Supporting Information

Optical control of the antigen translocation by synthetic photo-conditional viral inhibitors

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3. References
1. METHODS

1.1 Chemical synthesis of the photo-conditional viral immune inhibitors

All ICP47 peptides were synthesized on a pre-loaded Fmoc-L-Arg(Pbf)-TentaGel S AC resin (Rapp Polymere, Tübingen, Germany) as a solid support using a Liberty microwave peptide synthesizer (CEM, Kamp-Lintfort, Germany) with a standard protocol (54 W, 3 min, 75 °C). Coupling reactions were performed twice with 0.2 M of Fmoc-protected amino acid, 0.5 M HBTU, and HOBT•H₂O in the presence of 2 M DIPEA in NMP. Deprotection was achieved with 20% piperidine and 0.1 M HOBT•H₂O in DMF. The coupling of Fmoc-R-Anp and Fmoc-S-Anp (PepTech Corporation, Bedford, MA, USA) and of the subsequent amino acids of ICP472-13 and ICP472-14 was performed manually. Therefore, Fmoc-protected amino acid (3.0 eq), DIPEA (6.0 eq), and COMU (3.0 eq) were pre-activated in DMF (0.3 M) for 1-2 min and thereafter applied to the resin for 1 h at room temperature and under continuous shaking. Double coupling of Fmoc-protected R-Anp or S-Anp and triple coupling for all other Fmoc-protected amino acids were performed. Peptides were cleaved from the resin by incubation with a cleavage cocktail containing 92.5% (v/v) TFA, 2.5% (v/v) H₂O, 2.5% (v/v) thioanisol, and 2.5% (w/v) phenol for at least 8 h at room temperature. Cleaved peptides were precipitated in ice-cold Et₂O, pelleted, washed with ice-cold Et₂O, pelleted again, finally dissolved in tBuOH/H₂O (4:1, v/v), and afterwards lyophilized. Crude peptides were purified by reversed-phase (RP)-C₁₈ HPLC (PerfectSil C18 column 250 x 22 mm 300 ODS 5 μm, MZ Analysentechnik, Mainz, Germany; buffer A: 0.05% TFA (aq.); buffer B: MeCN + 0.05% TFA). Purity and identity of the peptides were confirmed by analytical RP-C₁₈ HPLC, MALDI-TOF-MS and ESI-MS.

1.2 Photo-cleavage of ICP47

pc-ICP47 was photo-cleaved at 365 nm using a LED lamp (Thorlab, CS2010 UV Curing System) with an intensity of 185 mW cm⁻². Photo-cleavage was carried out at room temperature with various illumination times. Qualitative analysis of photo-cleavage was realized by analytical RP-C₁₈ HPLC (Method 1, Table S1). The fragments were assigned by ESI-MS.

1.3 Secondary structure analysis of ICP47 bound to liposomes

Measurements were performed on a CD spectrometer (Jasco J-710) in a 1 mm quartz cuvette (V = 120 μl) at 25 °C under a constant nitrogen flow. 10 μM of the ICP47 variant was pre-incubated with liposomes for 1 h. Subsequently, CD spectra were recorded ten times from λ = 190 - 260 nm. Data sets were analyzed using DiChroWeb server.¹ A mixture of E. coli polar lipids in CHCl₃ and DOPS in CHCl₃ was transferred into a small round flask. After solvent removal under vacuum, the flask was flushed with argon. The lipid mixture was resuspended in PBS (final lipid concentration 2 mg/ml) and sonicated for 30 min in an ultrasonic bath. The lipid solution was snap-frozen in liquid nitrogen and thawed four time at 25 °C. Liposomes were extruded 15 times through a polycarbonate filter (400 nm pore size; Avestin, Mannheim, Germany) and size distribution was determined by nanoparticle tracking (NanoSight LM14, Nanosight Ltd.).

1.4 Peptide binding

For the filter-based peptide competition assay, core-TAP₁₅₅Venus/core-TAP₂₅₇Cerulean containing crude membranes (70 μg of total protein per sample) from Pichia pastoris were incubated with increasing concentrations of the respective ICP47 variant in PBS for 1 h at 4 °C. Reporter peptide C4F (1 μM) was added to the solution, followed by incubation for 15 min at 4 °C.
After photo-cleavage of the respective pc-ICP47 for 2 x 3 min on ice, all samples were incubated for further 15 min at 4 °C. Filter plates (MultiScreen Filter Plate with Durapora Membrane, 0.65 μm, Merck Millipore) were pre-incubated with 200 μl of 0.3% (w/v) PEI in ddH₂O for 10 min at 4 °C and the peptide/membrane mixtures were applied onto the filter membranes. Free peptide was removed by washing twice with 250 μl of ice-cold PBS pH 7.4. The filter membranes were incubated with 300 μl of solubilization buffer (1% w/v SDS in PBS, pH 7.4) for 10 min at room temperature, then transferred to Eppendorf tubes, and boiled for 5 min at 95 °C to denature the fluorescent proteins fused to TAP1/2. The membranes were spun down and the fluorescence of C4F in the supernatant was quantified by a fluorescence plate reader (λ_{ex/em} 485/520 nm). The inhibition constant Kᵢ was calculated by utilizing the Cheng-Prusoff equation, whereby the appropriate IC₅₀ value was deduced from the dose response curve.² The data represent biological triplicates with mean ± SD.

1.5 Microsome preparation

Microsomes from human B-cell lymphoma Raji cells were prepared as described.³ Briefly, 800 ml cell suspension (2 g cells) were harvested by centrifugation (700 x g, 4 min, 4 °C) and resuspended in 8 ml of microsome buffer 1 (50 mM TEA, 50 mM KAc, 6 mM Mg(ClO₄)₂, 1 mM DTT, 250 mM sucrose, 0.5 mM PMSF, pH 7.5). For cell lysis, cells were disrupted with a tight glass douncer. Cell debris was removed by centrifugation (1,000 x g, 10 min, 4 °C). The supernatant was applied to a 1.3 M sucrose solution and centrifuged for 2.5 h (200,000 x g, 4 °C). The pellet was resuspended in microsome buffer 1 and layered onto a 0.5 M sucrose solution. Microsomes were harvested for 20 min at 300,000 x g at 4 °C. The pellet was resuspended in ice-cold microsome buffer 2 (50 mM TEA, 0.5 mM DTT, 250 mM sucrose, pH 7.5) and aliquots were snap-frozen in liquid nitrogen and stored at -80 °C.

1.6 Peptide transport

To analyze peptide transport, 1 μM of NST-F in 5 mM MgCl₂, PBS pH 7.0 was incubated with Raji microsomes (1 mg/ml total protein) and 5 μM of ICP47 or pc-ICP47 for 15 min on ice. For photo-cleavage, samples were illuminated for additional 2 x 3 min on ice. For equal conditions, samples without photo-cleavage were kept on ice during this procedure. Peptide transport was started with 3 mM of ATP or ADP for 10 min at 37 °C. The reaction was stopped with 500 μl of ice-cold stop buffer (10 mM EDTA in PBS pH 8.0). Microsomes were pelleted by centrifugation (20,000 x g, 8 min) and finally lysed in 800 μl of lysis buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 1 mM MnCl₂, 1% NP-40) for 15 min at room temperature. After debris was pelleted at 20,000 x g for 8 min, the supernatant was incubated with 50 μl of Concanavalin A beads (50% w/v in lysis buffer) for 1 h at 4 °C. Thereafter, Concanavalin A beads were washed twice with 1 ml of lysis buffer and finally incubated with 250 μl of elution buffer (lysis buffer supplemented with 200 mM of methyl-α-D-glycopyranoside) for 30 min at room temperature to elute the bound peptide. Fluorescence of NST-F was detected at λ_{ex/em} 485/520 nm with an ELISA reader. The data represent biological triplicates with mean ± SD.

1.7 TAP stability

Purified coreTAP1-mVenuse/coreTAP2-mCerulean from Pichia pastoris was incubated with 1 μM of ICP47₂₋₅₋₅ as well as pc-ICP47₂₋₅₋₅ for 5 min on ice to allow ICP47 binding. If not otherwise stated, photo-stimulation was performed for 5 min on ice. Thereafter, samples were incubated for 5 min at 40 °C. Thermostability was analyzed by fluorescence-based size-exclusion chromatography (FSEC) with regard to monodispersity of the purified TAP
complex. The fluorescence of mCerulean was used as read-out. SEC running buffer was composed of 20 mM HEPES/NaOH, pH 7.4, 200 mM NaCl, 50 mM KCl, 5% (v/v) glycerol, and 0.05% (w/v) GDN. SEC was performed with a Shodex semi-micro KW 404-4F (4.66 x 300 mm) column.

1.8 Isolation of CD14+ mononuclear cells
Buffy coats were diluted 1:4 (v/v) with PBS and pipetted onto a layer of Ficoll solution (Biocoll, δ = 1.077 g/ml, isotonic, Biochrom AG, Berlin, Germany). Human peripheral blood mononuclear cells (PBMCs) were isolated after gradient centrifugation at 900 x g for 20 min at room temperature. Further enrichment of CD14+ monocytes was performed by magnetic-activated cell sorting (MACS) positive selection using human anti-CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). Enrichment was performed according to the manufacturer’s protocol of Miltenyi Biotec using LS-columns. Briefly, PBMCs were first washed with MACS rinsing buffer. PBMCs were then resuspended in 40 µl of MACS rinsing buffer and 10 µl microbeads per 10^7 PBMCs, incubated for 20 min at 4 °C in the dark. After further washing steps, cell suspension was applied on a LS-column and CD14+ cells were enriched. Informed consent was obtained from all donors as approved by the Ethics Committee from the DRK-Blutspendedienst Baden-Württemberg/Hessen and subject data were treated as confidential information protected by medical confidentiality.

1.9 Single cell-based translocation assay
Raji cells or CD14+ human monocytes (~200,000 cells per sample) in PBS were centrifuged at 300 x g for 5 min at 4 °C. After aspiring the supernatant, cells were incubated for 15 min at 4 °C with 4-5 µg/ml streptolysin O (Abcam, ab63978). Thereafter, cells were centrifuged at 700 x g for 1.5 min and the supernatant was discarded. 50 µl of the transport mixture (10 mM ATP/ADP, 10 mM MgCl2, 30 nM NST-F or NST-AF647), in the absence or presence of the respective ICP47 (5 µM), were added to each probe and cells were incubated for 10 min at 37 °C. Samples, which had not been treated with pc-ICP47, were incubated on ice for 6 min, whereas samples bearing pc-ICP47 were kept on ice while photo-cleavage was performed for 2 x 3 min. Cells were subsequently incubated for 15 min at 37 °C. The reaction was stopped by adding 20 mM EDTA in PBS and samples were analyzed by FACS Aria III (BD Biosciences) with λex/em 494/520 nm (NST-F) as well as λex/em 650/668 nm (NST-AF647). Data analysis was performed with FlowJo V10 to determine the mean fluorescence intensity (MFI). The statistics are based on two independently performed experiments in biological duplicates resulting in n = 4. Data are represented in mean ± SD utilizing one-way analysis of variance (ANOVA) with Tukey’s test (P<0.001). Analysis was performed with GraphPad Prism5.

1.10 Cell line identity
Raji cells (ICLAC: CVCL_0511) were obtained from DSMZ (cat no. ACC 319) with guarantee for no cross-contamination. Additionally, Raji cells have been tested for mycoplasma contamination.4
2. SUPPLEMENTARY TABLES AND FIGURES

Table S1. RP-C\textsubscript{18} HPLC gradient.

<table>
<thead>
<tr>
<th>Method 1</th>
<th></th>
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<tbody>
<tr>
<td>t (min)</td>
<td>0.05% TFA (aq.) (%)</td>
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<tr>
<td>0.0</td>
<td>95</td>
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<td>20.0</td>
<td>30</td>
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<td>95</td>
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<td>28.0</td>
<td>95</td>
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Table S2. Secondary structure analysis.

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<th>Compound</th>
<th>α-helical content (%)</th>
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<tr>
<td>ICP47\textsubscript{2-34}</td>
<td>61</td>
</tr>
<tr>
<td>pc-ICP47\textsubscript{2-34}\textsuperscript{14R-Anp}</td>
<td>60</td>
</tr>
<tr>
<td>pc-ICP47\textsubscript{2-34}\textsuperscript{14S-Anp}</td>
<td>56</td>
</tr>
<tr>
<td>pc-ICP47\textsubscript{2-34}\textsuperscript{15R-Anp}</td>
<td>65</td>
</tr>
<tr>
<td>pc-ICP47\textsubscript{2-34}\textsuperscript{15S-Anp}</td>
<td>64</td>
</tr>
<tr>
<td>ICP47\textsubscript{2-55}</td>
<td>56</td>
</tr>
<tr>
<td>pc-ICP47\textsubscript{2-55}\textsuperscript{15R-Anp}</td>
<td>60</td>
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Table S3. Photo-cleavage analysis.

<table>
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<th>Compound</th>
<th>t\textsubscript{\textgamma} (s)</th>
<th>Conversion (%)</th>
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<tr>
<td>pc-ICP47\textsubscript{2-34}\textsuperscript{14R-Anp}</td>
<td>17 ± 3</td>
<td>97.0</td>
</tr>
<tr>
<td>pc-ICP47\textsubscript{2-34}\textsuperscript{14S-Anp}</td>
<td>13 ± 1</td>
<td>97.5</td>
</tr>
<tr>
<td>pc-ICP47\textsubscript{2-34}\textsuperscript{15R-Anp}</td>
<td>12 ± 1</td>
<td>97.0</td>
</tr>
<tr>
<td>pc-ICP47\textsubscript{2-34}\textsuperscript{15S-Anp}</td>
<td>13 ± 2</td>
<td>90.3</td>
</tr>
<tr>
<td>pc-ICP47\textsubscript{2-55}\textsuperscript{15R-Anp}</td>
<td>14 ± 2</td>
<td>91.0</td>
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</table>
**Table S4. Inhibitory constants.**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC$_{50}$ (µM)</th>
<th>$K_i$ (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICP47$_{2-34}$</td>
<td>2.1 ± 0.2</td>
<td>100 ± 11</td>
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<tr>
<td>pc-ICP47$_{2-34}^{14R}$-Anp</td>
<td>11.6 ± 0.7</td>
<td>550 ± 35</td>
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<tr>
<td>pc-ICP47$_{2-34}^{145}$-Anp</td>
<td>10.5 ± 0.5</td>
<td>498 ± 22</td>
</tr>
<tr>
<td>pc-ICP47$_{2-34}^{15R}$-Anp</td>
<td>2.5 ± 0.2</td>
<td>125 ± 8</td>
</tr>
<tr>
<td>pc-ICP47$_{2-34}^{155}$-Anp</td>
<td>2.6 ± 0.2</td>
<td>121 ± 9</td>
</tr>
<tr>
<td>ICP47$_{2-55}$</td>
<td>2.8 ± 0.2</td>
<td>134 ± 10</td>
</tr>
<tr>
<td>pc-ICP47$_{2-55}^{15R}$-Anp</td>
<td>3.7 ± 0.3</td>
<td>176 ± 14</td>
</tr>
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</table>
Figure S1. Purity and identity of ICP47<sub>2-34</sub>.

RP-C<sub>18</sub> HPLC analysis of ICP47<sub>2-34</sub> (purity > 97%) was performed with Method 1 (Table S1). ESI-MS analysis revealed a molecular weight for ICP47<sub>2-34</sub>: 3881.9 Da (calc.), 3883.0 ± 1.4 Da (deconvoluted).
**Figure S2.** Purity and identity of pc-ICP47\(_{2-34}\) variants.

RP-C\(_{18}\) HPLC analysis of pc-ICP47\(_{2-34}\)\(^{14}\text{S}-\text{Anp}\) (purity > 90%), pc-ICP47\(_{2-34}\)\(^{14}\text{R}-\text{Anp}\) (purity > 93%), as well as pc-ICP47\(_{2-34}\)\(^{15}\text{S}-\text{Anp}\) (purity > 94%) was performed with Method 1 (Table S1). ESI-MS analysis showed consistent molecular weights for all photo-conditional viral inhibitors. pc-ICP47\(_{2-34}\)\(^{14}\text{S}-\text{Anp}\): 3959.9 Da (calc.), 3961.6 ± 0.3 Da (deconvoluted). pc-ICP47\(_{2-34}\)\(^{14}\text{R}-\text{Anp}\): 3959.9 Da (calc.), 3961.6 ± 0.4 Da (deconvoluted). pc-ICP47\(_{2-34}\)\(^{15}\text{S}-\text{Anp}\): 3942.9 Da (calc.), 3944.4 ± 0.1 Da (deconvoluted).
**Figure S3.** High-resolution MS analysis of pc-ICP47$_{2-34}$ variants.

The isotope distribution of the respective pc-ICP47$_{2-34}$ was analyzed by MALDI-TOF MS and is in agreement with the calculated mass.
Figure S4. Purity and identity of ICP47_{2-55} and pc-ICP47_{2-55}.

The purity and identity of both peptides were confirmed by RP-C_{18} HPLC (upper panel) and ESI-MS (lower panel), respectively. Mass spectrometry analysis revealed a deconvoluted molecular weight of 6277.8 ± 1.3 Da (6279.1 Da calc.) for ICP47_{2-55} and 6355.6 ± 1.1 Da (6354.3 Da calc.) for pc-ICP47_{2-55}^{15R-Anp}. 
Figure S5. Secondary structure analysis of ICP47_{2-34}, ICP47_{2-55}, and their photo-conditional variants.

The respective ICP47 (10 µM) was added to *E. coli* polar lipids/DOPS (3:1) liposomes (size distribution 335 ± 165 nm). After incubation for 1 h at room temperature, CD spectra were recorded ten times. Two distinct minima at ~210 nm and ~224 nm were identified for all engineered viral factors, indicative for an α-helical structure.
Figure S6. Photolytic analysis of pc-ICP47$_{2-34}$ variants.

Time-dependent photo-cleavage of pc-ICP47$_{2-34}^{145\text{-Anp}}$ ($t_{1/2} = 13 \pm 1$ s, 97.5% conversion), pc-ICP47$_{2-34}^{14R\text{-Anp}}$ ($t_{1/2} = 17 \pm 3$ s, 97.0% conversion), as well as pc-ICP47$_{2-34}^{155\text{-Anp}}$ ($t_{1/2} = 13 \pm 2$ s, 90.3% conversion) was monitored by RP-C$_{18}$ HPLC with Method 1 (Table S1). Photofragmentation was performed at 365 nm (185 mW cm$^{-2}$) for the indicated time intervals. Photolytic scission followed a mono-exponential decay.
Figure S7. ESI-MS analysis of photo-fragmented pc-ICP472-34.

Photo-cleavage (5 min at 365 nm; 185 mW cm$^{-2}$) of all pc-ICP47$_{2-34}$ variants led to two fragments (A, B). Fragment assignment was performed via ESI-MS analysis of pc-ICP47$_{2-34}$ fragments from residue 2-13/14 (dark grey, Fragment A) as well as 15/16-34 (light grey, Fragment B). Calculated molecular weights (MW$_{calc}$) and deconvoluted molecular weights (MW$_{decon}$) are in excellent agreement. All photo-conditional viral inhibitors showed the expected fragmentation pattern, demonstrating the correct photo-scission at the Anp moiety.
Figure S8. Photo-conditional peptide transport by TAP.

Translocation of NST-F was analyzed in Raji microsomes before and after photo-stimulation at 365 nm for 5 min. Peptide transport was carried out in the presence of 3 mM of Mg-ATP or Mg-ADP, 1 μM of NST-F, 5 μM of unmodified or photo-conditional viral inhibitor for 10 min at 37 °C. The amount of N-core glycosylated reporter peptide was quantified. In the presence of unmodified ICP47-2-34 and its photo-conditional variants, peptide translocation was effectively blocked to a different extent, depending on the used pc-ICP47-2-34. Upon photolysis of the respective pc-ICP47-2-34, peptide transport was restored to ~ 90% for all viral factors. *: photo-cleavage of pc-ICP47-2-34 prior use in the assay.
Figure S9. Light-controlled peptide binding to TAP.

(a) Binding of the reporter peptide C4F was competed with increasing concentrations of ICP47_{2-55} or pc-ICP47_{2-55}^{15R-Anp}. Inhibition constants of ICP47_{2-55} (K_i = 134 ± 10 nM) and pc-ICP47_{2-55} (K_i = 176 ± 14 nM) towards TAP were in a similar range. (b) Peptide binding was competed with 25 µM of ICP47_{2-55} as well as pc-ICP47_{2-55}^{15R-Anp}. Photo-stimulation was performed at 365 nm for 5 min. Recovery to ~ 80% of peptide binding activity was observed.
Figure S10. Time-dependent photolysis of pc-ICP47$_{2-55}^{15}R$-Anp.

Photo-cleavage was analyzed by RP-C$_{18}$ HPLC at 0-300 s of illumination time at 365 nm. Starting material followed a mono-exponential decay with a $t_{1/2} = 14 \pm 2$ s upon photo-stimulation, yielding 91% conversion.
Figure S11. ESI-MS analysis of photo-fragmented pc-ICP472-55$^{15\text{R-Anp}}$.

Photo-cleavage of pc-ICP472-55$^{15\text{R-Anp}}$ led to two fragments (A, B). Fragment assignment was performed via ESI-MS analysis of pc-ICP472-55$^{15\text{R-Anp}}$ fragments from residue 2-14 (dark grey, Fragment A) as well as 16-55 (light grey, Fragment B). Calculated molecular weights (MW$_{\text{calc}}$) and deconvoluted molecular weights (MW$_{\text{decon}}$) are in very good agreement, demonstrating correct photo-scission at the light-sensitive Anp.
Figure S12. Light-modulated thermostability of ICP47-TAP complexes.

Purified TAP1-mVenus/TAP2-mCerulean were incubated with 1 µM of ICP472-55 (a) or 1 µM of pc-ICP472-5515R-Anp (b) for 5 min at 4 °C, followed by 5 min at 40 °C, and analyzed by multicolor-fluorescence size exclusion chromatography (MC-FSEC, mCerulean fluorescence). Photo-stimulation was performed for 5 min immediately after incubation at 4 °C. Monodispersity of the elution profile of TAP1/2 and, hence thermostability were achieved for ICP472-55 and its photo-conditional variant. After photo-stimulation, the ICP472-55-TAP complex is still stable, whereas the pc-ICP472-5515R-Anp-TAP complex dissociates.
**Figure S13.** Cell viability of Raji cells upon photo-stimulation.

(a) Raji cells were exposed to 365 nm (185 mW cm\(^{-2}\)) for 0, 1, and 5 min, incubated for 20 min at 37 °C to simulate the time frame of the TAP assay and measured by flow cytometry. The percentage of live and dead cell population based on the forward-sideward scatter gating does not significantly differ between non-illuminated, 1 min and 5 min illuminated cells. (b) Cell viability was assessed by propidium iodine (PI) staining, typically applied for live-dead-cell discrimination, showing no increase of the PI positive population upon illumination. (c) The percentage of PI positive cells for non-illuminated, 1 min and 5 min illuminated cells is in a similar range. These data demonstrate that illumination at 365 nm has no significant impact on the cell viability within the time frame of 20 min, which is the time interval required for the TAP-dependent peptide translocation assay.
Figure S14. Photo-stimulated peptide translocation in primary human monocytes.

(a) In the presence of ICP47_{2-34} and pc-ICP47_{2-34}^{15R-Anp}, peptide transport was diminished to background level. In contrast to ICP47_{2-34}, photo-cleavage of pc-ICP47_{2-34}^{15R-Anp} restored peptide translocation in primary human immune cells. As controls, semi-permeabilized, ATP treated, and NST-F treated monocytes were analyzed. (b) Summary of light-stimulated peptide translocation in semi-permeabilized monocytes. Data represent mean ± SD (n = 2, biological duplicates).
3. REFERENCES