest an event following virus attachment that does not require any protein synthesis is involved. The two possibilities are that the virus binds to a cell surface receptor that sends a signal or that a molecule released into the cell following infection triggers the event, perhaps causing vesicles to fuse with the plasma membrane. The latter explanation of a molecule released into the cell from the virion fits well with the effect of AraC that we observe at very late time point points. In this case, a

late protein that becomes incorporated into the virion may also be present in the cytoplasm at a level sufficient to trigger the event and its new production at late time points sustains the upregulation by acting from inside the cell. VACV carries a number of proteins in the virion and within the lateral bodies (30), such as VH1, which are known to modulate a range of cellular functions as well as set up the cell for virus replication (31). It then seems paradoxical that UV-inactivation would prevent the increase in 4C7-signal. We did observe that partial UV-inactivation (where plaque production was diminished, but not absent) reduced, but did not eliminate, the upregulation of the 4C7 signal (data not shown). It is possible that our UV-inactivation is so extensive that it prevents virus attachment or the release of the proteins from the lateral bodies, post fusion (31, 32). Another explanation would be that the effector molecule is UV-sensitive itself.

The difference in the ability of VACV strains to mediate the increase in 4C7 signal suggests that it is linked to a process other than the replication of the virus. If there is any advantage for the virus to produce the effect on the 4C7-reactive molecule *in vivo,* it is likely to manipulate the host immune response to the virus. Such immuno-modulating functions are often different among strains of the virus. We observed that infection by WR and IHD-W resulted in increased 4C7 staining as compared to infection with Copenhagen virus in Cos-7 cells. We, and others, have noted that there are differences in the requirements to infect different cells types, with lymphocytes being quite refractory to infection (19, 33). We observed differences in the virus strains ability to infect 221 cells that necessitated comparing strains in Cos-7 cells only. It might then seem logical that there would be a relationship in what is required to infect 221 cells and other lymphoid

cells types and upregulating expression of a 4C7-reactive molecule. However, WR and IDH-W infection of Cos-7 cells resulted in increased 4C7 staining compared to infection with Copenhagen virus, which does not correlate with the ability of these viruses to infect 221 cells. Another possibility is that the ability to alter the 4C7-signal is related to the pathway that virus uses to enter cells, which can vary with the strain of virus. WR is dependent on endocytosis of the virus as a first step because it expresses fusion-inhibitors in the viral membrane that are inactivated when the virus is in the acidic environment of the endosome (34-36). In contrast, Copenhagen and IHD-W are able to efficiently fuse directly at the cell membrane (37-39). Since only Copenhagen did not increase the 4C7-signal, there appears not to be a relationship between the known entry pathway and the effect on 4C7-signal. It is worth noting IHD-W was derived from WR (40, 41) and therefore in most respects WR and IHD-W are more similar to each other than to Copenhagen. Differences between Copenhagen and WR, or between WR and IHD-W may prove useful in the future to pinpoint the viral proteins involved.

It is very difficult to speculate on the biological significance of the increased detection at the cell surface by 4C7 after infection without knowing what the 4C7 antibody is binding. None-the-less, it is interesting that a molecule was externalized so rapidly following infection and sustained over time. The only surface protein that we are aware of that stays constant or occasionally increases during a poxvirus infection is the transferrin receptor, CD71, which is a highly stable and recycled receptor (42, 43). In addition to an increase on the cell surface, the characteristics of the molecule we detect has some similarity with CD71 in terms of stability and potentially are within the recycling pathway

for plasma membrane proteins to some extent (see Figure 4b). Identifying additional molecules that behave in a similar manner to the 4C7-ligand might provide clues to vesicles or pathways involved.

In summary, we have determined that CLEC2D mRNA is rapidly lost in VACV infected cells but there is a molecule that is rapidly mobilized to the cell surface that could be related to CLEC2D based on the cross-reactivity of the antibody. We propose that this is the first demonstration of rapid surface upregulation of a host molecule following VACV infection that is likely caused by the release of a virion-associated molecule after fusion that differs among strains of VACV. Identifying the mysterious molecule will be necessary to understand the possible advantage to VACV that possess this feature and whether it is a pathway with potential for manipulation in design of VACV-based vaccines and therapies.

## ACKNOWLEDGEMENTS

We thank Dr. David Evans and Dr. Chad Irwin for providing myxoma and various strains of vaccinia virus, Dr. Michele Barry for the EGFP-Copenhagen, Dr. Luis Schang for the EGCG, and Dr. Fiona Culley for the LLT1-YFP construct. We are grateful to Drs. David Evans, Mary Hitt, David Marchant, Ryan Noyce, and Chad Irwin for their helpful comments.

## DISCLOSURE

The authors have no conflicts of interest to declare.

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**FIGURE LEGENDS**

**FIGURE 1:** Alignment of CLEC proteins with poxvirus sequences. Original blasts were done within the Poxvirus Bioinformatics Resource Centre (http://www.poxvirus.org/index.asp?bhcp=1). Alignments were done using Cobalt (Blastp constrained alignment). Cysteines are highlighted in yellow (- = gap, . = amino acid identity, blue text in the viral sequence denotes residues with chemical similarity). The residues in red in LLT1/CLEC2D-1 are those known to be important for receptor binding. The viral sequences were obtained from GenBank: GER91-010 = Cowpox Acc#ABD97357; A40R VACV-WR Acc#YP_233047; DPV = Deerpox W848_83-142 Acc#YP_227519.

**FIGURE 2:** VACV *infection causes upregulation of the 4C7-signal.* 221 cells were infected with EGFP-VACV WR at an MOI of 10 for twelve hours and stained with 4C7 for flow analysis. (a) Representative EGFP and surface 4C7 staining. (b) Average MFI of surface 4C7 staining following infection. (c) 4C7 staining on 221 cells treated with media, UV inactivated VACV or EGFP-VACV WR for twelve hours at an MOI of 5.

**FIGURE 3:** *Time course analysis of 4C7 signal and CLEC2D transcript*. 221 cells were infected with EGFP-VACV WR at various MOIs and for the indicated times, and stained with 4C7 for flow analysis. (a) GFP expression of infected 221 cells over time. (b) MFI of surface 4C7 staining on 221 cells over time. (c) Representative RT-PCR of mRNA

extracted from 221 cells following infection over time. n = 3 experiments each done in triplicate, all data points included. (d) Representative EGFP and surface 4C7 staining for NIH 3T3 cells infected with EGFP-VACV at an MOI of 5 for four hours and stained with 4C7 for flow analysis. n = 3 experiments done in triplicate.

**FIGURE 4:** *4C7 detects an intracellular molecule.* (a) Representative surface and intracellular 4C7 staining on 221 cells by flow cytometry. (b) Confocal microscopy of intracellular 4C7 staining in 221 cells (60x magnification). n = 3 experiments all done in triplicate. (c) Surface staining of 293T cells transfected with an LLT1-YFP plasmid for 48 hours with various anti-CLEC2D antibodies. n = 1 experiment. 221 cells were infected with EGFP-VACV WR for two hours at an MOI of 5. (d) Representative surface staining of 221 cells for various anti-CLEC2D antibodies. (e) Average MFI on 221 cells with various CLEC2D antibodies. n = 3 experiments all done in triplicate, all data points included. **$p < 0.001$.

**FIGURE 5:** *VACV induced 4C7 upregulation does not require protein synthesis.* 221 cells were treated with cyclohexamide (CHX) for one hour at 37°C then infected with EGFP-VACV WR at an MOI of 5. (a) Representative EGFP and surface 4C7 staining on CHX treated and untreated 221 cells. (b) Average MFI of surface 4C7 staining on 221 treated with cyclohexamide. n = 3 experiments all done in triplicate; all data points included. **$p < 0.001$. 221 cells were treated with AraC for five minutes at 37°C then infected with EGFP-

VACV WR for twelve hours at an MOI of 10. (c) Representative EGFP and 4C7 surface staining on 221 cells over time. n = 2 experiments. (d) Representative EGFP and surface 4C7 staining on 221 cells following infection. (e) Average MFI of surface 4C7 staining on 221 cells following infection. n = 3 experiments done in duplicate, all data point included. **$p < 0.001$ and ***$p < 0.0001$.

**FIGURE 6:** *Viral binding is required for upregulation of the 4C7 signal.* (a) Representative EGFP and surface 4C7 staining of 221 cells infected with EGFP-VACV WR for five minutes at an MOI of 5. (b) Average MFI of 4C7 surface staining following a five minute infection on 221 cells in warm media or cooled on ice for 10 minutes. EGFP-VACV WR was pre-treated with media, DMSO or EGCG for 10 minutes at 37$^{o}$C and 12 minutes on ice. 221 cells were then infected with treated EGFP-VACV at an MOI of 5 for five minutes. (c) Representative EGFP expression in 221 cells infected with EGFP-VACV treated with media (untreated), DMSO or EGCG. (d) Representative surface 4C7 staining on 221 cells infected with treated EGFP-VACV. (e) Average MFI of surface 4C7 staining on 221 cells with EGCG treated EGFP-VACV. n = 3 experiments each done in triplicate, all data points included. *$p < 0.01$, **$p < 0.001$ and ***$p < 0.0001$.

**FIGURE 7:** *Comparison of 4C7 expression by active infection with Myxoma.* (a) Representative mCherry and 4C7 staining on 221 cells infected with mCherry-MYX for

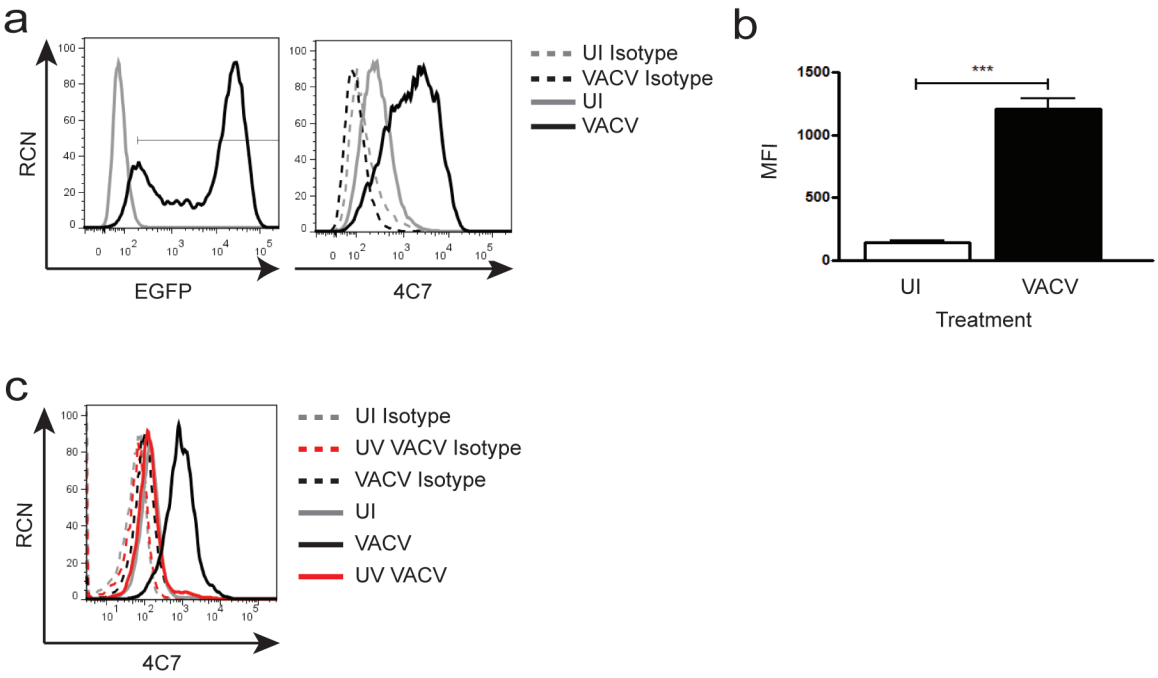
four hours at an MOI of 10. (b) Average MFI of surface 4C7 staining on 221 cells following infection. n = 3 experiments done in triplicate, all data points included.

**FIGURE 8:** *The extent of the 4C7 signal upregulation varies with VACV strains.* 221 cells were infected with EGFP expressing VACV strains for four hours at an MOI of 10. (a) Representative EGFP expression in 221 cells following infection. (b) Representative 4C7 surface staining on 221 cells following infection. n = 2 experiments done in duplicate. (c) Cos-7 cells were infected with EGFP expressing VACV strains for two hours at an MOI of 5. Representative EGFP expression in Cos-7 cells following infection. (d) Representative 4C7 surface staining on Cos-7 cells following infection. (e) Average MFI of surface 4C7 staining on Cos-7 cells following infection. n = 3 experiments done in duplicate, all data point included. *$p < 0.01$ and **$p < 0.001$.

## SUPPORTING INFORMATION

Supporting Information Figure 1: *NKR-P1A function is not altered with VACV-infected 221 cells.*


Clrb 1 MCVTKASLPMLSPTGSPQEVEVGKILQGKRHGTISPESCAKLYCYYGVIMVLTVAVIALSVALSATKTEQIPVNKTYAAC 80
GER91-010 1 ----------MFIMRELV.RV..VI.SLSL.SSFLVICFIEHRCFKEETR.KI. 44
Clrb 81 PQNWIGVENKCFYFSEYPSNWTFAQAFCMAQEAQLARFDNQDELNFLMRYKANFDSWIGLHRESSEHPWKWTDNTEYNNT 160
GER91-010 45 ...T.S.N.I.Y....DTT...ASFEY.KNMNGR..CII.-.TFPKIC...GT..Y.V..Y.TN.QS..LNC..KP...I 123
Clrb 161 IPIRGEERFAYLNNNGISSTRIYSLRMWICSKLNSYSLHCQTPFFPS 207
GER91-010 124 .....V.DY.....TA...AD.R..EVPMVDY 160
LLT1 1 MHDSNNVE-KDITPSELPANPGCLHS---KEHSIKATLIW--------RLFFLIMFLTIIVCGMVAALSAIRA-NCHQE 66
DPV- 1 .FPELKMAL.PT.EIVEMGKDD.RDTNSD..TETQKNYVQFTSFVTPEK.YCC.ITIC.LITINLVPIIILM.FKSDT. 80
LLT1 67 PSVCLQAACPESWIGFQRKCFYFSDDTKNWTSSQRFCDSQDADLAQVESFQELNFLLRYKGPSDHWIGLSREQGQ-PWKW 145
DPV- 81 .TI-KYVT..KG....GY......E.S....FGNT..T.LG.T.VK..TEE.....K...DS.......N..SSNH.... 159
LLT1 146 INGTEWTRQFPILGAGECAYLNDKGASSARHYTERKWICSKSDIHV---------- 191
DPV- 160 A.D.NYNSS.V.T.T.......IRI..S.V.AN.....TYTNIYIPSNNSYNYP 216
A40R 1 ---------- ----------MNKHKTDYAGYACCVICGLIVGIIFTATLLKVVERKLVHTPSIDKT 46
hNKG2A 1 [27]KSSILATEQEITYAEL[9]QGNDKTYHCKDLPSAPEKLIVGILGIICLILMASVVTIVVIPSTLIQRHNNS.LNTR 109
LLT1 1 MHDSNNVEKDITPSEL PANPGCLHSKE-HSIKATLIWRLFFLIMFLTIIVCGMVAALSAIRANCHQEP.VCLQ 72
A40R 47 IKDAYIREDCPTDWISYNNKCIHLSTDRKTWEEGRNACKALNPNSDLIKIETPNELSFLRSIRR--GYWVGESEILNQT- 123
hNKG2A 110 TQK.RHCGH..EE..T.S.S.YYIGKE.R....SLL..TS--K..S.LS.DNEE.MK..-..ISPSS-.I.VFR--.SSH 183
LLT1 73 AA-------..ES..GFQR..FYF.D.T.N.TSSQRF.DS--QDA..AQV.SFQ.LN..LRYKGPSDH.I.LSR--E.G- 140
A40R 124 TPYNFIAKNATKNGTK----------KRKYICSTTNTPKLHSCYTI 159
hNKG2A 184 H.WVTMNGL.F.HEI.DSDNAELNCAVLQVNRLKSAQCGSSII.H.KHKL--- 233
LLT1 141 Q.WKW.NGTEWTRQFPILGAGE--CAYLNDKGASSARHYTE..W...KSDIHV 191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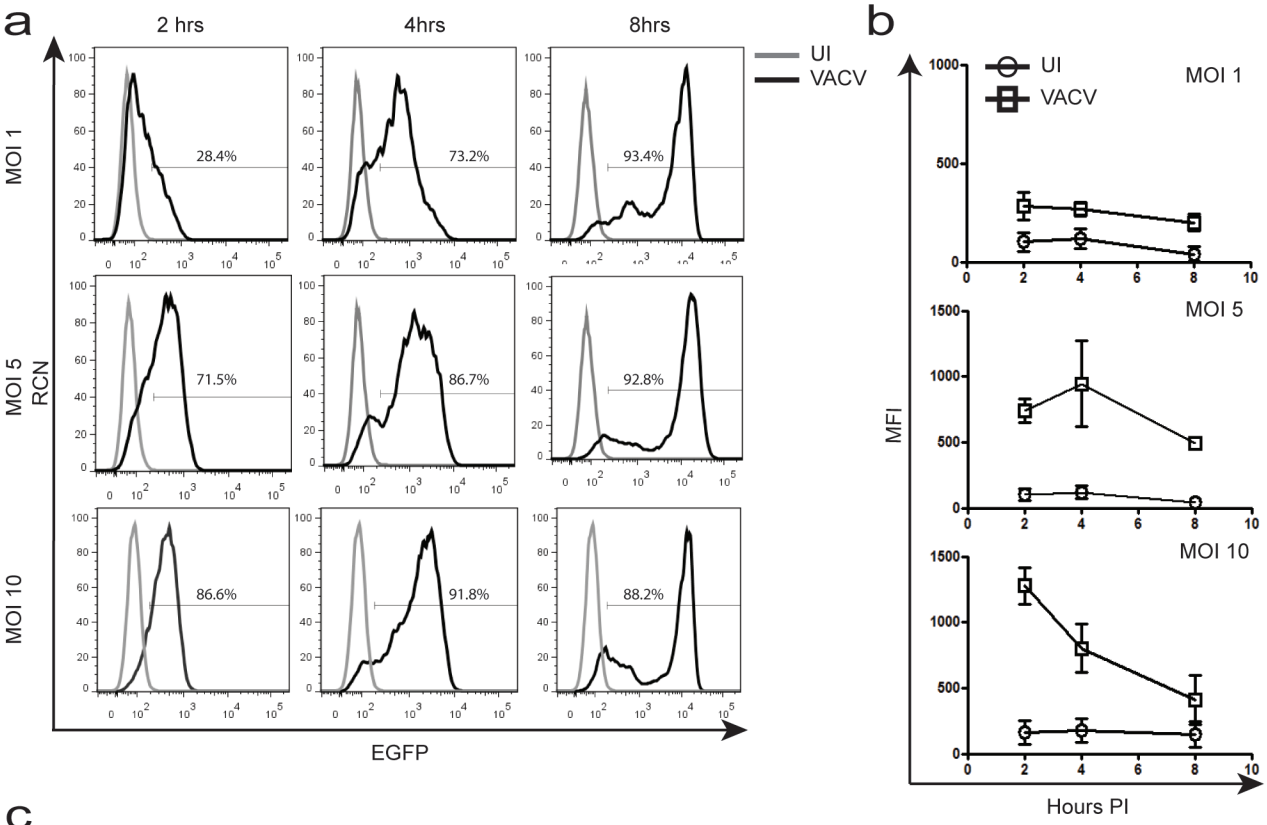
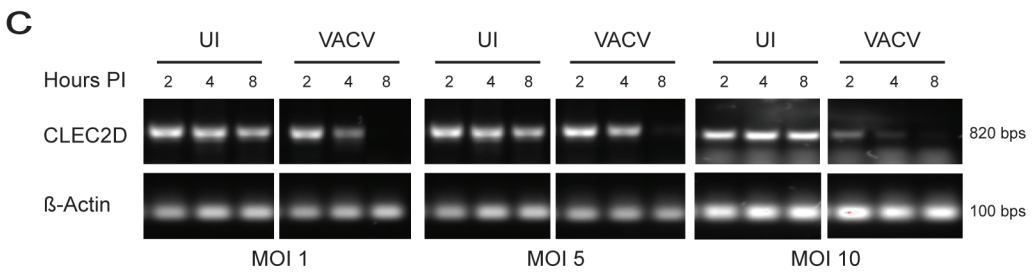
UV VACV

Figure 2


a
2 hrs
4hrs
8hrs
UI
VACV
MOI 1
MOI 5
MOI 10
RCN
28.4%
73.2%
93.4%
71.5%
86.7%
92.8%
86.6%
91.8%
88.2%
EGFP
b
UI
VACV
MOI 1
MOI 5
MOI 10
MFI
Hours PI


c
UI
VACV
Hours PI
2 4 8
CLEC2D
ß-Actin
820 bps
100 bps
MOI 1
MOI 5
MOI 10

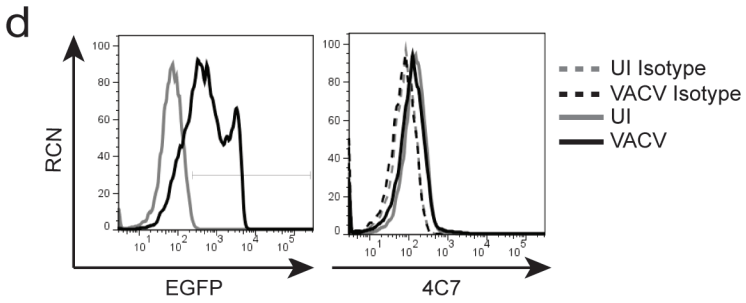

d
RCN
UI Isotype
VACV Isotype
UI
VACV
EGFP
4C7

Figure 3

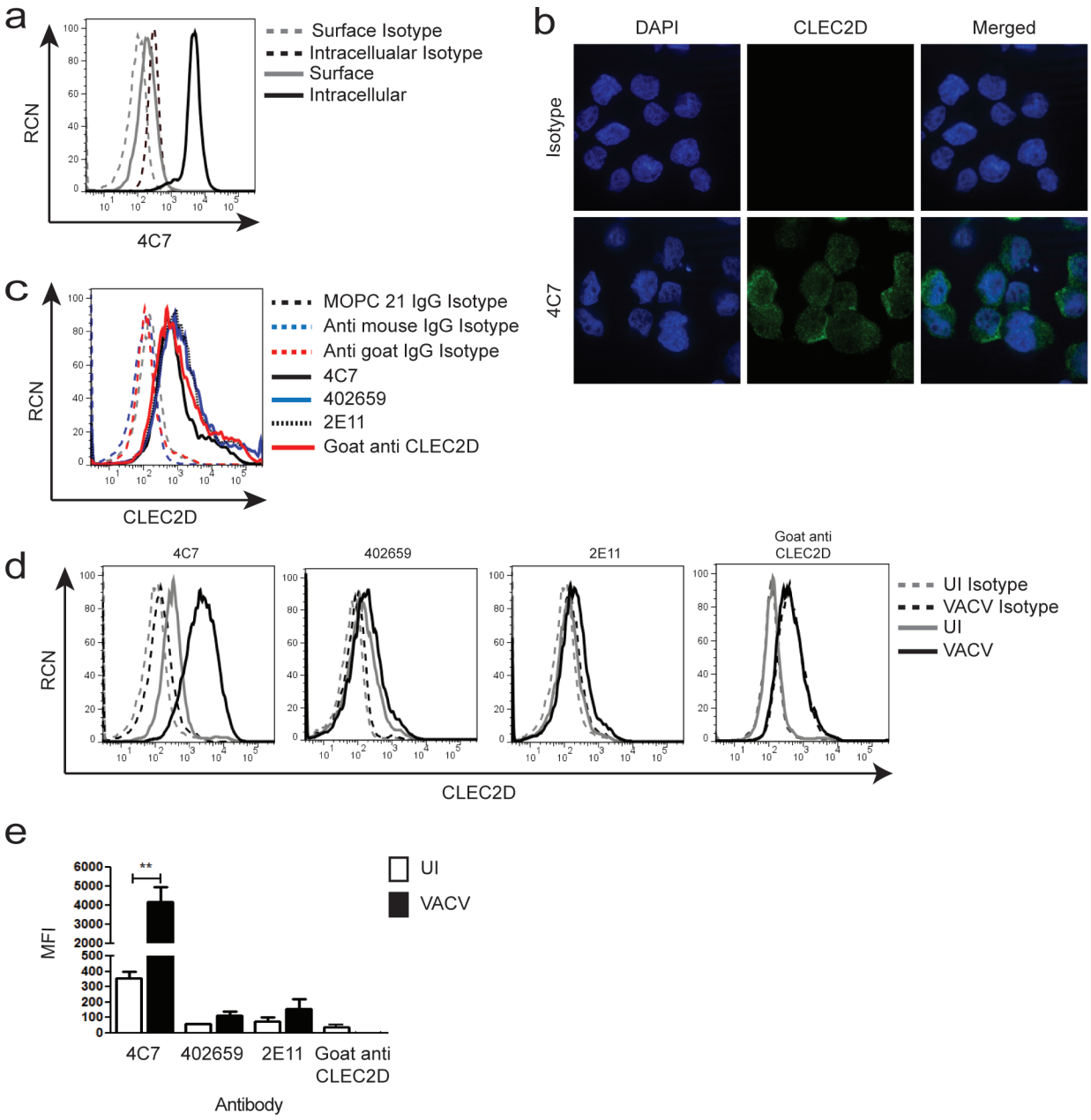

a
RCN
4C7
Surface Isotype
Intracellualar Isotype
Surface
Intracellular
b
DAPI
CLEC2D
Merged
Isotype
4C7
c
RCN
CLEC2D
MOPC 21 IgG Isotype
Anti mouse IgG Isotype
Anti goat IgG Isotype
4C7
402659
2E11
Goat anti CLEC2D
d
4C7
402659
2E11
Goat anti CLEC2D
RCN
CLEC2D
UI Isotype
VACV Isotype
UI
VACV
e
MFI
**
UI
VACV
4C7
402659
2E11
Goat anti CLEC2D
Antibody

Figure 4

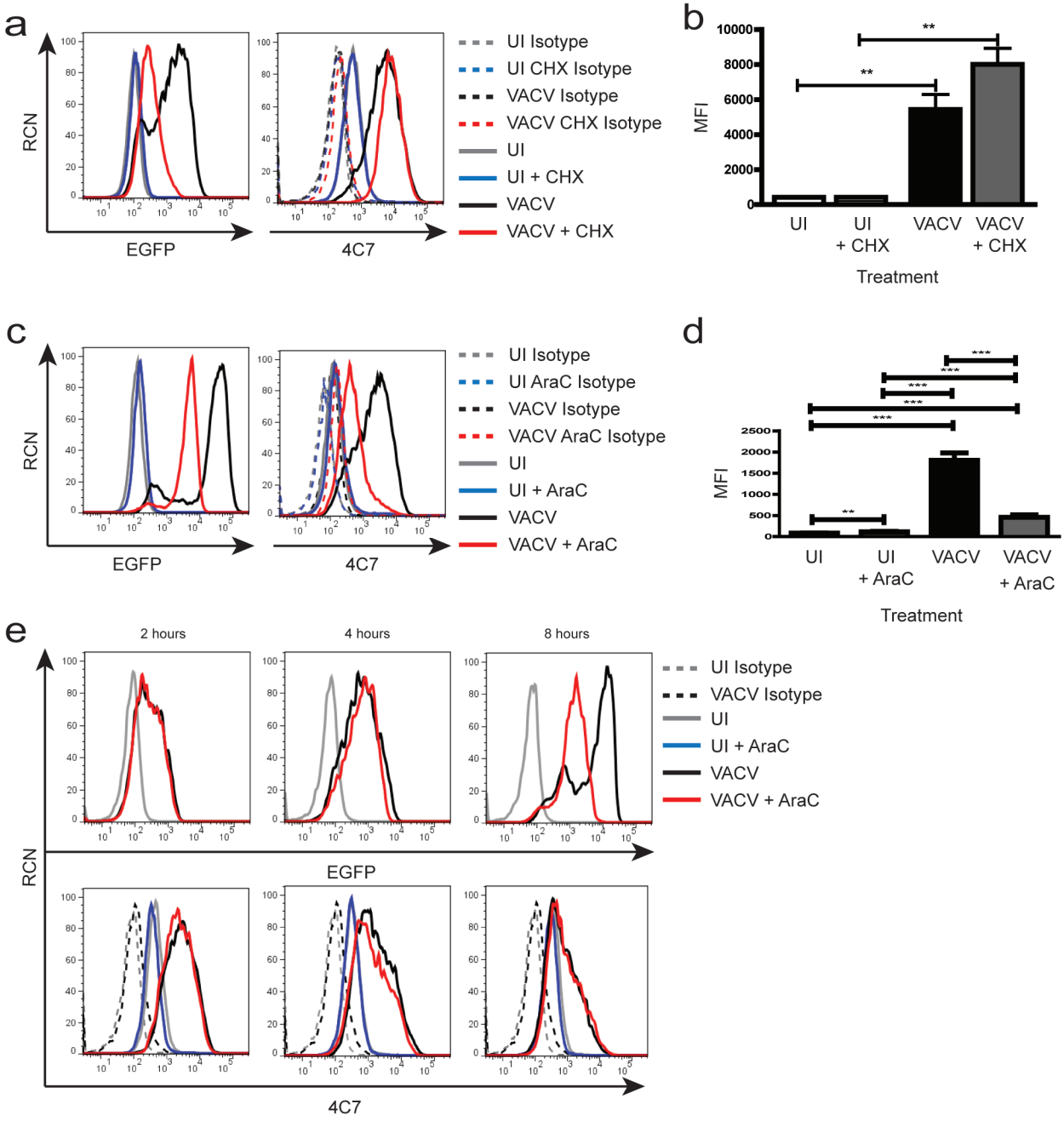

a
RCN
EGFP
4C7
UI Isotype
UI CHX Isotype
VACV Isotype
VACV CHX Isotype
UI
UI + CHX
VACV
VACV + CHX
b
MFI
UI
UI + CHX
VACV
VACV + CHX
Treatment
c
RCN
EGFP
4C7
UI Isotype
UI AraC Isotype
VACV Isotype
VACV AraC Isotype
UI
UI + AraC
VACV
VACV + AraC
d
MFI
UI
UI + AraC
VACV
VACV + AraC
Treatment
e
2 hours
4 hours
8 hours
RCN
EGFP
4C7
UI Isotype
VACV Isotype
UI
UI + AraC
VACV
VACV + AraC

Figure 5

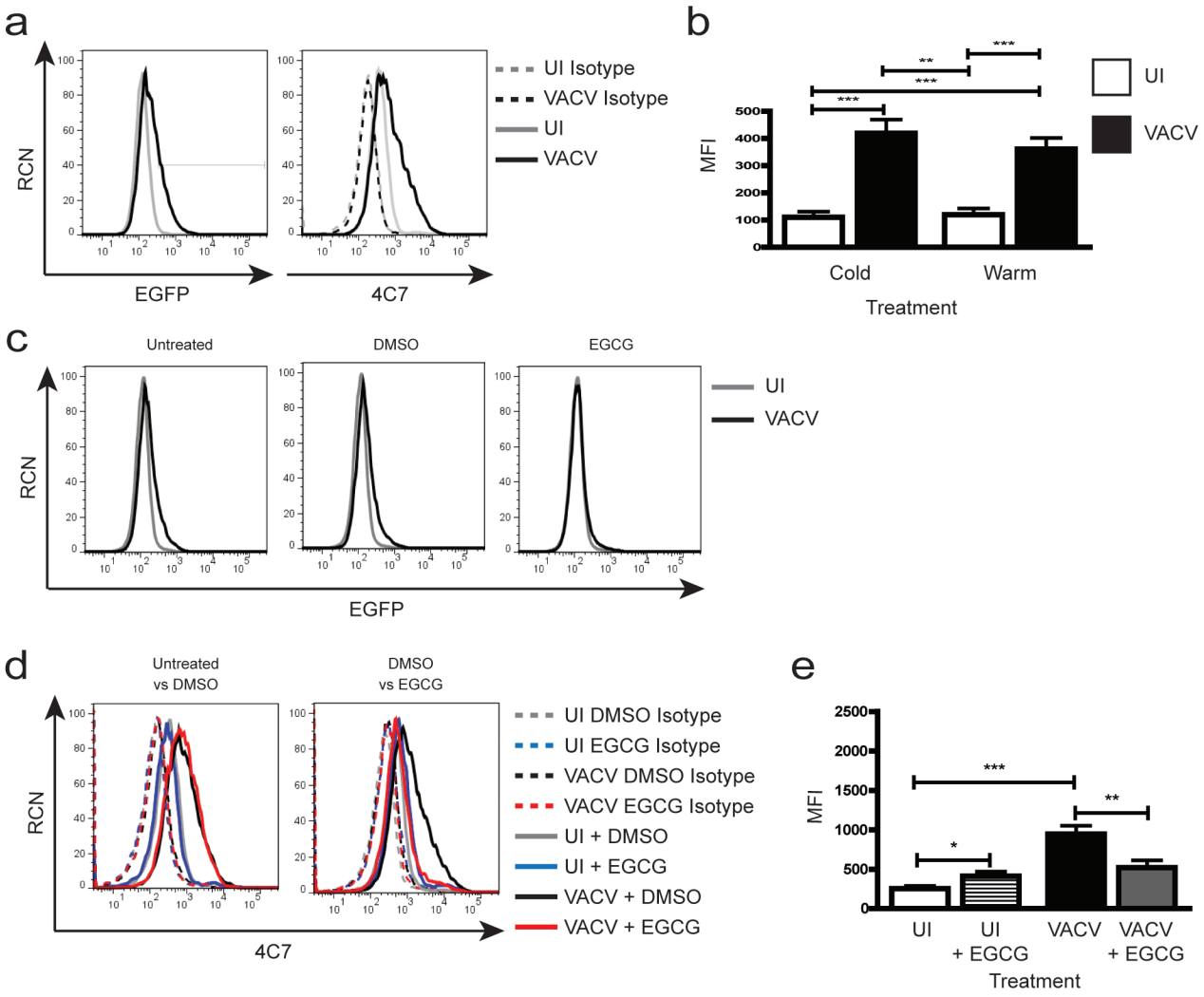

a
RCN
EGFP
4C7
UI Isotype
VACV Isotype
UI
VACV
b
MFI
Cold
Warm
Treatment
UI
VACV
c
Untreated
DMSO
EGCG
RCN
EGFP
UI
VACV
d
Untreated vs DMSO
DMSO vs EGCG
RCN
4C7
UI DMSO Isotype
UI EGCG Isotype
VACV DMSO Isotype
VACV EGCG Isotype
UI + DMSO
UI + EGCG
VACV + DMSO
VACV + EGCG
e
MFI
UI
UI + EGCG
VACV
VACV + EGCG
Treatment

Figure 6

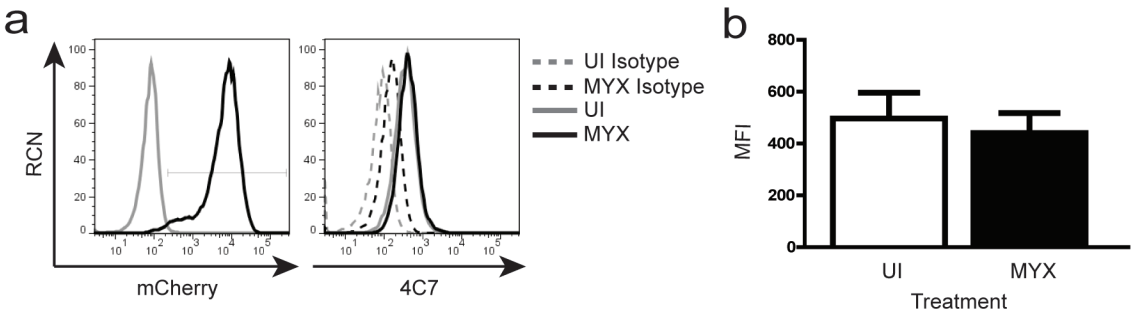

a
RCN
mCherry
4C7
UI Isotype
MYX Isotype
UI
MYX
b
MFI
800
600
400
200
0
UI
MYX
Treatment

Figure 7

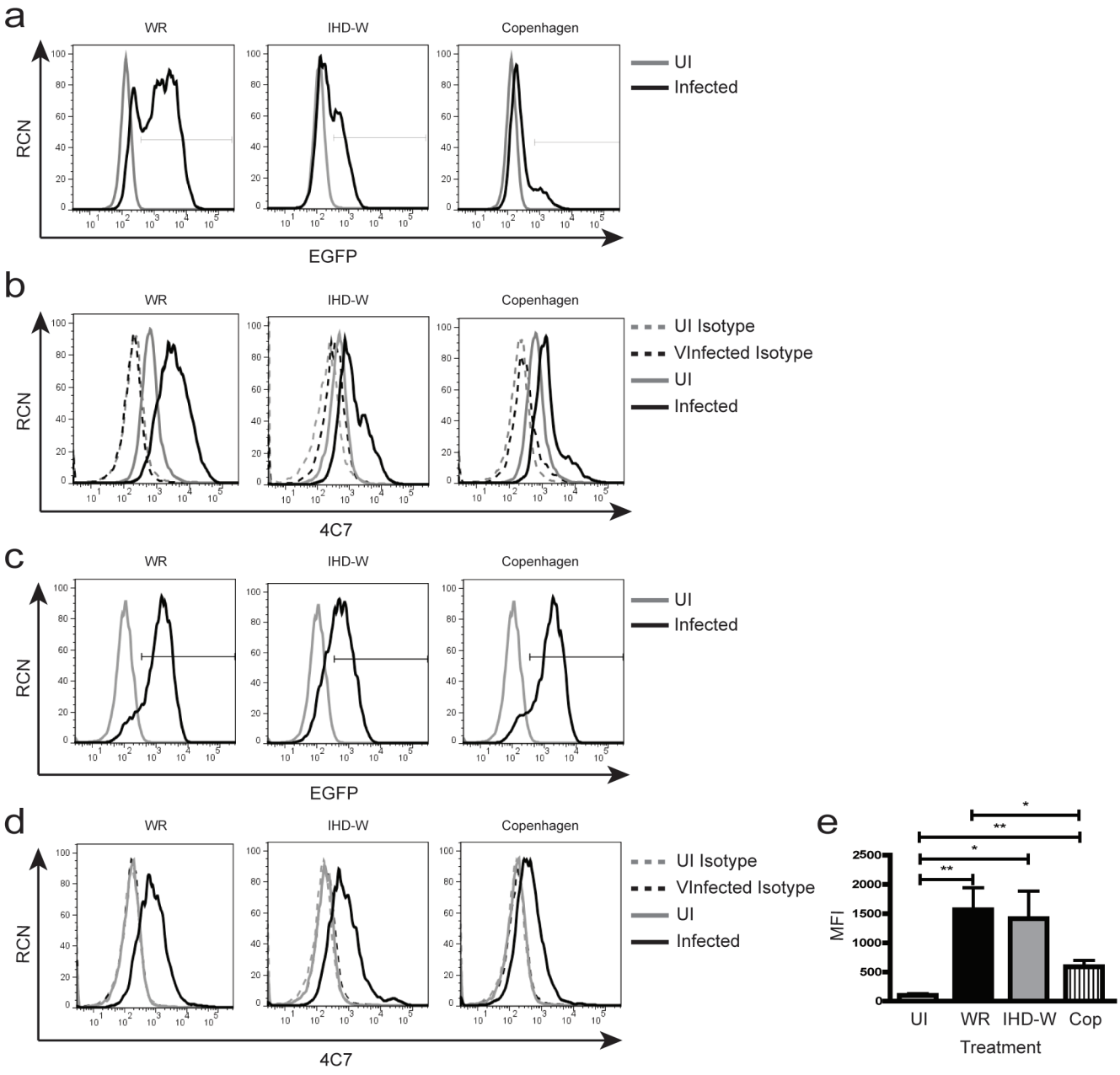
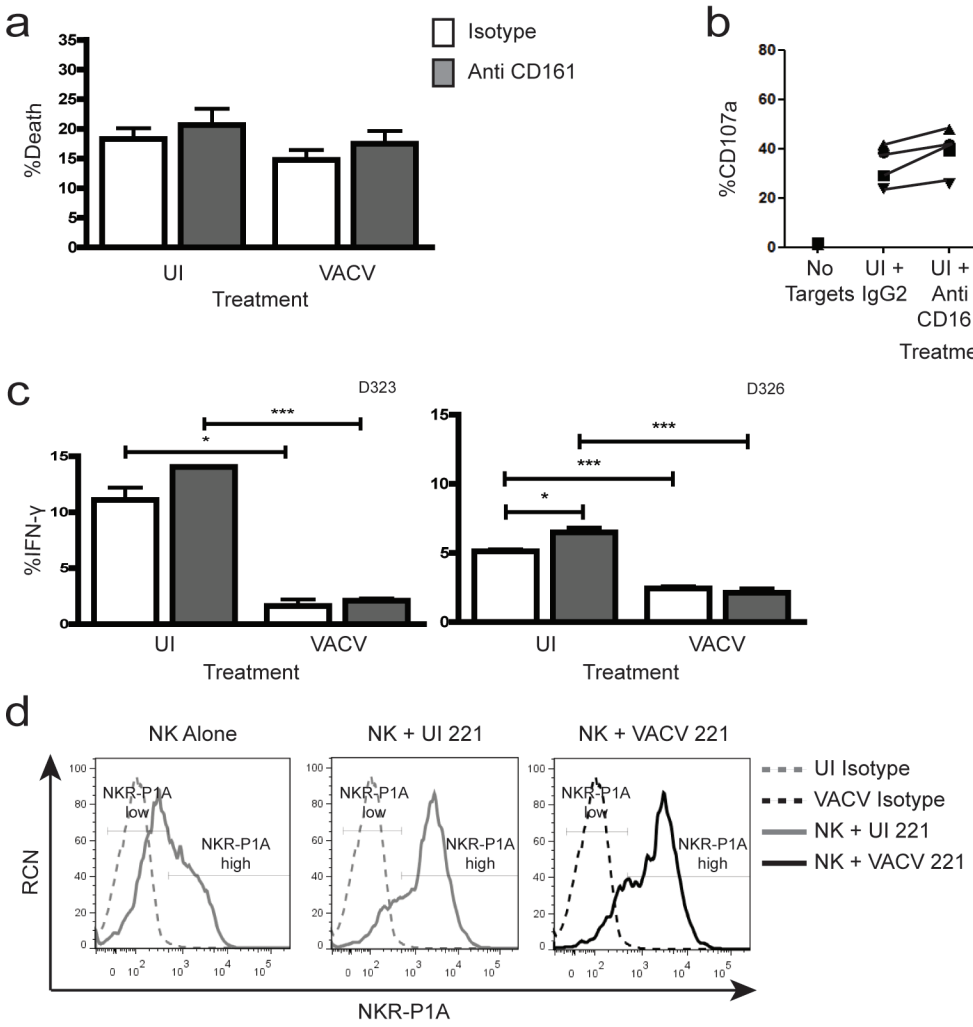

a
WR
IHD-W
Copenhagen
RCN
EGFP
UI
Infected
b
WR
IHD-W
Copenhagen
RCN
4C7
UI Isotype
VInfected Isotype
UI
Infected
c
WR
IHD-W
Copenhagen
RCN
EGFP
UI
Infected
d
WR
IHD-W
Copenhagen
RCN
4C7
UI Isotype
VInfected Isotype
UI
Infected
e
MFI
UI
WR
IHD-W
Cop
Treatment

Figure 8




**SUPPORTING INFORMATION FIGURE 1:** *NKR-P1A function is not altered with VACV-infected 221 cells*. Primary NK cells were isolated from PBMC and incubated with VACV infection 221 cells at a 3:1 E:T ratio, with and without blocking NKR-P1A receptor with anti-CD161. (a) Average percentage of 221 cell death by NK cells from three different donors. n = 2 experiments per donor each done in triplicate. (b) Average percentage of CD107a on NKR-PIA+ NK cells incubated with 221 cells from 4 donors D303 (●) n = 3 experiments each done in triplicate, D227 (▲) n = 1 experiment done in triplicate, D311 (▼) n = 2 experiments each done in triplicate, D323 (■) n = 2 experiments each done in triplicate. All points included. (c) Percentage of NK cells from different donors expressing IFN-γ following incubation with 221 cells. n = a single experiment performed in triplicates on 2 donors on two separate days. Isotype controls in white boxes and anti-CD161 in dark grey boxes. (d) Representative histograms of NKR-P1A staining on primary IL-2 stimulated NK cells with or without target cells. *p > 0.01 and ***p > 0.0001.