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Chikungunya virus envelope protein E2 provides a vector for targeted antigen delivery to human dermal CD14⁺ dendritic cells.

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1006 words

TO THE EDITOR

Therapeutic modulation of human cutaneous antigen-presenting cells (APCs) that control the fate of T cells represents an interesting option for immunotherapy. As they capture glycosylated proteins via C-type lectins, lectin-targeting antibodies have been used to specifically convey antigens to APCs (Romani *et al.*, 2012;Stoitzner *et al.*, 2014;Kastenmuller *et al.*, 2014). Beyond potential therapeutic applications, APC-targeting strategies can be harnessed to acquire fundamental knowledge about their function (Flacher *et al.*, 2014). In human skin, epidermal Langerhans cells (LCs), CD1c⁺ dermal type-2 conventional dendritic cells (cDC2) and CD141⁺ dermal cDC1 (Nestle *et al.*, 2009;Schlitzer *et al.*, 2015) can migrate to lymph nodes and stimulate T cells. Dermal APCs (dAPCs) include two CD14⁺ subsets that express the lectins Mannose Receptor (MR)/CD206 and DC-specific intracellular adhesion molecules-3 grabbing non-integrin (DC-SIGN)/CD209 (Klechevsky *et al.*, 2008;McGovern *et al.*, 2014). During skin explant culture, CD14⁺ dermal DCs (dDCs) migrate into the supernatant, whereas sessile dermal macrophages require enzymatic digestion to be isolated. Still, CD14⁺ dDCs and dermal macrophages display strong similarities (McGovern *et al.*, 2014). They are both considered permanent dermal residents that exert local functions in pathogen capture (e.g. arthropod-borne viruses) (Kwan *et al.*, 2008;Schaeffer *et al.*, 2015) and may interact with skin-resident memory T cells (TRM) (Clark, 2015). Subset differences might reflect their origin, i.e. monocyte-derived dDCs vs. embryonically seeded resident macrophages (Ginhoux and Guilliams, 2016;McGovern *et al.*, 2014). Because tools to specifically target CD14⁺ dAPCs remain scarce (Duinkerken *et al.*, 2019), we propose here a previously unreported approach based on recombinant arboviral envelope proteins.

Chikungunya virus (CHIKV) of the flavivirus genus of *Togaviridae* family is introduced into the dermis during blood meals of mosquitoes. CHIKV infection triggers high fever, skin rash and persistent arthralgia. Our finding that CD14⁺ dAPCs are preferentially infected by Dengue virus (Kwan *et al.*, 2008;Schaeffer *et al.*, 2015) prompted the idea that they may represent an immediate CHIKV target. CD14⁺ dAPCs isolated from human dermis, monocyte-derived DCs (MDDCs) and dermal-like macrophages (MDdMs) were exposed to CHIKV-06.049 strain (MOI=2.5) and compared with HeLa cells, in which vigorous viral replication is expected (Sourisseau *et al.*, 2007). CD14⁺ dAPCs and their monocyte-derived equivalent MDdMs were permissive CHIKV target cells, as well as MDDCs, which resemble the inflammatory DCs recruited to the site of infection (**Figure 1a**).

We hypothesized that APCs express endocytic receptors of viral envelope proteins. CHIK-sE2, a soluble form of CHIKV envelope glycoprotein E2, was fused to the SNAP proprietary enzyme and produced in insect cells to retain specific N-glycosylations that may be recognized by lectins (**Figure 1b**). In surface plasmon resonance assays, CHIK-sE2 displayed high affinity for MR and DC-SIGN (**Figure S1a/b**), with dissociation constants of 3.9nM and 51.1nM, respectively (**Figure S1c**). CHIK-sE2 was then covalently conjugated via its enzymatic SNAP moiety to AlexaFluor488 fluorochrome (**Figure 1c**). Since MDdMs and MDDCs, but not

monocytes, express high levels of lectins (Kwan *et al.*, 2008), we assessed their binding of the recombinant viral glycoprotein. CHIK-sE2-A488 only labelled MDdMs and MDDCs, and an anti-CHIK-sE2 antibody diminished binding to MDDCs (**Figure 1d/e**). Moreover, fluorescence microscopy revealed intracellular vesicles containing CHIK-sE2-A488 within MDdMs and MDDCs, but not in monocytes (**Figure 1f**). Incubation with CHIK-sE2 had no influence on MDDC maturation, estimated by CD86 expression (**Figure S2**).

To evaluate whether CHIK-sE2 can target CD14⁺ dAPCs within the skin, we compared its capture by different primary cutaneous APCs and non-hematopoietic cells. Epidermal cell suspensions, fibroblasts or purified CD14⁺ dAPCs and CD1c⁺ dDCs were incubated with CHIK-sE2-A488 and binding efficacy was measured by flow cytometry. CD14⁺ dAPCs displayed clear binding of CHIK-sE2-A488, as opposed to keratinocytes, LCs, fibroblasts and CD1c⁺ dDCs (**Figure 1g**). Anti-CHIK-sE2 mAb inhibited the interaction with CD14⁺ dAPCs. Quantitative analyses on skin samples from different individuals confirmed that only CD14⁺ dAPCs could efficiently capture CHIK-sE2-A488 (**Figure 1h**). CHIK-sE2-A488 was detected as condensed spots in the cytoplasm of CD14⁺ dAPCs, reflecting endocytic uptake (**Figure 1i**).

We next tested whether CHIK-sE2 could be used to convey antigens to CD14⁺ dDCs, which stimulate T cells more potently than macrophages (McGovern *et al.*, 2014). To assess binding to CD14⁺ dDCs in comparison with the other skin DC subsets, we cultured skin explants for 3 days and collected LCs, CD14⁻ dDCs and CD14⁺ dDCs from the supernatant (**Figure 2a**). When the cell suspension was exposed to CHIK-sE2-A488, only CD14⁺ dDCs significantly captured (**Figure 2b/c**) and internalized (**Figure 2d, Video S3**) the fluorescent protein. Mannan potently decreased CHIK-sE2-A488 binding, and inhibition assays using anti-lectin mAb suggested that MR is a more efficient CHIK-sE2 receptor than DC-SIGN (**Figure 2e**).

To address antigen presentation, we used a CD4⁺ T-cell clone with defined specificity for HLA-DR1-restricted antigenic peptide of the Epstein-Barr virus Nuclear Antigen-1 (EBNA1) (Rühl *et al.*, 2019). Total migratory APCs or CD14⁺ dDCs from HLA-DR1⁺ skin explants (5 donors out of 34) were loaded with CHIK-sE2 conjugated to EBNA1 peptide (**Figure 2f**). APCs were then cocultured with CFSE-labelled, EBNA1-specific CD4⁺ T cells. After 5 days, proliferation and intracellular IFN- γ production were quantified by flow cytometry. CHIK-sE2-EBNA1-exposed APCs from HLA-DR1 donors strongly enhanced proliferation (**Figure 2g**) and triggered IFN- γ production (**Figure 2h**) of EBNA1-specific T cells. However, T cells did not proliferate when cultured with mismatched, non-HLA-DR1 APCs (**Figure 2i**). Targeting purified CD14⁺ dDCs with CHIK-sE2-EBNA1 was sufficient to promote EBNA1-specific T-cell proliferation. Yet, IFN- γ production was not significantly enhanced, possibly because CD14⁺ dDCs produce anti-inflammatory IL-10 and display low CD86 expression (Klechevsky *et al.*, 2008).

Cutaneous TRM are derived from effector T cells that colonize the skin to mediate adaptive immune responses (Clark, 2015). CD14⁺ dAPCs might be critically involved during secondary exposure to cognate antigen because they also permanently reside in the skin and can reactivate memory T cells (McGovern *et al.*,

2014). CD14⁺ dAPCs targeting by CHIK-sE2 could be particularly useful to investigate and influence these interactions for therapeutic purposes. Moreover, our work suggests that arboviruses may represent a natural reservoir of molecules targeting different skin APCs. They may be derived into a specific delivery system for antigens but also adjuvants, silencing RNA or toxins, thereby harnessing the properties of skin APCs for immunotherapy or vaccination.

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Data availability

No datasets were generated or analyzed during the current study.

Conflict of Interest Statement

The authors declare no conflict of interest.

Acknowledgements

PD, CGM and VF designed the research. JR and CM provided EBNA-1-specific T cell lines and methods for antigen-specific assays. JBM and OC produced antigenic peptides. OC performed surface plasmon resonance assays. AB, MK, MC, AS, AH, HHG, CGM and VF performed experiments. AB, MK, CGM and VF analyzed the data. AB, PD, CGM and VF wrote the manuscript. The authors wish to thank Dr. Teunis Geijtenbeek (University of Amsterdam, NL) for anti-DC-SIGN mAb clone AZN-D1, as well as Drs. Sylvie Tourne and Henri De La Salle (Etablissement Français du Sang, Strasbourg, France) for their advice on HLA typing assays. This work was

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CRedit statement

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FIGURE LEGENDS

Figure 1: CD14⁺ dAPCs are infected by Chikungunya virus and selectively capture CHIK-sE2 recombinant envelope protein

(a) HeLa cells, MDdMs, MDDCs and CD14⁺ dAPCs were exposed to CHIKV at an MOI of 2.5. After 2h, the cells were washed and cultured for 24h. The cell supernatant was harvested and viral progeny titrated on AP-61 cells by a plaque assay. **(b)** Schematic representation of CHIK-sE2 cloned in frame with a SNAP-tag and produced in S2 insect cells. Red residues depict N-glycosylation sites N264, N274 and N346. **(c)** Covalent coupling to CHIK-sE2 occurs when the SNAP enzymatic moiety reacts with benzylguanine (BG)-coupled AlexaFluor488, generating fluorescent CHIK-sE2-A488. **(d)** Interaction of CHIK-sE2-A488 with monocytes, MDdMs and MDDCs. The cells were incubated for 30 min at 37°C with 150nM CHIK-sE2-A488 or left untreated, then analyzed by flow cytometry. Representative histograms are shown. For MDDCs, CHIK-sE2 was preincubated with or without anti-CHIK-sE2 mAb. **(e)** Relative mean fluorescence intensity (MFI) of CHIK-sE2-A488-treated cells vs. untreated cells (n=4-6 donors). **(f)** Confocal microscopy images of monocytes, MDdMs and MDDCs incubated with CHIK-sE2-A488. Counterstaining of CD14 or HLA-DR allowed visualization of the cell surface. DAPI labels nuclei in blue. Scale bars: 10µm. **(g)** Healthy human skin samples were from donors undergoing plastic surgery. Epidermal cell suspensions were prepared by trypsin digestion. HLA-DR⁺ CD1a⁺ LCs and HLA-DR⁻ CD1a⁻ keratinocytes were identified by flow cytometry. The dermis was digested by collagenase and CD1c⁺ dDCs were isolated by anti-CD1c-coupled magnetic beads. After 2 days of culture, the dermal fibroblasts were identified as HLA-DR⁻ CD14⁻ adherent cells and the non-adherent CD14⁺ dAPCs were isolated by anti-CD14-coupled magnetic beads. 10⁵ enriched cells were incubated in serum-free medium for 1h at 37°C with 150nM CHIK-sE2-A488 or left untreated. Where indicated, CHIK-sE2-A488 was preincubated for 20min with anti-CHIK-sE2 mAb before addition of CD14⁺ dAPCs. Cells were stained with antibodies against HLA-DR, CD1a, CD14 and CD1c and analyzed by flow cytometry. **(h)** Relative MFI comparing the fluorescence intensity of CHIK-sE2-A488-treated vs. untreated cells (n=3-6 donors). **(i)** Confocal microscopy images of CD14⁺ dAPCs exposed to CHIK-sE2-A488 for 30 min at 37°C. Cells were labelled for cell-surface HLA-DR, fixed and visualized by fluorescence microscopy. DAPI labels nuclei in blue. Scale bars: 10µm. Data were obtained from at least three different donors and are presented as mean ±SEM. Statistical comparisons: ANOVA followed by post-hoc tests (ns: non significant, *p<0.05, **p<0.01, ***p<0.001).

Figure 2: CHIK-sE2 conveys antigenic peptide to CD14⁺ dDCs for presentation to CD4⁺ T cells. (a) Whole skin pieces were floated on complete culture medium for 3 days. 10⁵ total migratory cells were incubated alone or with 150nM CHIK-sE2-A488 in serum-free medium for 1h at 37°C. Cells were then stained with antibodies against HLA-DR, Langerin/CD207, DC-SIGN/CD209, and CD14 and analyzed by flow cytometry. **(b)** Representative histograms of cells treated with CHIK-sE2-A488 or left untreated. **(c)** Relative MFI comparing

the fluorescence intensity of CHIK-sE2-A488 vs. untreated cells (n=14 donors). **(d)** CD14⁺ dDCs enriched by CD14 MACS were incubated with CHIK-sE2-A488 for 1h at 37°C, fixed with 4% PFA and stained for CD14. Fluorescent CHIK-sE2 in intracellular compartments was visualized by spinning-disk confocal microscopy. Scale bar: 10µm. A video rendition is provided as Figure S3. **(e)** Skin migratory DCs (n=4-10 donors) were incubated for 30min at 37°C with 1mM mannan, 10µg/mL purified antibodies against DC-SIGN (clones AZN-D1 or 111H2.02), MR (clone 19.2), combinations of anti-MR and anti-DC-SIGN (total concentration 20µg/mL) or the corresponding isotype controls. CHIK-sE2-A488 binding was quantified as described above and expressed as % of MFI obtained for untreated cells. **(f)** Schematic representation of EBNA1-derived peptide coupling to CHIK-sE2. The selected peptide contains the cognate HLA-DR1-restricted CD4⁺ T cell epitope (underlined). **(g)** EBV-specific T-cell proliferation induced by HLA-DR1-matched skin APCs with and without CHIK-sE2-EBNA1 loading (200nM) was measured by CFSE dilution assays. **(h)** IFN-γ production was assessed by intracellular cytokine staining in proliferating EBNA1-specific CD4⁺ T cells after cognate peptide restimulation of co-cultures with the HLA-DR1-matched skin APCs with and without CHIK-sE2-EBNA1 loading. **(i)** EBV-specific T-cell proliferation by HLA-DR-mismatched skin APCs was measured as in (g). Representative flow cytometry plots are shown for (g, h, i). Data were obtained from at least three different donors and are presented as mean ±SEM. Statistical comparisons: ANOVA followed by post-hoc tests (nd, not determined, ns: non significant, *p<0.05, **p<0.01, ***p<0.001).

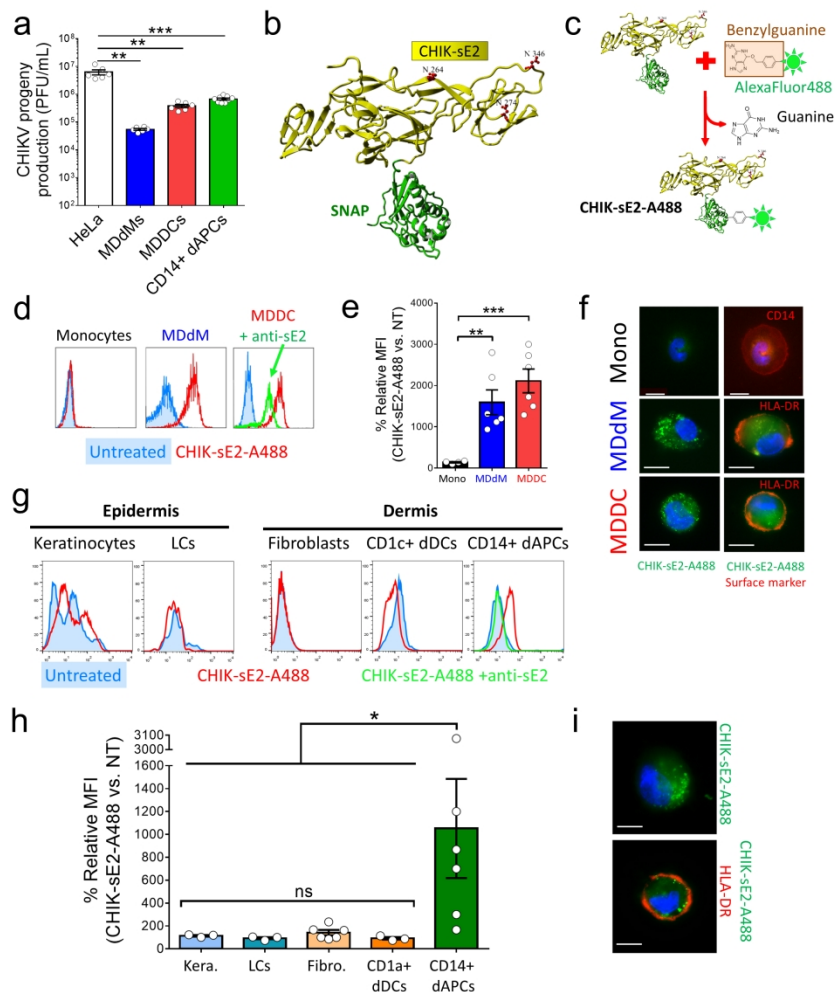


Figure 1

Figure 1: CD14+ dAPCs are infected by Chikungunya virus and selectively capture CHIK-sE2 recombinant envelope protein

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samples were from donors undergoing plastic surgery. Epidermal cell suspensions were prepared by trypsin digestion. HLA-DR⁺ CD1a⁺ LCs and HLA-DR⁻ CD1a⁻ keratinocytes were identified by flow cytometry. The dermis was digested by collagenase and CD1c⁺ dDCs were isolated by anti-CD1c-coupled magnetic beads. After 2 days of culture, the dermal fibroblasts were identified as HLA-DR⁻ CD14⁻ adherent cells and the non-adherent CD14⁺ dAPCs were isolated by anti-CD14-coupled magnetic beads. 105 enriched cells were incubated in serum-free medium for 1h at 37°C with 150nM CHIK-sE2-A488 or left untreated. Where indicated, CHIK-sE2-A488 was preincubated for 20min with anti-CHIK-sE2 mAb before addition of CD14⁺ dAPCs. Cells were stained with antibodies against HLA-DR, CD1a, CD14 and CD1c and analyzed by flow cytometry. (h) Relative MFI comparing the fluorescence intensity of CHIK-sE2-A488-treated vs. untreated cells (n=3-6 donors). (i) Confocal microscopy images of CD14⁺ dAPCs exposed to CHIK-sE2-A488 for 30 min at 37°C. Cells were labelled for cell-surface HLA-DR, fixed and visualized by fluorescence microscopy. DAPI labels nuclei in blue. Scale bars: 10µm. Data were obtained from at least three different donors and are presented as mean ±SEM. Statistical comparisons: ANOVA followed by post-hoc tests (ns: non significant, *p<0.05, **p<0.01, ***p<0.001).

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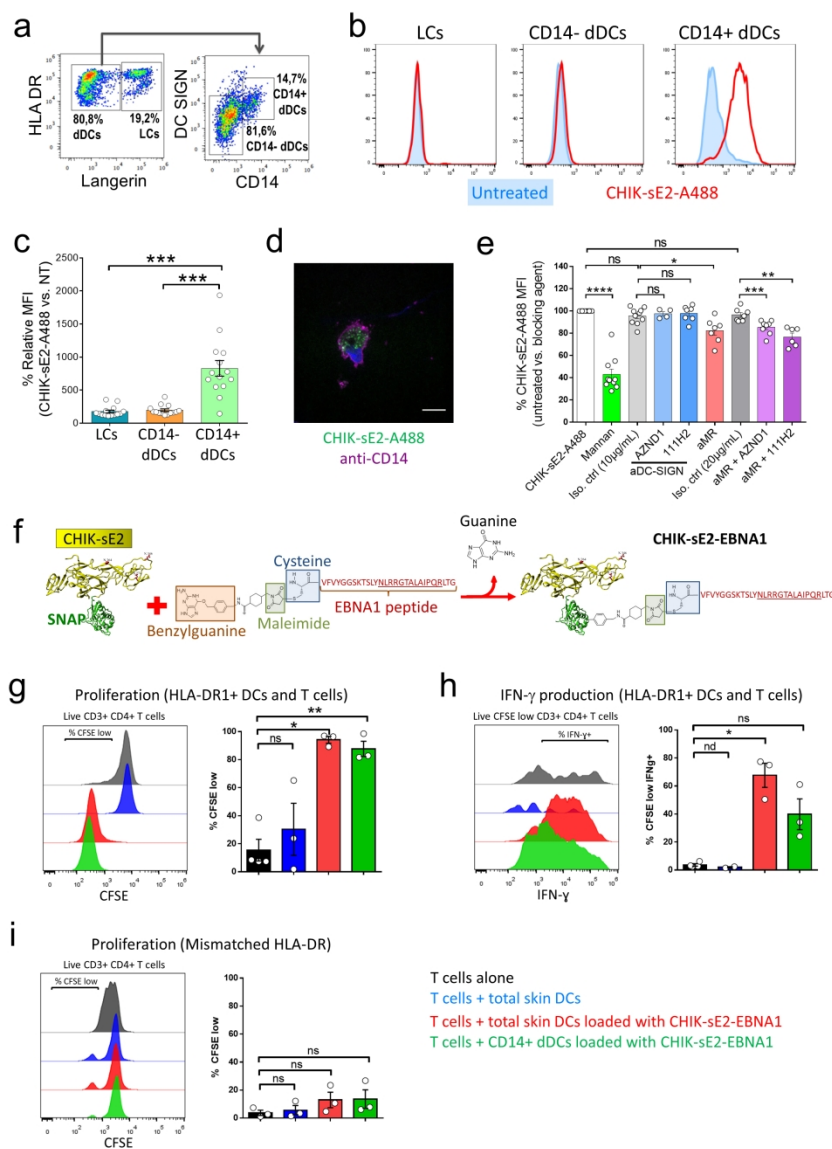


Figure 2

Figure 2: CHIK-sE2 conveys antigenic peptide to CD14+ dDCs for presentation to CD4+ T cells. (a) Whole skin pieces were floated on complete culture medium for 3 days. 105 total migratory cells were incubated alone or with 150nM CHIK-sE2-A488 in serum-free medium for 1h at 37°C. Cells were then stained with antibodies against HLA-DR, Langerin/CD207, DC-SIGN/CD209, and CD14 and analyzed by flow cytometry. (b) Representative histograms of cells treated with CHIK-sE2-A488 or left untreated. (c) Relative MFI comparing the fluorescence intensity of CHIK-sE2-A488 vs. untreated cells (n=14 donors). (d) CD14+ dDCs enriched by CD14 MACS were incubated with CHIK-sE2-A488 for 1h at 37°C, fixed with 4% PFA and stained for CD14. Fluorescent CHIK-sE2 in intracellular compartments was visualized by spinning-disk confocal microscopy. Scale bar: 10µm. A video rendition is provided as Figure S3. (e) Skin migratory DCs (n=4-10 donors) were incubated for 30min at 37°C with 1mM mannan, 10µg/mL purified antibodies against DC-SIGN (clones AZN-D1 or 111H2.02), MR (clone 19.2), combinations of anti-MR and anti-DC-SIGN (total concentration 20µg/mL) or the corresponding isotype controls. CHIK-sE2-A488 binding was quantified as described above and expressed as % of MFI obtained for untreated cells. (f) Schematic representation of EBNA1-derived peptide coupling to CHIK-sE2. The selected peptide contains the cognate HLA-DR1-restricted

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SUPPLEMENTARY FIGURE LEGENDS

Figure S1: DC-SIGN and MR are receptors for CHIK-sE2. Affinity of CHIK-sE2 for immobilized MR **(a)** and DC-SIGN **(b)** were measured by surface plasmon resonance. Sensorgrams depict association and dissociation to DC-SIGN (0.1, 0.2, 0.4, 0.8, and 1.6 μ M of CHIK-E2) and MR (3.1, 6.3, 12.5, 25, 50 and 100nM of CHIK-sE2). **(c)** Constants for the binding assays. The K_D for DC-SIGN and MR were 51.1nM and 3.9nM, respectively.

Figure S2: CHIK-sE2 does not promote DC maturation. Immature MDDCs were incubated for 24h with 150nM CHIK-sE2 or 100ng/mL LPS. Maturation was quantified by flow cytometry from the MFI of CD86 stainings. Representative histograms are shown. Data were obtained from six different donors and are presented as mean \pm SEM. Statistical comparisons: Wilcoxon tests (ns: non significant, * $p < 0.05$).

Figure S3: Z-stack video rendition of CD14⁺ dDCs loaded with CHIK-sE2-A488. CD14⁺ dDCs enriched by CD14 MACS were incubated with 150nM CHIK-sE2-A488 in serum-free medium for 1h at 37°C, then fixed with 4% PFA and counterstained with anti-CD14 to highlight the cell surface (magenta). Fluorescent CHIK-sE2 (green) was visualized in intracellular compartments of targeted cells by spinning-disk confocal microscopy.

SUPPLEMENTARY MATERIAL AND METHODS

Antibodies and media

The following antibodies were used for flow cytometry: HLA-DR (clones Tü39 or L243), CD206/MR (19.2), CD209/DC-SIGN (DCN46), CD1a (HI149), CD86 (FUN-1), IFN- γ (B27), CD3 (UCHT1) from BD, CD14 (MEM-18, Immunotools), CD1c (AD5-8E7) and CD4 (REA623) from Miltenyi Biotec, and CD207/Langerin (DCGM4, Dendritics). Staining buffer was PBS containing 2% fetal calf serum (FCS; Dutscher) and 2.5mM EDTA (SE buffer). Dead cells were labelled by Fixable Viability Dye-eFluor450 (eBioscience) or Sytox Red dye (Invitrogen). Cells were collected on FACSCalibur (BD) or Gallios (Beckman-Coulter) flow cytometers. Data were analysed with CellQuest (BD), Kaluza (Beckman-Coulter) or FlowJo (Treestar) softwares. Complete medium was RPMI1640 supplemented with 10 μ g/mL gentamycin, 100 units/mL penicillin, 100 μ g/mL streptomycin, 10mM HEPES (all from Lonza) and 10% FCS.

Primary cell isolation and culture

Fresh abdominal skin was obtained from patients undergoing abdominoplasty with written informed consent and institutional review board approval, in agreement with the Helsinki Declaration and French legislation.

Enzymatic digestion: Subcutaneous fat was removed and the remainder was incubated for 1h at 37°C in PBS containing 0.5% trypsin XI (Sigma-Aldrich). The epidermis was then peeled off and agitated in complete medium for 10min to liberate LCs and keratinocytes. The dermis was cut into small pieces and digested overnight with 1mg/mL collagenase I (Invitrogen) and 50µg/mL DNase (Sigma-Aldrich) at 37°C and 5% CO₂ in complete medium. The digested dermis was filtered in SE buffer with 50µm cell strainers and dermal cDC2 were isolated by anti-CD1c-coupled magnetic beads (Miltenyi Biotec). Remaining cells were incubated for 48h in a 175cm² culture flask (50x10⁶ cells/50mL complete medium). CD14⁺ dAPCs were isolated from the loosely adherent cells using anti-CD14-conjugated magnetic beads (Miltenyi Biotec). After an overnight culture in dermal fibroblast-conditioned culture medium at 10⁶ cells/mL, CD14⁺ dAPCs were sorted by flow cytometry as CD14⁺ HLA-DR^{low} CD86^{low} cells.

Skin explant cultures: 2cm² biopsies of ~1mm thickness were placed at air-liquid interface onto 40µm cell strainers in 6mL complete medium. After 3 days, migratory cells were harvested from the culture supernatant. Resident memory T cells were excluded by selection with anti-CD3 magnetic beads (Miltenyi-Biotec). Where indicated, CD14⁺ dDCs were separated with anti-CD14 magnetic beads (Miltenyi-Biotec).

Monocyte-derived DCs and macrophages

Monocyte-derived dermal-like macrophages (MDdMs) and MDDCs were generated from blood monocytes as described (Kwan *et al.*, 2008). Briefly, 2x10⁶ monocytes, purified from adult blood by counterflow centrifugal elutriation (Etablissement Français du Sang, Strasbourg, France) were cultured in 5mL complete medium in the presence of GM-CSF (50ng/mL) and IL-4 (10ng/mL) for MDDCs, or GM-CSF (25ng/mL), IL-10 (25ng/mL) and M-CSF (10ng/mL) for MDdMs. On day 3, the culture was refreshed with 1mL of complete medium containing GM-CSF (50ng/mL) and IL-4 (10ng/mL) for MDDCs, or GM-CSF (12.5ng/mL) and IL-10 (12.5ng/mL) for MDdMs. On day 5, cells were characterized by flow cytometry for expression of CD1a, CD14 and DC-SIGN.

Cell lines

HeLa and Vero cells were cultured at 37°C and 5% CO₂ in DMEM (Invitrogen) supplemented with 10% FCS and antibiotics. *Aedes pseudoscutellaris* AP-61 mosquito cells were cultured at 27°C in Leibovitz's L-15 medium (Invitrogen) supplemented with 10% FCS, 1% tryptose phosphate broth (Eurobio) and antibiotics.

Viral infections

The CHIKV-06.049 virus strain was isolated from a young adult during the 2006 outbreak of Chikungunya fever in La Réunion island (Schuffenecker *et al.*, 2006). Virus stocks were produced on mosquito AP-61 cells (Brehin *et al.*, 2008) and all infectivity titers were expressed as plaque-forming units on Vero cells. 5x10⁴ HeLa cells, CD14⁺ dAPCs, MDdMs and MDDC were exposed to CHIKV at an MOI of 2.5, or mock-infected, in RPMI1640 supplemented with 2% FCS for 2h at 37°C. Virus was

removed by extensive washing and cells were kept for 22h in their respective culture media. The supernatants were used for titer analysis by the plaque-forming assay on Vero cells.

Recombinant CHIK-sE2 protein production

Stably transfected *Drosophila melanogaster* S2 cell line inducible for the secretion of the ectodomain of CHIKV envelope E2 protein (CHIK-sE2; residues 4-364) were described elsewhere (Aubry *et al.*, 2015). Briefly, a SNAP adaptor sequence (New England Biolabs) was fused in-frame with the C-terminus of recombinant CHIK-sE2, resulting in a chimeric protein that was purified from the supernatants of S2 cells. SNAP is the catalytic site of O⁶-alkylguanine-DNA-alkyltransferase. 3D rendition of CHIK-sE2-SNAP was created with the Yasara software based on the available crystal structures (CHIK-sE2: PDB 3J2W; SNAP-tag: PDB 3KZY).

Surface plasmon resonance

Surface plasmon resonance experiments were performed using a Biacore 3000. Extracellular domains of DC-SIGN[Lys62-Ala404] and MR[Leu19-Lys1383] (R&D Systems) were immobilized on a CM5 sensor chip (GE-Healthcare) by injecting 70µL of recombinant proteins (50µg/mL in formate buffer, pH 4.3) onto the surface activated with N-Ethyl-N'-dimethylaminopropyl carbodiimide/N-hydroxysuccinimide. 20µL of ethanolamine hydrochloride, pH 8.5, saturated the free activated sites of the matrix. Biosensor assays were performed with HEPES-buffered-saline (10mM HEPES, 150mM sodium chloride, pH 7.4) containing 0.005% surfactant P20 and 5mM CaCl₂, as running buffer. All binding experiments were carried out at 25°C with a constant flow rate of 20µL/min. CHIK-sE2 dissolved in the running buffer was injected for 3min, followed by a dissociation phase of 3min. The sensor chip surface was regenerated after each experiment by injecting 10µL EDTA 0.5M pH 8.0. The kinetic parameters were calculated using the BIAeval 4.1 software. Analysis was performed using the simple Langmuir binding 1:1 model with separate k_a/k_d (k_{on}/k_{off}). The specific binding profiles were obtained after subtracting the response signal from the control channel and from blank-buffer injection. The fitting to each model was judged by the chi-square value and randomness of residue distribution compared to the theoretical model.

CHIK-sE2 binding studies

Benzylguanine (BG)-AlexaFluor488 fluorochrome (New England Biolabs) was covalently linked to CHIK-sE2 via the SNAP moiety according to the manufacturer's recommendations. 10⁵ cells were incubated in 200µL RPMI1640 alone or with 2µg of CHIK-sE2-A488 (final concentration 150nM) for 30min at 37°C. The cells were then washed and labelled for cell surface markers in SE buffer and analyzed by flow cytometry. Where indicated, CHIK-sE2 (1.2µg) was pre-incubated for 30min at 4°C with anti-E2 monoclonal antibody 3E4 (0.9µg) to verify specificity of binding (Brehin *et al.*, 2008). To quantify binding efficiency, the mean fluorescent intensity (MFI) of CHIK-sE2-A488-exposed cells was calculated relatively to the MFI of untreated cells, which was set at 100%. For internalization studies by confocal

microscopy, cells exposed to CHIK-sE2-A488 were washed in SE buffer and counterstained with antibodies against HLA-DR or CD14 for 25min on ice. The cells were washed twice, fixed in 4% formaldehyde for 20min at RT, and the secondary goat-anti-mouse AlexaFluor594 antibody (Invitrogen) was added and incubated for 30min on ice. After washing, nuclei were labelled with DAPI. Samples were mounted on glass slides in fluorescence mounting medium (Dako). Images taken with Zeiss confocal microscopes LSM510 and Axio Observer Z1 were analyzed with ImageJ. For maturation assays, MDDCs were incubated with 150nM CHIK-sE2 or 100ng/mL ultrapure K12 LPS (Invivogen) for 24h, then CD86 expression was quantified by flow cytometry.

Peptide synthesis

Peptides were assembled using optimized Fmoc chemistry protocols with a multichannel peptide synthesizer (Neimark and Briand, 1993). Side-chain deprotection and cleavage of peptides from the solid support was performed by treatment with reagent K (82.5% TFA v/v, 5% phenol w/v, 5% water v/v, 5% thioanisole v/v, 2.5% 1,2-ethanedithiol v/v) for 150min at 20°C. Peptides were purified by reversed-phase HPLC (RP-HPLC) using a Beckman preparative HPLC system (Gagny, France) on a Nucleosil C18 (1 x 30cm) column, (Macherey Nagel, Hoerd, France). The elution was achieved with a linear gradient of aqueous 0.1% TFA and 0.08% TFA in acetonitrile at a flow rate of 6mL/min with UV detection at 230nm. The purity of peptides was controlled by analytical RP-HPLC on a Beckman instrument (Gagny, France) with a Nucleosil C18 5µm column (150 x 4.6mm) using a linear gradient of 0.1% TFA in water and acetonitrile containing 0.08% TFA at a flow rate of 1.2mL/min. The integrity of each peptide was assessed by LC/MS using a Thermo Finigan LCQ.

The Epstein-Barr virus Nuclear Antigen-1 (EBNA1)-derived peptide (507-535; CVFVYGGSKTSLYNLRRGTALAIPQRLTG) was bound to BG-maleimide (New England Biolabs) by reacting with the thiol group of the N-terminal cysteine residue, before purification by HPLC.

Blocking CHIK binding

Skin migratory DCs were incubated for 30min at 37°C with 1mM mannan (Sigma-Aldrich), 10µg/mL purified antibodies against DC-SIGN (clone AZN-D1, Dr. Teunis Geijtenbeek, Amsterdam, NL (Geijtenbeek *et al.*, 2000); clone 111H2.02, Dendritics, France (Canard *et al.*, 2011)), MR (clone 19.2, BD), combinations of anti-MR and anti-DC-SIGN (total concentration 20µg/mL) or the corresponding isotype controls. Then, 150nM CHIK-sE2-A488 was added for 1h and its binding was quantified by flow cytometry.

Expansion of EBNA1-specific CD4⁺ T memory cells

Allogeneic PBMCs were purified from buffy coats of healthy blood donors (EFS) by Ficoll gradient (Sigma-Aldrich). 5x10³ EBNA1-specific CD4⁺ T cell (CK-NLR clone, HLA-DR1-restricted) cells/well were cocultured with 10⁵ irradiated PBMCs (3000rad) and 2x10³ irradiated, EBV-infected autologous B-LCL (20000rad) in RPMI1640 supplemented with 8% PHS (pooled human sera, Corning), 150U/mL IL-2

(Novartis), 1 μ g/mL Phytohemagglutinin-L (Sigma-Aldrich), and gentamicin. After 5 days, half of the medium was refreshed with RPMI1640 + 8% PHS + gentamycin + 450U/mL IL-2. Cells were monitored daily and split into the same medium and new 96 well-plates if necessary, until freezing at day 14 (Rühl *et al.*, 2019).

Antigen presentation assays

Genomic DNA was extracted from 40-80mg of skin using Nucleospin columns (Macherey-Nagel) and HLA haplotype of donors was determined with SSP HLA DQDR Combitray kit (Olerup). BG-EBNA1 peptide (250pmol) was coupled to CHIK-sE2 (200pmol) via the SNAP moiety in PBS. CHIK-sE2-EBNA1 (200nM) was incubated with 5x10⁵ migratory skin DCs or CD14⁺ dDCs for 1h in RPMI1640 medium. 10⁴ T-cell depleted, CHIK-EBNA1-loaded skin DCs or CD14⁺ dDCs from HLA-DR1 donors were cocultured in 200 μ L RPMI1640 + 8% PHS in 96-well U-bottom plates with 5x10⁴ EBNA1-specific CD4⁺ T cells previously labelled with 0.5 μ M carboxyfluorescein succinimidyl ester (CFSE). After 5 days, T cells were restimulated 6h with EBNA1 peptide (1 μ g/mL), including 4h with 10 μ g/mL brefeldin A and 1 μ M monensin (BD). T cells were stained for CD3 and CD4, then fixed and permeabilized with BD Cytotfix/Cytoperm Kit, stained with anti-IFN- γ antibody and analyzed by flow cytometry. T-cell proliferation was determined by CFSE dilution.

Statistical analyses

Data from different experimental conditions were tested by ANOVA, followed by relevant post-hoc multiple comparisons or by Wilcoxon tests, performed with Prism 6.0 software (GraphPad). Values of p below 0.05 were considered significant.

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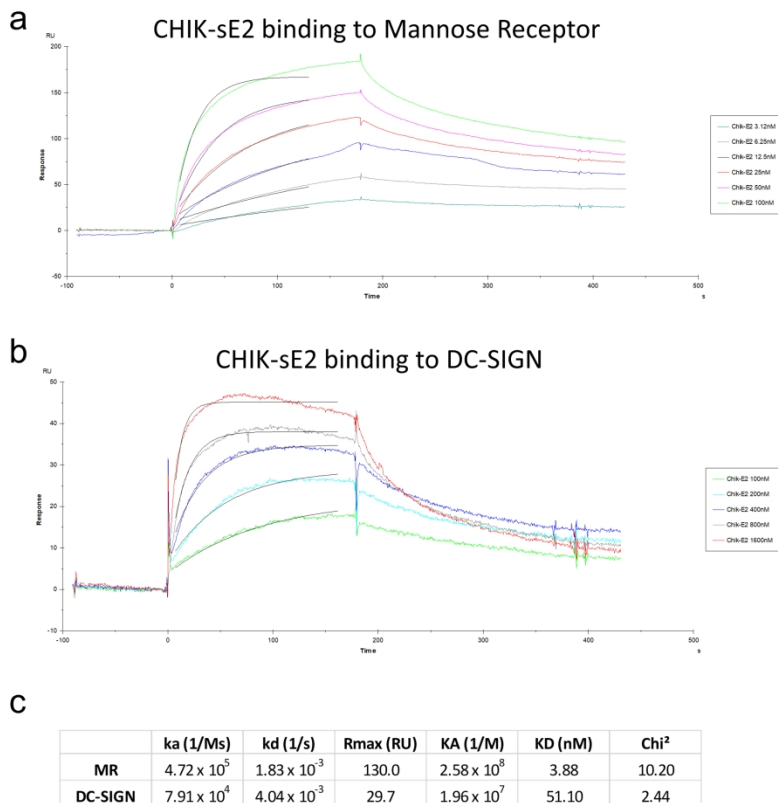
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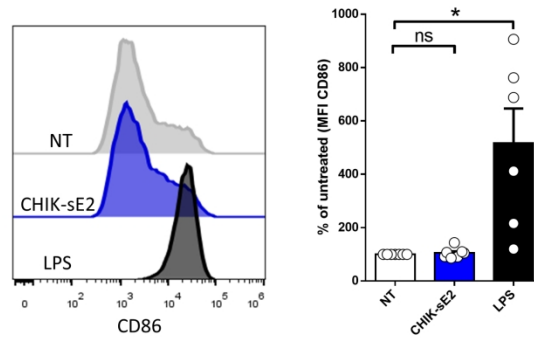
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Supplementary Figure S1

Figure S1: DC-SIGN and MR are receptors for CHIK-sE2. Affinity of CHIK-sE2 for immobilized MR (a) and DC-SIGN (b) were measured by surface plasmon resonance. Sensorgrams depict association and dissociation to DC-SIGN (0.1, 0.2, 0.4, 0.8, and 1.6 μ M of CHIK-E2) and MR (3.1, 6.3, 12.5, 25, 50 and 100nM of CHIK-sE2). (c) Constants for the binding assays. The KD for DC-SIGN and MR were 51.1nM and 3.9nM, respectively.

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Supplementary Figure S2

Figure S2: CHIK-sE2 does not promote DC maturation. Immature MDDCs were incubated for 24h with 150nM CHIK-sE2 or 100ng/mL LPS. Maturation was quantified by flow cytometry from the MFI of CD86 stainings. Representative histograms are shown. Data were obtained from six different donors and are presented as mean \pm SEM. Statistical comparisons: Wilcoxon tests (ns: non significant, * $p < 0.05$).

201x288mm (300 x 300 DPI)

NOTICE TO EDITORS AND REVIEWERS:

The authors have provided 1 supplementary video file. Please request this video file from the editorial office by emailing jidoffice2@sidnet.org.

Chikungunya virus envelope protein E2 provides a vector for targeted antigen delivery to human dermal CD14⁺ dendritic cells.

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1005 words

TO THE EDITOR

Therapeutic modulation of human cutaneous antigen-presenting cells (APCs) that control the fate of T cells represents an interesting option for immunotherapy. As they capture glycosylated proteins via C-type lectins, lectin-targeting antibodies have been used to specifically convey antigens to APCs (Romani *et al.*, 2012;Stoitzner *et al.*, 2014;Kastenmuller *et al.*, 2014). Beyond potential therapeutic applications, APC-targeting strategies can be harnessed to acquire fundamental knowledge about their function (Flacher *et al.*, 2014). In human skin, epidermal Langerhans cells (LCs), CD1c⁺ dermal type-2 conventional dendritic cells (cDC2) and CD141⁺ dermal cDC1 (Nestle *et al.*, 2009;Schlitzer *et al.*, 2015) can migrate to lymph nodes and stimulate T cells. Dermal APCs (dAPCs) include two CD14⁺ subsets that express the lectins Mannose Receptor (MR)/CD206 and DC-specific intracellular adhesion molecules-3 grabbing non-integrin (DC-SIGN)/CD209 (Klechevsky *et al.*, 2008;McGovern *et al.*, 2014). During skin explant culture, CD14⁺ dermal DCs (dDCs) migrate into the supernatant, whereas sessile dermal macrophages require enzymatic digestion to be isolated. Still, CD14⁺ dDCs and dermal macrophages display strong similarities (McGovern *et al.*, 2014). They are both considered permanent dermal residents that exert local functions in pathogen capture (e.g. arthropod-borne viruses) (Kwan *et al.*, 2008;Schaeffer *et al.*, 2015) and may interact with skin-resident memory T cells (TRM) (Clark, 2015). Subset differences might reflect their origin, i.e. monocyte-derived dDCs vs. embryonically seeded resident macrophages (Ginhoux and Guilliams, 2016;McGovern *et al.*, 2014). Because tools to specifically target CD14⁺ dAPCs remain scarce (Duinkerken *et al.*, 2019), we propose here a novel approach based on recombinant arboviral envelope proteins.

Chikungunya virus (CHIKV) of the flavivirus genus of *Togaviridae* family is introduced into the dermis during blood meals of mosquitoes. CHIKV infection triggers high fever, skin rash and persistent arthralgia. Our finding that CD14⁺ dAPCs are preferentially infected by Dengue virus (Kwan *et al.*, 2008;Schaeffer *et al.*, 2015) prompted the idea that they may represent an immediate CHIKV target. CD14⁺ dAPCs isolated from human dermis, monocyte-derived DCs (MDDCs) and dermal-like macrophages (MDdMs) were exposed to CHIKV-06.049 strain (MOI=2.5) and compared with HeLa cells, in which vigorous viral replication is expected (Sourisseau *et al.*, 2007). CD14⁺ dAPCs and their monocyte-derived equivalent MDdMs were permissive CHIKV target cells, as well as MDDCs, which resemble the inflammatory DCs recruited to the site of infection (**Figure 1A**).

We hypothesized that APCs express endocytic receptors of viral envelope proteins. CHIK-sE2, a soluble form of CHIKV envelope glycoprotein E2, was fused to the SNAP proprietary enzyme and produced in insect cells to retain specific N-glycosylations that may be recognized by lectins (**Figure 1B**). In surface plasmon resonance assays, CHIK-sE2 displayed high affinity for MR and DC-SIGN (**Figure S1A/B**), with dissociation constants of 3.9nM and 51.1nM, respectively (**Figure S1C**). CHIK-sE2 was then covalently conjugated via its enzymatic SNAP moiety to AlexaFluor488 fluorochrome (**Figure 1C**). Since MDdMs and MDDCs, but not

monocytes, express high levels of lectins (Kwan *et al.*, 2008), we assessed their binding of the recombinant viral glycoprotein. CHIK-sE2-A488 only labelled MDdMs and MDDCs, and an anti-CHIK-sE2 antibody diminished binding to MDDCs (**Figure 1D/E**). Moreover, fluorescence microscopy revealed intracellular vesicles containing CHIK-sE2-A488 within MDdMs and MDDCs, but not in monocytes (**Figure 1F**). Incubation with CHIK-sE2 had no influence on MDDC maturation, estimated by CD86 expression (**Figure S2**).

To evaluate whether CHIK-sE2 can target CD14⁺ dAPCs within the skin, we compared its capture by different primary cutaneous APCs and non-hematopoietic cells. Epidermal cell suspensions, fibroblasts or purified CD14⁺ dAPCs and CD1c⁺ dDCs were incubated with CHIK-sE2-A488 and binding efficacy was measured by flow cytometry. CD14⁺ dAPCs displayed clear binding of CHIK-sE2-A488, as opposed to keratinocytes, LCs, fibroblasts and CD1c⁺ dDCs (**Figure 1G**). Anti-CHIK-sE2 mAb inhibited the interaction with CD14⁺ dAPCs. Quantitative analyses on skin samples from different individuals confirmed that only CD14⁺ dAPCs could efficiently capture CHIK-sE2-A488 (**Figure 1H**). CHIK-sE2-A488 was detected as condensed spots in the cytoplasm of CD14⁺ dAPCs, reflecting endocytic uptake (**Figure 1I**).

We next tested whether CHIK-sE2 could be used to convey antigens to CD14⁺ dDCs, which stimulate T cells more potently than macrophages (McGovern *et al.*, 2014). To assess binding to CD14⁺ dDCs in comparison with the other skin DC subsets, we cultured skin explants for 3 days and collected LCs, CD14⁺ dDCs and CD14⁺ dDCs from the supernatant (**Figure 2A**). When the cell suspension was exposed to CHIK-sE2-A488, only CD14⁺ dDCs significantly captured (**Figure 2B/C**) and internalized (**Figure 2D, Video S3**) the fluorescent protein. Mannan potently decreased CHIK-sE2-A488 binding, and inhibition assays using anti-lectin mAb suggested that MR is a more efficient CHIK-sE2 receptor than DC-SIGN (**Figure 2E**).

To address antigen presentation, we used a CD4⁺ T-cell clone with defined specificity for HLA-DR1-restricted antigenic peptide of the Epstein-Barr virus Nuclear Antigen-1 (EBNA1) (Rühl *et al.*, 2019). Total migratory APCs or CD14⁺ dDCs from HLA-DR1⁺ skin explants (5 donors out of 34) were loaded with CHIK-sE2 conjugated to EBNA1 peptide (**Figure 2F**). APCs were then cocultured with CFSE-labelled, EBNA1-specific CD4⁺ T cells. After 5 days, proliferation and intracellular IFN- γ production were quantified by flow cytometry. CHIK-sE2-EBNA1-exposed APCs from HLA-DR1 donors strongly enhanced proliferation (**Figure 2G**) and triggered IFN- γ production (**Figure 2H**) of EBNA1-specific T cells. However, T cells did not proliferate when cultured with mismatched, non-HLA-DR1 APCs (**Figure 2I**). Targeting purified CD14⁺ dDCs with CHIK-sE2-EBNA1 was sufficient to promote EBNA1-specific T-cell proliferation. Yet, IFN- γ production was not significantly enhanced, possibly because CD14⁺ dDCs produce anti-inflammatory IL-10 and display low CD86 expression (Klechevsky *et al.*, 2008).

Cutaneous TRM are derived from effector T cells that colonize the skin to mediate adaptive immune responses (Clark, 2015). CD14⁺ dAPCs might be critically involved during secondary exposure to cognate antigen because they also permanently reside in the skin and can reactivate memory T cells (McGovern *et*

al., 2014). CD14⁺ dAPCs targeting by CHIK-sE2 could be particularly useful to investigate and influence these interactions for therapeutic purposes. Moreover, our work suggests that arboviruses may represent a natural reservoir of molecules targeting different skin APCs. They may be derived into a specific delivery system for antigens but also adjuvants, silencing RNA or toxins, thereby harnessing the properties of skin APCs for immunotherapy or vaccination.

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Data availability

No datasets were generated or analyzed during the current study.

Conflict of Interest Statement

The authors declare no conflict of interest.

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PD, CGM and VF designed the research. JR and CM provided EBNA-1-specific T cell lines and methods for antigen-specific assays. JBM and OC produced antigenic peptides. OC performed surface plasmon resonance assays. AB, MK, MC, AS, AH, HHG, CGM and VF performed experiments. AB, MK, CGM and VF analyzed the data. AB, PD, CGM and VF wrote the manuscript. The authors wish to thank Dr. Teunis Geijtenbeek (University of Amsterdam, NL) for anti-DC-SIGN mAb clone AZN-D1, as well as Drs. Sylvie Tourne and Henri De La Salle (Etablissement Français du Sang, Strasbourg, France) for their advice on HLA typing assays. This

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FIGURE LEGENDS

Figure 1: CD14⁺ dAPCs are infected by Chikungunya virus and selectively capture CHIK-sE2 recombinant envelope protein

(A) HeLa cells, MDdMs, MDDCs and CD14⁺ dAPCs were exposed to CHIKV at an MOI of 2.5. After 2h, the cells were washed and cultured for 24h. The cell supernatant was harvested and viral progeny titrated on AP-61 cells by a plaque assay. **(B)** Schematic representation of CHIK-sE2 cloned in frame with a SNAP-tag and produced in S2 insect cells. Red residues depict N-glycosylation sites N264, N274 and N346. **(C)** Covalent coupling to CHIK-sE2 occurs when the SNAP enzymatic moiety reacts with benzylguanine (BG)-coupled AlexaFluor488, generating fluorescent CHIK-sE2-A488. **(D)** Interaction of CHIK-sE2-A488 with monocytes, MDdMs and MDDCs. The cells were incubated for 30 min at 37°C with 150nM CHIK-sE2-A488 or left untreated, then analyzed by flow cytometry. Representative histograms are shown. For MDDCs, CHIK-sE2 was preincubated with or without anti-CHIK-sE2 mAb. **(E)** Relative mean fluorescence intensity (MFI) of CHIK-sE2-A488-treated cells vs. untreated cells (n=4-6 donors). **(F)** Confocal microscopy images of monocytes, MDdMs and MDDCs incubated with CHIK-sE2-A488. Counterstaining of CD14 or HLA-DR allowed visualization of the cell surface. DAPI labels nuclei in blue. Scale bars: 10µm. **(G)** Healthy human skin samples were from donors undergoing plastic surgery. Epidermal cell suspensions were prepared by trypsin digestion. HLA-DR⁺ CD1a⁺ LCs and HLA-DR⁻ CD1a⁻ keratinocytes were identified by flow cytometry. The dermis was digested by collagenase and CD1c⁺ dDCs were isolated by anti-CD1c-coupled magnetic beads. After 2 days of culture, the dermal fibroblasts were identified as HLA-DR⁻ CD14⁻ adherent cells and the non-adherent CD14⁺ dAPCs were isolated by anti-CD14-coupled magnetic beads. 10⁵ enriched cells were incubated in serum-free medium for 1h at 37°C with 150nM CHIK-sE2-A488 or left untreated. Where indicated, CHIK-sE2-A488 was preincubated for 20min with anti-CHIK-sE2 mAb before addition of CD14⁺ dAPCs. Cells were stained with antibodies against HLA-DR, CD1a, CD14 and CD1c and analyzed by flow cytometry. **(H)** Relative MFI comparing the fluorescence intensity of CHIK-sE2-A488-treated vs. untreated cells (n=3-6 donors). **(I)** Confocal microscopy images of CD14⁺ dAPCs exposed to CHIK-sE2-A488 for 30 min at 37°C. Cells were labelled for cell-surface HLA-DR, fixed and visualized by fluorescence microscopy. DAPI labels nuclei in blue. Scale bars: 10µm. Data were obtained from at least three different donors and are presented as mean ±SEM. Statistical comparisons: ANOVA followed by post-hoc tests (ns: non significant, *p<0.05, **p<0.01, ***p<0.001).

Figure 2: CHIK-sE2 conveys antigenic peptide to CD14⁺ dDCs for presentation to CD4⁺ T cells. (A) Whole skin pieces were floated on complete culture medium for 3 days. 10⁵ total migratory cells were incubated alone or with 150nM CHIK-sE2-A488 in serum-free medium for 1h at 37°C. Cells were then stained with antibodies against HLA-DR, Langerin/CD207, DC-SIGN/CD209, and CD14 and analyzed by flow cytometry. **(B)**

Representative histograms of cells treated with CHIK-sE2-A488 or left untreated. **(C)** Relative MFI comparing the fluorescence intensity of CHIK-sE2-A488 vs. untreated cells (n=14 donors). **(D)** CD14⁺ dDCs enriched by CD14 MACS were incubated with CHIK-sE2-A488 for 1h at 37°C, fixed with 4% PFA and stained for CD14. Fluorescent CHIK-sE2 in intracellular compartments was visualized by spinning-disk confocal microscopy. A video rendition is provided as Figure S3. **(E)** Skin migratory DCs (n=4-10 donors) were incubated for 30min at 37°C with 1mM mannan, 10µg/mL purified antibodies against DC-SIGN (clones AZN-D1 or 111H2.02), MR (clone 19.2), combinations of anti-MR and anti-DC-SIGN (total concentration 20µg/mL) or the corresponding isotype controls. CHIK-sE2-A488 binding was quantified as described above and expressed as % of MFI obtained for untreated cells. **(F)** Schematic representation of EBNA1-derived peptide coupling to CHIK-sE2. The selected peptide contains the cognate HLA-DR1-restricted CD4⁺ T cell epitope (underlined). **(G)** EBV-specific T-cell proliferation induced by HLA-DR1-matched skin APCs with and without CHIK-sE2-EBNA1 loading (200nM) was measured by CFSE dilution assays. **(H)** IFN-γ production was assessed by intracellular cytokine staining in proliferating EBNA1-specific CD4⁺ T cells after cognate peptide restimulation of co-cultures with the HLA-DR1-matched skin APCs with and without CHIK-sE2-EBNA1 loading. **(I)** EBV-specific T-cell proliferation by HLA-DR-mismatched skin APCs was measured as in (G). Representative flow cytometry plots are shown for G, H, I. Data were obtained from at least three different donors and are presented as mean ±SEM. Statistical comparisons: ANOVA followed by post-hoc tests (nd, not determined, ns: non significant, *p<0.05, **p<0.01, ***p<0.001).

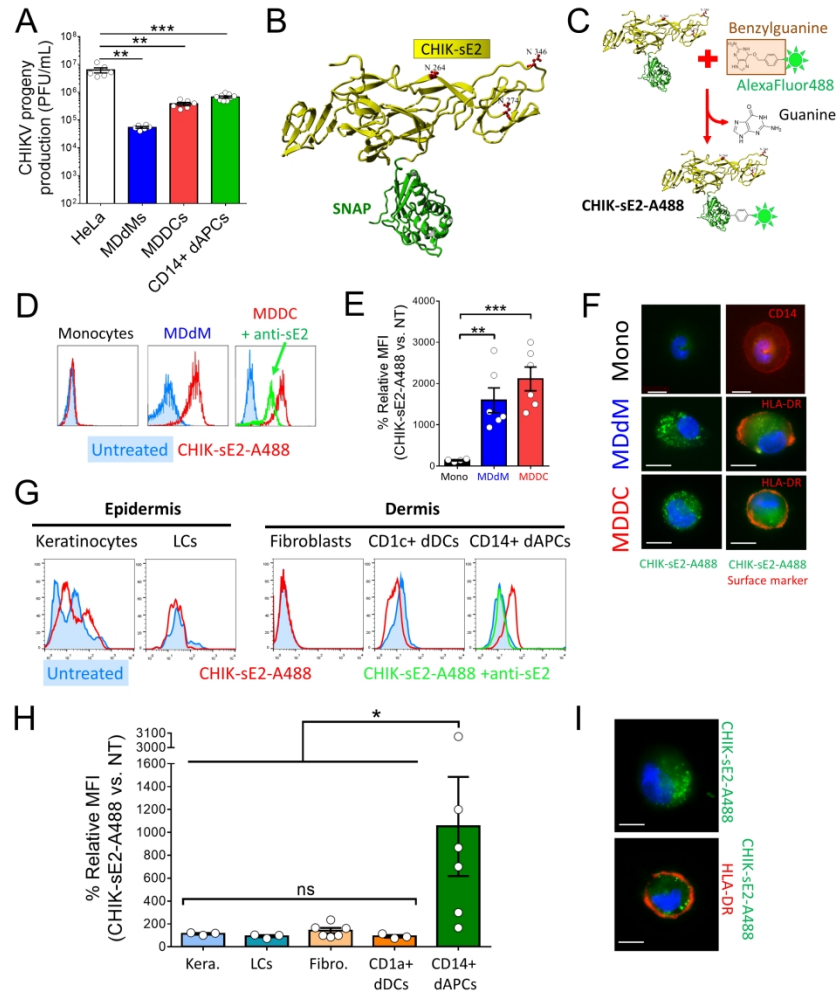


Figure 1

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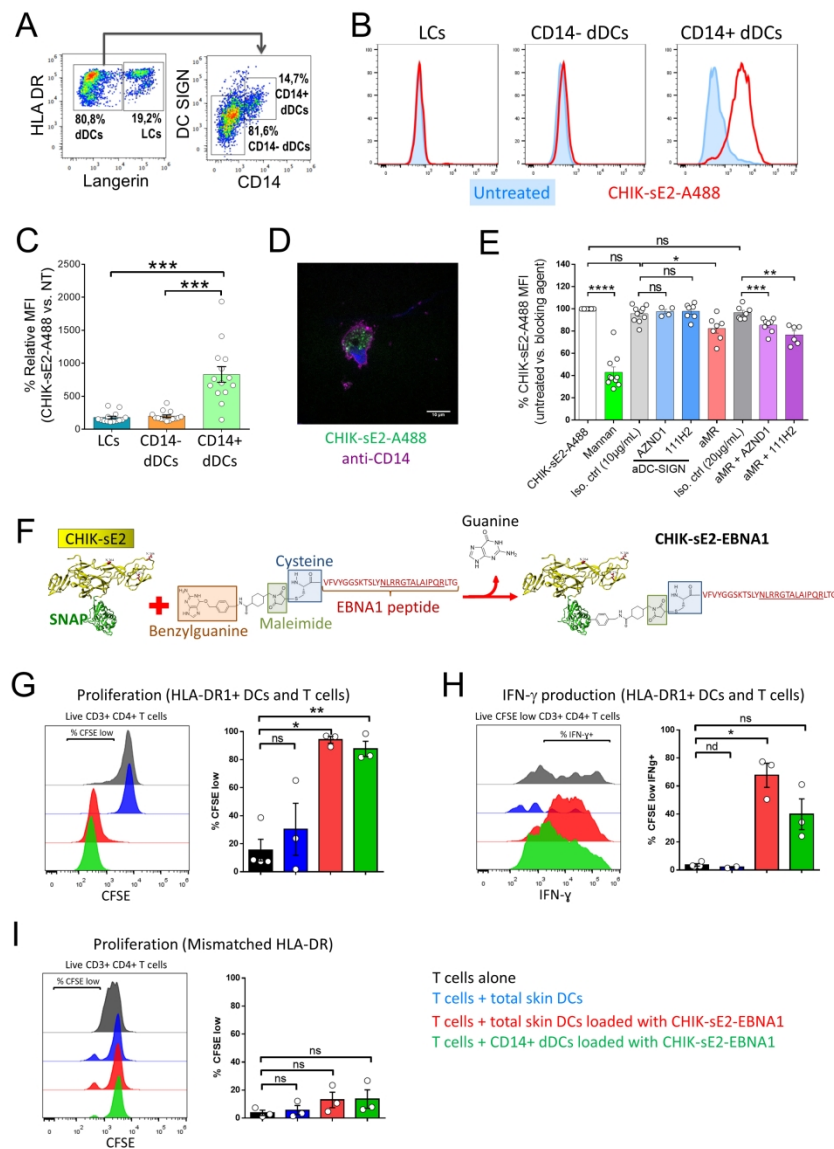


Figure 2

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(B) Representative histograms of cells treated with CHIK-sE2-A488 or left untreated. (C) Relative MFI comparing the fluorescence intensity of CHIK-sE2-A488 vs. untreated cells (n=14 donors). (D) CD14+ dDCs enriched by CD14 MACS were incubated with CHIK-sE2-A488 for 1h at 37°C, fixed with 4% PFA and stained for CD14. Fluorescent CHIK-sE2 in intracellular compartments was visualized by spinning-disk confocal microscopy. A video rendition is provided as Figure S3. (E) Skin migratory DCs (n=4-10 donors) were incubated for 30min at 37°C with 1mM mannan, 10µg/mL purified antibodies against DC-SIGN (clones AZN-D1 or 111H2.02), MR (clone 19.2), combinations of anti-MR and anti-DC-SIGN (total concentration 20µg/mL) or the corresponding isotype controls. CHIK-sE2-A488 binding was quantified as described above and expressed as % of MFI obtained for untreated cells. (F) Schematic representation of EBNA1-derived peptide coupling to CHIK-sE2. The selected peptide contains the cognate HLA-DR1-restricted CD4+ T cell

epitope (underlined). (G) EBV-specific T-cell proliferation induced by HLA-DR1-matched skin APCs with and without CHIK-sE2-EBNA1 loading (200nM) was measured by CFSE dilution assays. (H) IFN- γ production was assessed by intracellular cytokine staining in proliferating EBNA1-specific CD4⁺ T cells after cognate peptide restimulation of co-cultures with the HLA-DR1-matched skin APCs with and without CHIK-sE2-EBNA1 loading. (I) EBV-specific T-cell proliferation by HLA-DR-mismatched skin APCs was measured as in (G). Representative flow cytometry plots are shown for G, H, I. Data were obtained from at least three different donors and are presented as mean \pm SEM. Statistical comparisons: ANOVA followed by post-hoc tests (nd, not determined, ns: non significant, * p <0.05, ** p <0.01, *** p <0.001).

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SUPPLEMENTARY FIGURE LEGENDS

Figure S1: DC-SIGN and MR are receptors for CHIK-sE2. Affinity of CHIK-sE2 for immobilized MR (A) and DC-SIGN (B) were measured by surface plasmon resonance. Sensorgrams depict association and dissociation to DC-SIGN (0.1, 0.2, 0.4, 0.8, and 1.6 μ M of CHIK-E2) and MR (3.1, 6.3, 12.5, 25, 50 and 100nM of CHIK-sE2). (C) Constants for the binding assays. The KD for DC-SIGN and MR were 51.1nM and 3.9nM, respectively.

Figure S2: CHIK-sE2 does not promote DC maturation. Immature MDDCs were incubated for 24h with 150nM CHIK-sE2 or 100ng/mL LPS. Maturation was quantified by flow cytometry from the MFI of CD86 stainings. Representative histograms are shown. Data were obtained from six different donors and are presented as mean \pm SEM. Statistical comparisons: Wilcoxon tests (ns: non significant, * p <0.05).

Figure S3: Z-stack video rendition of CD14⁺ dDCs loaded with CHIK-sE2-A488. CD14⁺ dDCs enriched by CD14 MACS were incubated with 150nM CHIK-sE2-A488 in serum-free medium for 1h at 37°C, then fixed with 4% PFA and counterstained with anti-CD14 to highlight the cell surface (magenta). Fluorescent CHIK-sE2 (green) was visualized in intracellular compartments of targeted cells by spinning-disk confocal microscopy.

SUPPLEMENTARY MATERIAL AND METHODS

Antibodies and media

The following antibodies were used for flow cytometry: HLA-DR (clones Tü39 or L243), CD206/MR (19.2), CD209/DC-SIGN (DCN46), CD1a (HI149), CD86 (FUN-1), IFN- γ (B27), CD3 (UCHT1) from BD, CD14 (MEM-18, Immunotools), CD1c (AD5-8E7) and CD4 (REA623) from Miltenyi Biotec, and CD207/Langerin (DCGM4, Dendritics). Staining buffer was PBS containing 2% fetal calf serum (FCS; Dutscher) and 2.5mM EDTA (SE buffer). Dead cells were labelled by Fixable Viability Dye-eFluor450 (eBioscience) or Sytox Red dye (Invitrogen). Cells were collected on FACSCalibur (BD) or Gallios (Beckman-Coulter) flow cytometers. Data were analysed with CellQuest (BD), Kaluza (Beckman-Coulter) or FlowJo (Treestar) softwares. Complete medium was RPMI1640 supplemented with 10 μ g/mL gentamycin, 100 units/mL penicillin, 100 μ g/mL streptomycin, 10mM HEPES (all from Lonza) and 10% FCS.

Primary cell isolation and culture

Fresh abdominal skin was obtained from patients undergoing abdominoplasty with written informed consent and institutional review board approval, in agreement with the Helsinki Declaration and French legislation.

Enzymatic digestion: Subcutaneous fat was removed and the remainder was incubated for 1h at 37°C in PBS containing 0.5% trypsin XI (Sigma-Aldrich). The epidermis was then peeled off and agitated in complete medium for 10min to liberate LCs and keratinocytes. The dermis was cut into small pieces and digested overnight with 1mg/mL collagenase I (Invitrogen) and 50µg/mL DNase (Sigma-Aldrich) at 37°C and 5% CO₂ in complete medium. The digested dermis was filtered in SE buffer with 50µm cell strainers and dermal cDC2 were isolated by anti-CD1c-coupled magnetic beads (Miltenyi Biotec). Remaining cells were incubated for 48h in a 175cm² culture flask (50x10⁶ cells/50mL complete medium). CD14⁺ dAPCs were isolated from the loosely adherent cells using anti-CD14-conjugated magnetic beads (Miltenyi Biotec). After an overnight culture in dermal fibroblast-conditioned culture medium at 10⁶ cells/mL, CD14⁺ dAPCs were sorted by flow cytometry as CD14⁺ HLA-DR^{low} CD86^{low} cells.

Skin explant cultures: 2cm² biopsies of ~1mm thickness were placed at air-liquid interface onto 40µm cell strainers in 6mL complete medium. After 3 days, migratory cells were harvested from the culture supernatant. Resident memory T cells were excluded by selection with anti-CD3 magnetic beads (Miltenyi-Biotec). Where indicated, CD14⁺ dDCs were separated with anti-CD14 magnetic beads (Miltenyi-Biotec).

Monocyte-derived DCs and macrophages

Monocyte-derived dermal-like macrophages (MDdMs) and MDDCs were generated from blood monocytes as described (Kwan *et al.*, 2008). Briefly, 2x10⁶ monocytes, purified from adult blood by counterflow centrifugal elutriation (Etablissement Français du Sang, Strasbourg, France) were cultured in 5mL complete medium in the presence of GM-CSF (50ng/mL) and IL-4 (10ng/mL) for MDDCs, or GM-CSF (25ng/mL), IL-10 (25ng/mL) and M-CSF (10ng/mL) for MDdMs. On day 3, the culture was refreshed with 1mL of complete medium containing GM-CSF (50ng/mL) and IL-4 (10ng/mL) for MDDCs, or GM-CSF (12.5ng/mL) and IL-10 (12.5ng/mL) for MDdMs. On day 5, cells were characterized by flow cytometry for expression of CD1a, CD14 and DC-SIGN.

Cell lines

HeLa and Vero cells were cultured at 37°C and 5% CO₂ in DMEM (Invitrogen) supplemented with 10% FCS and antibiotics. *Aedes pseudoscutellaris* AP-61 mosquito cells were cultured at 27°C in Leibovitz's L-15 medium (Invitrogen) supplemented with 10% FCS, 1% tryptose phosphate broth (Eurobio) and antibiotics.

Viral infections

The CHIKV-06.049 virus strain was isolated from a young adult during the 2006 outbreak of Chikungunya fever in La Réunion island (Schuffenecker *et al.*, 2006). Virus stocks were produced on mosquito AP-61 cells (Brehin *et al.*, 2008) and all infectivity titers were expressed as plaque-forming units on Vero cells. 5x10⁴ HeLa cells, CD14⁺ dAPCs, MDdMs and MDDC were exposed to CHIKV at an MOI of 2.5, or mock-infected, in RPMI1640 supplemented with 2% FCS for 2h at 37°C. Virus was

removed by extensive washing and cells were kept for 22h in their respective culture media. The supernatants were used for titer analysis by the plaque-forming assay on Vero cells.

Recombinant CHIK-sE2 protein production

Stably transfected *Drosophila melanogaster* S2 cell line inducible for the secretion of the ectodomain of CHIKV envelope E2 protein (CHIK-sE2; residues 4-364) were described elsewhere (Aubry *et al.*, 2015). Briefly, a SNAP adaptor sequence (New England Biolabs) was fused in-frame with the C-terminus of recombinant CHIK-sE2, resulting in a chimeric protein that was purified from the supernatants of S2 cells. SNAP is the catalytic site of O⁶-alkylguanine-DNA-alkyltransferase. 3D rendition of CHIK-sE2-SNAP was created with the Yasara software based on the available crystal structures (CHIK-sE2: PDB 3J2W; SNAP-tag: PDB 3KZY).

Surface plasmon resonance

Surface plasmon resonance experiments were performed using a Biacore 3000. Extracellular domains of DC-SIGN[Lys62-Ala404] and MR[Leu19-Lys1383] (R&D Systems) were immobilized on a CM5 sensor chip (GE-Healthcare) by injecting 70µL of recombinant proteins (50µg/mL in formate buffer, pH 4.3) onto the surface activated with N-Ethyl-N'-dimethylaminopropyl carbodiimide/N-hydroxysuccinimide. 20µL of ethanolamine hydrochloride, pH 8.5, saturated the free activated sites of the matrix. Biosensor assays were performed with HEPES-buffered-saline (10mM HEPES, 150mM sodium chloride, pH 7.4) containing 0.005% surfactant P20 and 5mM CaCl₂, as running buffer. All binding experiments were carried out at 25°C with a constant flow rate of 20µL/min. CHIK-sE2 dissolved in the running buffer was injected for 3min, followed by a dissociation phase of 3min. The sensor chip surface was regenerated after each experiment by injecting 10µL EDTA 0.5M pH 8.0. The kinetic parameters were calculated using the BIAeval 4.1 software. Analysis was performed using the simple Langmuir binding 1:1 model with separate k_a/k_d (k_{on}/k_{off}). The specific binding profiles were obtained after subtracting the response signal from the control channel and from blank-buffer injection. The fitting to each model was judged by the chi-square value and randomness of residue distribution compared to the theoretical model.

CHIK-sE2 binding studies

Benzylguanine (BG)-AlexaFluor488 fluorochrome (New England Biolabs) was covalently linked to CHIK-sE2 via the SNAP moiety according to the manufacturer's recommendations. 10⁵ cells were incubated in 200µL RPMI1640 alone or with 2µg of CHIK-sE2-A488 (final concentration 150nM) for 30min at 37°C. The cells were then washed and labelled for cell surface markers in SE buffer and analyzed by flow cytometry. Where indicated, CHIK-sE2 (1.2µg) was pre-incubated for 30min at 4°C with anti-E2 monoclonal antibody 3E4 (0.9µg) to verify specificity of binding (Brehin *et al.*, 2008). To quantify binding efficiency, the mean fluorescent intensity (MFI) of CHIK-sE2-A488-exposed cells was calculated relatively to the MFI of untreated cells, which was set at 100%. For internalization studies by confocal

microscopy, cells exposed to CHIK-sE2-A488 were washed in SE buffer and counterstained with antibodies against HLA-DR or CD14 for 25min on ice. The cells were washed twice, fixed in 4% formaldehyde for 20min at RT, and the secondary goat-anti-mouse AlexaFluor594 antibody (Invitrogen) was added and incubated for 30min on ice. After washing, nuclei were labelled with DAPI. Samples were mounted on glass slides in fluorescence mounting medium (Dako). Images taken with Zeiss confocal microscopes LSM510 and Axio Observer Z1 were analyzed with ImageJ. For maturation assays, MDDCs were incubated with 150nM CHIK-sE2 or 100ng/mL ultrapure K12 LPS (Invivogen) for 24h, then CD86 expression was quantified by flow cytometry.

Peptide synthesis

Peptides were assembled using optimized Fmoc chemistry protocols with a multichannel peptide synthesizer (Neimark and Briand, 1993). Side-chain deprotection and cleavage of peptides from the solid support was performed by treatment with reagent K (82.5% TFA v/v, 5% phenol w/v, 5% water v/v, 5% thioanisole v/v, 2.5% 1,2-ethanedithiol v/v) for 150min at 20°C. Peptides were purified by reversed-phase HPLC (RP-HPLC) using a Beckman preparative HPLC system (Gagny, France) on a Nucleosil C18 (1 x 30cm) column, (Macherey Nagel, Hoerdt, France). The elution was achieved with a linear gradient of aqueous 0.1% TFA and 0.08% TFA in acetonitrile at a flow rate of 6mL/min with UV detection at 230nm. The purity of peptides was controlled by analytical RP-HPLC on a Beckman instrument (Gagny, France) with a Nucleosil C18 5µm column (150 x 4.6mm) using a linear gradient of 0.1% TFA in water and acetonitrile containing 0.08% TFA at a flow rate of 1.2mL/min. The integrity of each peptide was assessed by LC/MS using a Thermo Finigan LCQ.

The Epstein-Barr virus Nuclear Antigen-1 (EBNA1)-derived peptide (507-535; CVFVYGGSKTSLYNLRRGTALAIPQRLTG) was bound to BG-maleimide (New England Biolabs) by reacting with the thiol group of the N-terminal cysteine residue, before purification by HPLC.

Blocking CHIK binding

Skin migratory DCs were incubated for 30min at 37°C with 1mM mannan (Sigma-Aldrich), 10µg/mL purified antibodies against DC-SIGN (clone AZN-D1, Dr. Teunis Geijtenbeek, Amsterdam, NL (Geijtenbeek *et al.*, 2000); clone 111H2.02, Dendritics, France (Canard *et al.*, 2011)), MR (clone 19.2, BD), combinations of anti-MR and anti-DC-SIGN (total concentration 20µg/mL) or the corresponding isotype controls. Then, 150nM CHIK-sE2-A488 was added for 1h and its binding was quantified by flow cytometry.

Expansion of EBNA1-specific CD4⁺ T memory cells

Allogeneic PBMCs were purified from buffy coats of healthy blood donors (EFS) by Ficoll gradient (Sigma-Aldrich). 5x10³ EBNA1-specific CD4⁺ T cell (CK-NLR clone, HLA-DR1-restricted) cells/well were cocultured with 10⁵ irradiated PBMCs (3000rad) and 2x10³ irradiated, EBV-infected autologous B-LCL (20000rad) in RPMI1640 supplemented with 8% PHS (pooled human sera, Corning), 150U/mL IL-2

(Novartis), 1 μ g/mL Phytohemagglutinin-L (Sigma-Aldrich), and gentamicin. After 5 days, half of the medium was refreshed with RPMI1640 + 8% PHS + gentamycin + 450U/mL IL-2. Cells were monitored daily and split into the same medium and new 96 well-plates if necessary, until freezing at day 14 (Rühl *et al.*, 2019).

Antigen presentation assays

Genomic DNA was extracted from 40-80mg of skin using Nucleospin columns (Macherey-Nagel) and HLA haplotype of donors was determined with SSP HLA DQDR Combitray kit (Olerup). BG-EBNA1 peptide (250pmol) was coupled to CHIK-sE2 (200pmol) via the SNAP moiety in PBS. CHIK-sE2-EBNA1 (200nM) was incubated with 5x10⁵ migratory skin DCs or CD14⁺ dDCs for 1h in RPMI1640 medium. 10⁴ T-cell depleted, CHIK-EBNA1-loaded skin DCs or CD14⁺ dDCs from HLA-DR1 donors were cocultured in 200 μ L RPMI1640 + 8% PHS in 96-well U-bottom plates with 5x10⁴ EBNA1-specific CD4⁺ T cells previously labelled with 0.5 μ M carboxyfluorescein succinimidyl ester (CFSE). After 5 days, T cells were restimulated 6h with EBNA1 peptide (1 μ g/mL), including 4h with 10 μ g/mL brefeldin A and 1 μ M monensin (BD). T cells were stained for CD3 and CD4, then fixed and permeabilized with BD Cytotfix/Cytoperm Kit, stained with anti-IFN- γ antibody and analyzed by flow cytometry. T-cell proliferation was determined by CFSE dilution.

Statistical analyses

Data from different experimental conditions were tested by ANOVA, followed by relevant post-hoc multiple comparisons or by Wilcoxon tests, performed with Prism 6.0 software (GraphPad). Values of p below 0.05 were considered significant.

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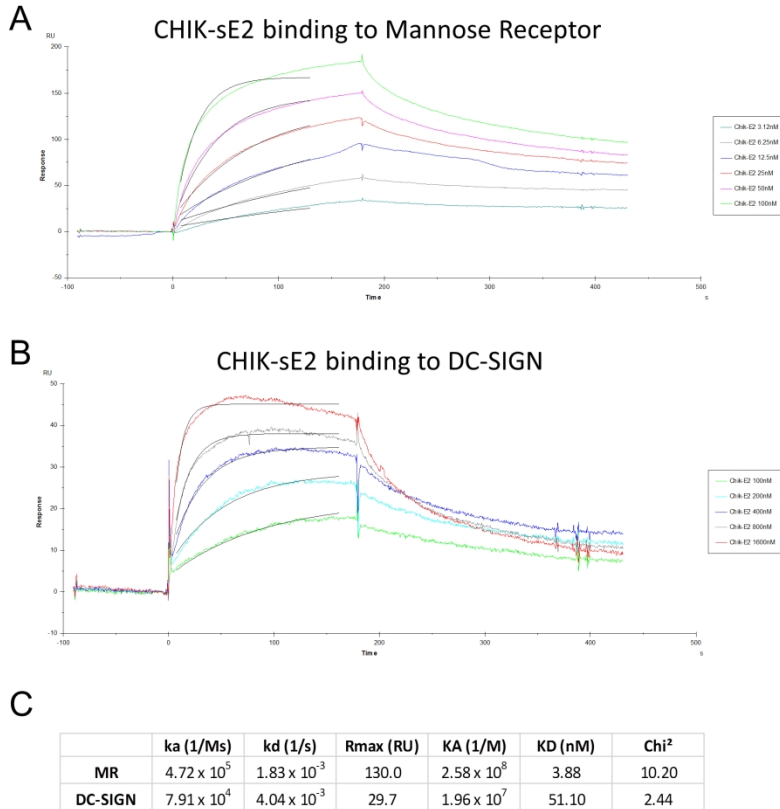
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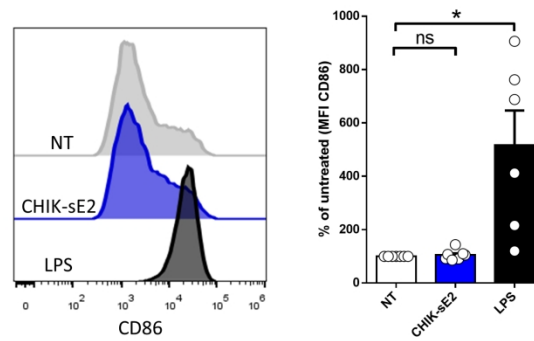
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Supplementary Figure S1

Figure S1: DC-SIGN and MR are receptors for CHIK-sE2. Affinity of CHIK-sE2 for immobilized MR (A) and DC-SIGN (B) were measured by surface plasmon resonance. Sensorgrams depict association and dissociation to DC-SIGN (0.1, 0.2, 0.4, 0.8, and 1.6 μ M of CHIK-E2) and MR (3.1, 6.3, 12.5, 25, 50 and 100nM of CHIK-sE2). (C) Constants for the binding assays. The KD for DC-SIGN and MR were 51.1nM and 3.9nM, respectively.

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Supplementary Figure S2

Figure S2: CHIK-sE2 does not promote DC maturation. Immature MDDCs were incubated for 24h with 150nM CHIK-sE2 or 100ng/mL LPS. Maturation was quantified by flow cytometry from the MFI of CD86 stainings. Representative histograms are shown. Data were obtained from six different donors and are presented as mean \pm SEM. Statistical comparisons: Wilcoxon tests (ns: non significant, * $p < 0.05$).

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