Figures and figure supplements

Composition and structure of synaptic ectosomes exporting antigen receptor linked to functional CD40 ligand from helper T cells

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Figure 1. CD40 dependent recruitment of CD40L to the IS and deposition in SE trail. (A) Schematic of PSLB and mature IS. (B) Detection of CD40L with the anti-CD40L clone 24–31 as a function of CD40 in the PSLB. T cells were allowed to form IS for 10 min in the presence of Alexa Fluor 647 anti-CD40L.
antibody and imaged by TIRFM. Data is pooled from five donors with each point being one cell. (C) Representative normalized maximum projections of Airyscan of CD4⁺ T (CellMask, cyan) cell and CD40L (anti-CD40L Alexa Fluor 647, Red hot) on PSLB in the presence/absence of UCHT1-Fab and CD40. Scale bar: 2 μm. (D) Representative horizontal planes (along the white lines depicted in (C)) of CD4⁺ T cells showing localization of CD40L within the cell volume. White squares represent the region of interest magnified on the right. (E) IS and kinapse stages of T cell interaction. Stages of TCR positive SE are released at the synaptic cleft upon mature IS formation. Following symmetry breaking the SE are partly dragged by the kinapse as they are left (Choudhuri et al., 2014). (F) Representative TIRFM of IS (top, 10 min incubation) and kinapse (bottom, 90 min incubation) showing CD40 clustering in PSLB coated with ICAM-1, UCHT1-Fab in the presence or absence of CD40. Following fixation and permeabilization cells were stained with anti-CD40L, scale bar: 5 μm. (G) Detection of CD40L with anti-CD40L mAb clone 24–31 in (F) (**** p ≤ 0.0001) nonparametric Mann-Whitney test (U test). Data is from five donors.

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**Figure 1—figure supplement 1.** Normalized maximum projections of Airyscan of CD40L (anti-CD40L Alexa Fluor 657, Red hot) within CD4+ T cell volume PSLB in the presence/absence of UCHT1-Fab and CD40. Scale bar: 5 μm.

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Figure 2. CD40L is incorporated in SE. (A) Schematic of BSLB (B) Schematic of T cell (green) interacting with BSLB with UCHT1-Fab (magenta) and SE on BSLB after T cell removal. (C) % CD40L and TCR transfer to BSLB coated with incremental levels of CD40. Data is from six donors. (D) Percentage Figure 2 continued on next page.
transfer of TCR, CD40L and CD4 to BSLB following electroporation of CD4⁺ T cells with either control CRISPR/Cas9-CD19gRNA RNP or CRISPR/Cas9-TSG101gRNA RNP, insert WB showing degree of knockdown using anti-TSG101 antibody. (E) Percentage transfer of TCR, CD40L and CD4 to BSLB following electroporation of CD4⁺ T cells with either control CRISPR/Cas9-CD19gRNA RNP or CRISPR/Cas9-VPS4bgRNA RNP, insert WB showing degree of knockdown using anti-VPS4b antibody. Multiple t-test $P$ values < 0.05 (*);<0.0001 (****) were considered significant; n.s. = non significant. (F) Heat maps showing the percentage of proteins transferred from T cells to BSLB. Data is from 10 donors. (G) Heat maps showing the percentage of proteins transferred from clone 35 to BSLB. Data is representative of 3 independent experiments with different clone 35 aliquots.

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Figure 2—figure supplement 1. Gating Strategy for SE capture on BSLB and representative histograms following CRISPR/Cas9 knockdown. (A) Gating Strategy for SE capture on BSLB. Forward/Right side scatter plot of beads and T cells following liberation of BSLB from EDTA/PBS. Beads have a

Figure 2—figure supplement 1 continued on next page
distinctive and narrow scatter signature, but this partly overlaps with cell debris. Beads are thus also positively discriminated from cell debris using Atto-488 lipids in the BSLB. Cells and conjugates are discriminated based on Atto-488-ve signal for cells and a combination of Atto-488+ve signal and higher light scattering signals for conjugates. (B) Representative histograms showing synaptic transfer of TCR and CD40L to BSLB following electroporation of CD4+ T cells with either control CRISPR/Cas9-CD19gRNA (green) RNP, CRISPR/Cas9-target gRNA RNP (magenta). DOI: https://doi.org/10.7554/eLife.47528.009
Figure 2—figure supplement 2. ICOS significantly increases CD40L transfer to BSLB. Per marker statistical analyses of heat maps shown in Figure 2D. 
P-values < 0.05 were considered significant. Repeat Measures ANOVA with Geisser-Greenhouse correction was performed. Significance was calculated.

Figure 2—figure supplement 2 continued on next page
Figure 2—figure supplement 2 continued

comparing in each bilayer composition group (either No accessory signal, CD40 + ICOSL, CD40 or ICOSL) at 500 and 5000 molec./μm² of UCHT1-Fab to No UCHT1-Fab. P values: *<0.05, **<0.005, ***<0.0005, ****<0.0001; n.s. = non significant.

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Figure 2—figure supplement 3. Efficient transfer of CD40L to BSLB at low UCHT1-Fab densities. (A) Percent of protein transfer (%) from human T cells to BSLBs containing increasing densities of UCHT1-Fab (0–1800 molec./μm²) and two different BSLB compositions: UCHT1-Fab + ICAM-1 (left) or UCHT1-Fab + ICAM-1 + CD40 + ICOSL (right). (B) Representative histograms showing as in A the synaptic transfer of proteins from T cells (gray background half offset histograms) to BSLBs (white background half-offset histograms) across increasing densities of UCHT1-Fab. (C) Molecules transferred to BSLB per molecule of TCRαβ transferred (marker to TCRαβ ratio). The Figure 2—figure supplement 3 continued on next page.
Figure 2—figure supplement 3 continued

First row depicts the mean absolute number of TCRαβ molecules per cell (first column) or per BSLB according to different UCHT1-Fab densities (columns 2–6) as reference of the number of molecules used in the calculation of molecular ratios (rows 2–7). Except for BST2, all the other markers reduce their transfer as the transfer of TCRαβ increases. The increased ratios of markers on BSLBs compared to the T cell PM is another form to depict the degree of protein enrichment in SE, but compared to the normal protein to TCRαβ ratio found on the PM of a non-activated T cell (shown are ratios for the mean values from six different donors). (D) CD4+ T cells were stained for the absolute quantification of surface markers using antibodies with known fluorescent dye to protein (F/P) ratios (See Supplementary file 2A). For analyses, dead cells were excluded with Propidium Iodide (0.5 μg/mL) during acquisition. Final molecules per cell were obtained by dividing the molecules of equivalent soluble fluorescent dye value (MESF/cell; interpolated from background-corrected GMFI values) by the F/P value of the relevant antibody. P values < 0.05 (*); <0.002 (**); <0.0002 (***); <0.0001 (****) were considered significant.

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Figure 2—figure supplement 4. ICOSL increases T cell: BSLB conjugate formation and ICOS transfer. (A) % T cell: BSLB conjugate formation in response to increasing densities of ICOSL. (B) % T cell: BSLB conjugate formation in response to increasing densities of UCHT1-Fab in the presence or absence of ICOSL. (C) BSLB composition and potential ICOS and BST2 engagement with TCRβ, CD40L, ICOS, BST2, CD4, CD6, CD63, CD81, CD82, and CD40 expression. (D) Representative images of TCRβ, CD40L, ICOS, BST2, CD4, CD6, CD63, CD81, and CD82 expression in response to increasing densities of ICOSL.
absence of accessory proteins ICOSL and or CD40. Multiple t test with false discovery rate of 1%. (C) Using a multicolor flow cytometry panel, the relative transfer of proteins (% of transfer) in response to increasing densities of ICOSL was determined. T cells were stimulated with BSLBs containing 200 molec./μm² of ICAM-1, 150 molec./μm² of UCHT1-Fab and 500 molec./μm² of CD40. After 1 hr, cell: bead conjugates were dissociated with cold 50 mM EDTA-PBS, and then stained with a multicolor panel using the same antibody clones described in Supplementary file 2A. We used CD4 as a control protein whose relative (% of total CD4 signal) transfer was not enriched in our single-color experiments (see Figure 2D). Each heat map square represent mean + /- SEM of data collected from 5 donors across two independent experiments. Shapiro-Wilk normality test. Non-Normally distributed data was analyzed using no matching or pairing Kruskal-Wallis test with Dunn’s multiple comparisons to the rank of conditions with no ICOSL. Normally distributed data was analyzed using one-way ANOVA with Holm-Sidak’s multiple comparison test to the mean of the no ICOSL group. p<0.03 was considered significant. *<0.03; **<0.002; ***<0.0002; ****<0.0001. (D) Representative off set histograms of BSLBs (white background) and T cells (gray background) analyzed after dissociation of conjugates. Histograms for controls stained with antibodies of appropriate isotype conjugated with the relevant fluorescent dye are shown in gray.

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Figure 2—figure supplement 5. BST2, CD63, CD81 and CD82 and CD40L localize to the synaptic cleft of UCHT-1 Fab stimulated cells. (A) Representative TIRF and IRM images showing that proteins characterized by flow cytometry as synaptically transferred and deposited on PSLB containing ICAM-1, UCHT1-Fab, CD40 and ICOSL. T cells were incubated for 1 hr at 37°C and 5% atmospheric CO₂, then cells were stained for 20 min.
at RT with anti-protein AF647 antibodies (5 μg/mL each). After two washes, cells were fixed with 4% PFA in PHEM buffer and imaged by TIRFM. (B) Pearson correlation of tetraspanins and CD40L with UCHT1-Fab as shown in A. (C) Representative TIRF and IRM images showing CD40L stained extracellular vesicles released by CD4⁺ T cells incubated for 15 min on PSLB coated with either ICAM-1 with UCHT1-Fab (top panels) or ICAM-1 with UCHT1-Fab and CD40 (bottom panels). Cells were incubated on the PSLB in the presence of anti-CD40L clone 24–31 fluorescently labeled with Alexa Fluor 647.

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Figure 3. CD40L, BST2, and ICOSL localize to the synaptic cleft. (A) Representative TIRFM images and IRM images showing staining of CD40L in the IS following incubation of HA specific clones on PSLB coated with ICAM-1 and either HLA-DR9 HA or HLA-DR9 CLIP monomers. Scale bar: 5 μm. (B) Figure 3 continued on next page

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Figure 3 continued

Detection of CD40L staining from (A) expressed as arbitrary unit (A.U.). (***p ≤ 0.0001) nonparametric Mann-Whitney test (U test). Data is from three experiments with different clone 35 aliquots. (C) Representative TIRFM and IRM images showing staining of BST2 in the IS following incubation of HA specific clones on PSLB coated with ICAM-1 and either His-tagged UCHT1-Fab, HLA-DR9HA or HLA-DR9CLIP monomers. Scale bar: 5 μm. (D) Pearson correlation of CD40L, BST2, ICOSL and ICAM-1 with HLA-DR9HA (as seen in C). (E) Percentage cells with or without cSMAC like structure (as seen in C) following incubation of HA specific clones on PSLB coated with ICAM-1 and either His-tagged UCHT1-Fab, HLA-DR9HA or HLA-DR9CLIP monomers in the presence or absence of ICOSL.

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**Figure 4.** Specificity of CD40L transfer- effects of bystander BSLB and general activation. (A) Representative flow cytometry histograms and percentage marker transfer (CD40L, TCR and CD81) of ‘cognate’ (UCHT1-Fab +ve Atto488; green) and bystander (UCHT1-Fab –ve Atto565; red) BSLB and T cells (blue). (B,C) Multiple t-test to compare the relative synaptic transfer (%) of CD40L (B) and TCRαβ (C) between cells pulsed for 30 min with PMA-Ionomycin (10 ng/mL and 0.5 μg/mL) and then incubated for another 90 min with agonistic BSLBs (increasing densities of UCHT-1 Fab, CD40 20 molec./μm², ICAM-1 200 molec./μm²) in the presence of the PMA-Ionomycin (values inside each cell represent mean percent synaptic transfer of 6 donors). Untreated cells were used as controls and as reference group for statistics; *, p<0.05; ***, p<0.001; ****p<0.0001 (data is from six donors).

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Figure 5. SE contains ESCRTs and TCR signalosome. (A) Proteins enriched by UCHT1-Fab on BSLB also containing ICAM-1, ICOSL and CD40. The network plot is based on known and predicted interactions from the STRING database (v11), with minimal confidence score of 0.4. Each protein

Figure 5 continued on next page
displayed > 1.75 log2 fold enrichment over BSLB coated with ICAM-1, ICOSL and CD40 in two independent experiments (three pooled donors/experiment). Node area represents protein LFQ intensity. Line thickness represents confidence score (0.4–0.999). False discovery rate (FDR) on peptide and protein level were set to 1%. (B) Modules identified in protein network shown in (A) determined using the Markov Cluster algorithm (inflation parameter: 2.5). Each colour represents a separate module (associated adjacency matrix). (C) Reactome analysis (reactome.org) of protein network shown in (A) reveals enrichment of TCR signaling, Vesicle-mediated transport and ESCRT pathways. (D) Representative TIRFM and IRM images showing staining of STAM2, EPN1 and ALIX on PSLB with UCHT1-Fab and ICAM-1, CD40 and ICOSL. T cells were incubated with PSLB at 37°C for 1 hr. Following fixation and permeabilization, cells were stained with relevant antibodies. (E) Average radial distribution in IS formed on PSLB containing ICAM-1 (black), ICOSL, CD40 and UCHT1-Fab (magenta) stained with antibodies to the candidate proteins (green). Data are from 3 donors and 50 cells; scale bar: 5 μm. (F) Percentage transfer of CD40L to BSLB following electroporation of CD4+ T cells with either control CRISPR/Cas9-CD19gRNA RNP or CRISPR/Cas9-VPS4bgRNA RNP. Multiple t-test, P values < 0.05 (*),<0.002 (**),<0.0002 (***)<0.0001 (****) were considered significant; n. s. = non significant.

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**Figure 5—figure supplement 1.** Recruitment of EPN1 to the plasma membrane. Airyscan confocal microscopy shows recruitment of EPN1 (green; white arrow) to the plasma membrane and at the synaptic cleft on PSLB in the presence of UCHT1-Fab (magenta) and CD40 (unlabeled).

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Figure 5—figure supplement 2. Transfer to BSLB following CRISPR/Cas9 RNP electroporation. (A) Percentage transfer of CD81, TCR, CD40L and CD4 to BSLB following electroporation of CD4+ T cells with either control CRISPR/Cas9-CD19gRNA RNP or CRISPR/Cas9-targetgRNA RNP. (B) WB showing degree of knockdown using anti-TSG101, anti-ALIX, anti-VPS4b, anti-EPN1 and anti-CHMP4b antibody.

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Figure 5—figure supplement 3. Effect of ADAM10 inhibitor and CRISPR/Cas9 electroporation on transfer to BSLB. (A) Representative histograms showing synaptic transfer of TCR, CD40L and CD81 to BSLB following treatment of CD4+ T cells with either vehicle control (green) or ADAM10/17 inhibitor (TAPI-1 acetate salt).

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Figure 5—figure supplement 3 continued

inhibitor (magenta). (B) Representative histograms showing level of knockdown of ADAM10 following electroporation of CD4+ T cells with either control CRISPR/Cas9-CD19gRNA RNP (gray) or CRISPR/Cas9-ADAM10 gRNA RNP (cyan). Representative histograms showing synaptic transfer of TCR, CD40L and CD81 to BSLB following electroporation of CD4+ T cells with either control CRISPR/Cas9-CD19gRNA RNP (green) or CRISPR/Cas9-ADAM10 gRNA RNP (magenta).

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Figure 6. Size distribution SE captured from immunological synapses on PSLB. (A) Schematic of SE deposition on PSLB. CD4⁺ T cell blasts were incubated for 90 min on supported lipid bilayers coated with ICAM-1 (200 molec./μm²), UCHT1-Fab (30 molec./μm²), CD40 (500 molec./μm²) and ICOSL.
Figure 6 continued

(200 molec./μm²), then T cells were removed with ice cold PBS and fixed with 4% PFA. (B) Representative dSTORM image of SE released by T cells were stained with WGA-CF568 to visualize the glycans (carried by lipids and proteins) on the surface of the SE. Examples of SE of different sizes, depicted by the white squares, are zoomed-in and shown with correspondent relative fluorescence intensity profiles. (C) Size distribution of SE released from all cells. Each symbol represents an SE (n = 1482). (D) Size distribution of SE released per cell. Each symbol represents the median size of SE released per cell (n = 40). (E) Number of SE released per cell. Each symbol represents the median number of SE released per cell. (F) Size distribution of TCRαβ microclusters from synapse and SE. Each symbol represents a microcluster. (G) Size distribution of TCRαβ microclusters from synapse and SE released per cell. Each symbol represents the mean size of SE released per cell. (H) Number of TCRαβ microclusters from synapse and SE released per cell. Each symbol represents the mean number of microclusters per cell. Lines and errors represent means ± SD.

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Figure 7. Nanoscale structure of SE. CD4+ T cell blasts were allowed to form IS for 90 min on PSLB with ICAM-1 (200 molec./μm²), ICSOL (200 molec./μm²), CD40 (500 molec./μm²) and UCHT1-Fab (30 molec./μm²) and then released with cold PBS and the SE were fixed, stained with mAb as indicated, and subjected to dSTORM analysis. (A) Representative dSTORM images showing TCR (green), CD40L (magenta) on CD81 (gray) labeled SE. Insets show examples of SEs containing only TCR, only CD40L or both proteins. (B) Percentage of SEs containing only TCR (green), only protein of interest (magenta), or containing both TCR and protein of interest (gray). (C) CBC histograms of the single-molecule distributions of the colocalization parameter. Bars represent means ± SD. The positive control is TCRαβ-AF488 and UCHT1-Fab-AF647, which are predicted to co-localize. (D) Cross-correlation analysis. (E) Nearest-neighbor distance (NND) analysis from data shown in B). Each symbol represents the median NND of all paired single-molecule localizations from EVs released per cell. Lines and errors represent means ± SD. Dashed line in panel (D) and (E) marks mean size of SE.

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Figure 7—figure supplement 1. TCR co-localization with BST2 and ICOS and segregation from CD40L. (A) Representative dSTORM images showing TCR (green) and CD40L (magenta) on WGA labeled SE (gray). Insets show examples of SEs containing only TCR, only CD40L or both proteins. (B) Figure 7—figure supplement 1 continued on next page
Multiple examples of SEs released by CD4⁺ T cells incubated for 90 min on PSLB coated with ICAM-1, UCHT1-Fab, CD40 and ICOSL. The SEs were stained with anti-TCRβ-AF488, WGA-CF568 to visualize the lipid membrane and with anti-CD40L-AF647, anti-ICOS-AF647 or anti-BST2-AF647.

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Figure 7—figure supplement 2. CD81 co-localization with TCR and CD40L. Multiple examples of SEs released by CD4+ T cells incubated for 90 min on P5LB coated with ICAM-1, UCHT1-Fab, CD40 and ICOSL. The SEs were stained with anti-TCRαβ-AF488, anti-CD81-AF568 to visualize the SE membrane and with anti-CD40L-AF647.

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Figure 7—figure supplement 3. Distinct cross-correlation distances for TCR with BST2 and ICOS versus CD40L. Examples of cross-correlation analysis for positive control data (A), between TCR and CD40L (B), TCR and ICOS (C), or between TCR and BST2 (D) from multiple cells.

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Figure 8. CD40L-positive SE left by T cells help DC and high-density vesicular CD40L is sufficient for DC maturation. (A, B) HLA-DR (A) or CD83 (B) expression on the surface of DCs stimulated for 24 hr on PSLB prepared as indicated to present SE. Each symbol represents the mean fluorescent intensity (MFI) of the indicated marker.

Figure 8 continued on next page
Figure 8 continued

Intensity of a cell (n ≥ 20) from five independent donors, which are represented by the different donors. Lines and errors represent means ± SD. ns, not significant; *, p ≤ 0.05; ***, p < 0.001; Kruskal-Wallis with Tukey’s post-hoc test. (C) After 24 hr of culture, the supernatant of DCs stimulated as in A) and B) were analyzed for the presence of different secreted factors. Results from seven independent donors. The fold-change was normalized to the results from the supernatant of DCs incubated with PSLB containing ICAM-1 alone. (D) For assessing the biological significance of presenting CD40L in vesicular structures compared to the proposed biologically active form of soluble trimeric CD40L [i, green], we developed 86 nm diameter synthetic unilamellar vesicles (SUV, Supplementary file 2D) using phospholipids either without (ii, gray) or with His-tag, and hence CD40L, binding activity (iii, blue). An equal total mass (Mass eq.) of N-terminal His-tagged recombinant human CD40L was either incubated with DOPC liposomes (mock-SUV [ii, gray]) or used to load 12.5% DOGS-NTA and make vesicular CD40L (SUV:CD40L [iii, green]). As control, a Mass eq. of CD40L was used as soluble, free protein in culture. (E) FCM analyses of moDCs stimulated for 24 hr with either SUVs containing increasing amounts of CD40L monomers (blue, approximately 0, 40, 80, 120, 190 and 230 CD40L molec./SUV respectively) or an equivalent concentration of soluble CD40L (green). Data represent fold change in the GMFIs for ICAM-1, CD80 and CD86 of treated moDCs over unpulsed controls. (F) As in B, moDCs were stimulated using SUV:CD40L at a final load of approximately 230 molec./SUV. An equivalent concentration of soluble CD40L either delivered alone or in combination with SUVs lacking NTA lipids (soluble CD40L + SUV) were used as controls. After 24 hr of stimulation moDCs were collected, FcR blocked, stained and analysed by multicolor FCM for the expression of maturation markers. Given the high variability of arbitrary fluorescence values, the response was normalized as a fold change compared to unpulsed, immature DC controls. Data represent mean ± /- SEM collected from five different donors and five independent experiments. One-way ANOVA and Dunn’s multiple comparisons test to the mock treated control was performed. P values < 0.05 (*); <0.002 (**); <0.0002 (***); <0.0001 (****) were considered significant.

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Figure 8—figure supplement 1. SE captured on PSLB efficiently activate moDCs. (A) Overlaid histograms illustrating the expression of ICAM-1, CD80, CD83 and CD86 maturation markers on DCs following 24 hr of stimulation on PSLBs loaded with SEs derived from CD4+ T cells. SE-coated PSLB were

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obtained after the removal of T cells from bilayers containing different stimuli (either A1, B1, C1 or C0). (B) Distribution of ICAM-1, CD80, CD83 and CD86 expression levels in DCs as shown in A. Each symbol represents a donor. (C) Statistical significances for the cytokines/chemokines from Figure 8C. ns, not significant; *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001; ****, p ≤ 0.0001. Kruskal-Wallis with Newman-Keuls post-hoc test.

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