Supplementary methods

Apoptosis/Necrosis assay

At 72h hours following activation, cells were collected and stained with Annexin-FITC and PI staining kit according to manufacturer's instructions (MBL technologies, Arlington, VA, USA, MEBCYTO® Apoptosis Kit Code No. 4700). The cells were then analyzed by flow cytometry. Single cells positive for Annexin but not PI were considered to be in early apoptotic stage, while cells positive for both Annexin and PI were considered to be in late apoptosis/necrosis stage.

Sub-differentiations induction

Following isolation and immediate activation, using either antigen-loaded DCs or activation beads, cytokines and neutralizing antibodies were added to the culture medium as follows: for Th1 differentiation IL-12 (10ng/ml) + anti-IL-4 (10μg/ml); for Th2 differentiation IL-4 (20ng/ml) + anti-IFNγ (10μg/ml) + anti-IL-12 (10μg/ml); for Treg differentiation TGFβ (10ng/ml) + IL-2 (5ng/ml) + anti-IL-4 (10μg/ml) + anti-IFNγ (10μg/ml) + anti-IL-12 (10μg/ml). Cells were then incubated for 5 days, followed by fixation, permeabilization and staining for CD4 and the relevant transcription factors (Tbet for Th1, GATA3 for Th2, and Foxp3 for Tregs) for flow cytometry analysis. All cytokines and antibodies were from BioLegend.

Restimulation and IFNγ analysis

T-cells were activated and induced to sub-differentiate to Th1 as described above. Following incubation for 7 days, cells were spun down and medium was replaced with medium containing PMA and ionomycin (BioLegend, BLG-423301, 1:500) for 4 hours of restimulation. Brefeldin (BioLegend, BLG420601) and Monensin (BioLegend, BLG420701) were then added for 2 hours (1:1000 each), for prevention of protein secretion. Cells were then collected, fixed/permeablized and stained for CD4 and IFNγ for flow cytometry analysis.

Supplementary Figures and Legends

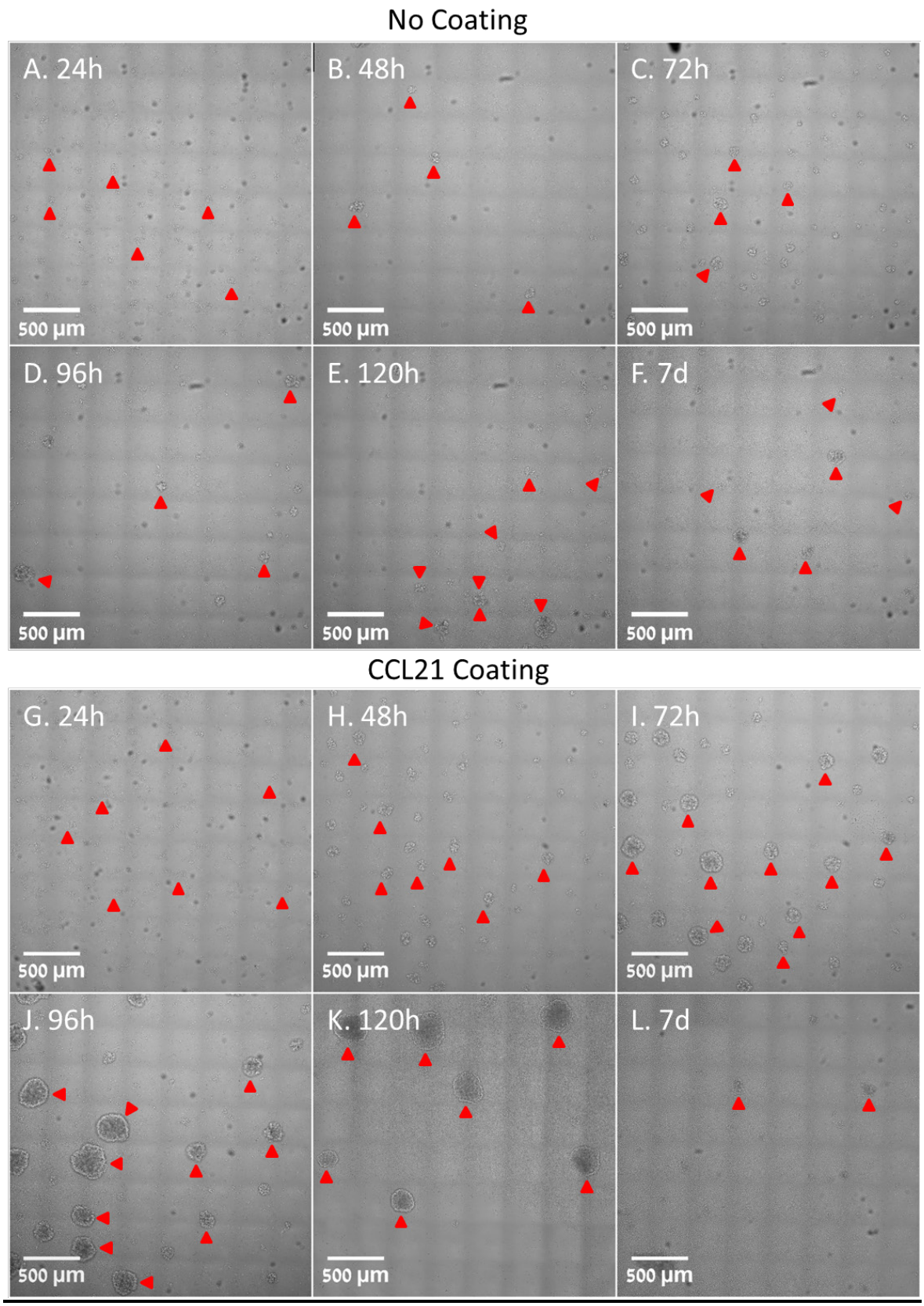

Figure S1 – Substrate-immobilized CCL21 induces transient formation of large T-cell clusters

Stitched images (8X8) of OT-II T-cells, cultured on uncoated substrate (A-F; controls), or substrate coated with CCL21 (G-L). Note that CCL21 coating induced very large clusters, compared to those formed on uncoated controls. The growth in cluster size persisted for 120 hours (K), then decreased dramatically from 7 days onwards (L). Representative clusters are marked with red arrowheads. Scale bars: 500μm.

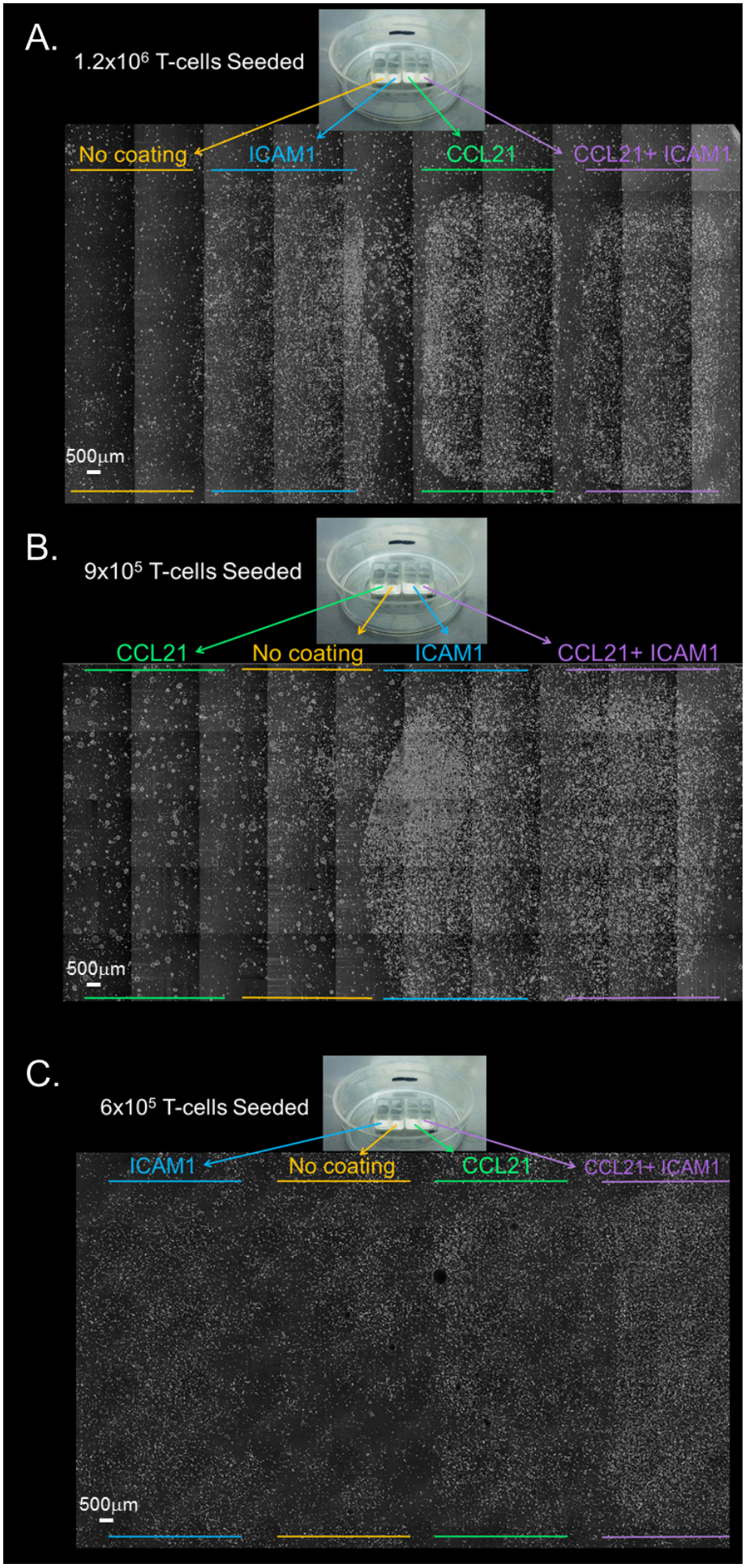
A.
1.2x10^6 T-cells Seeded
No coating
ICAM1
CCL21
CCL21+ ICAM1
500μm
B.
9x10^5 T-cells Seeded
CCL21
No coating
ICAM1
CCL21+ ICAM1
500μm
C.
6x10^5 T-cells Seeded
ICAM1
No coating
CCL21
CCL21+ ICAM1
500μm

Figure S2 – The combined effects of CCL21 and ICAM1 on cell morphology are confined to the functionalized areas along the substrate

Stitched images of the surfaces with cultured T-cells, 36 hours after their plating. Each area is marked beneath the horizontal line down to the bottom of the image, as follows: No coating in yellow; ICAM1 coating in blue; CCL21 coating in green; and CCL21 + ICAM1 coating in purple. (See experimental layout in Fig. 6A). T cells are confined mainly to the coated areas, which display a higher cell density (A-C; blue, green and purple, respectively) than that of the uncoated area (A-C; yellow). The induction of large clusters by CCL21 coating (A-C; green) and the cell spreading induced by ICAM1 (A-C; blue and purple), are confined to the coated area, and do not affect the morphology of T-cells in other areas that share the same original cell pool and culture medium. Similar findings were observed irrespective of the relative locations of the adhesive fields (A-C), as well as when different numbers of cells were seeded (A, $1.2x10^6$ cells; B, $9x10^5$ cells; and C, $6x10^5$ cells). Scale bars: 500μm.

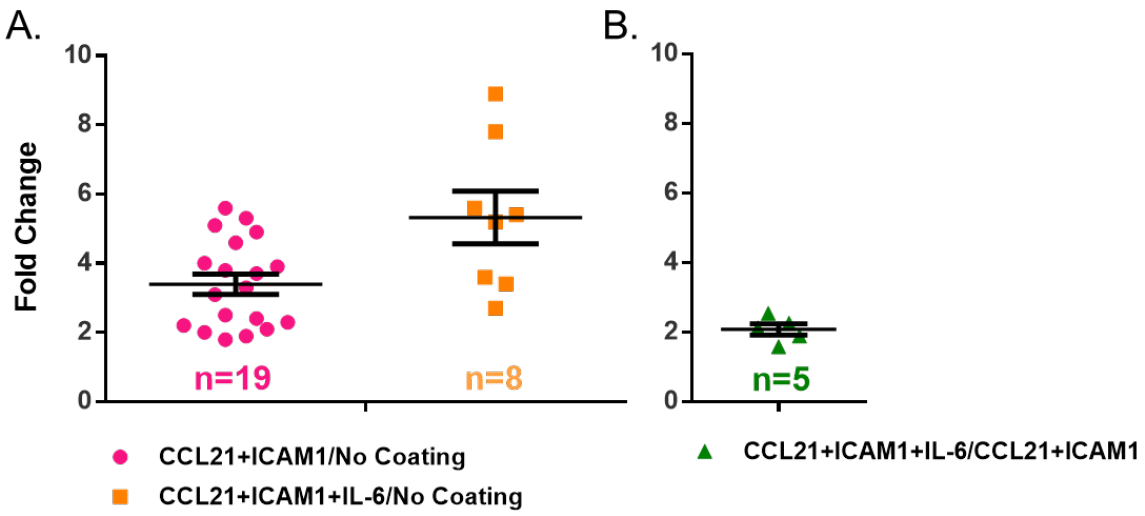

Figure S3 – Fold change in live T-cell numbers in all independent tests

Live T-cell numbers were measured using either metabolic or microscopic assays, with incubation times ranging from 72 hours up to 7 days, and seeded cell numbers ranging from 600 to 12,000 per well of 384/96-well plate. Each symbol denotes the result of one independent experiment: average of 3-10 replicates that were performed at the same date and with the same conditions (same initial cell number, incubation time and format, and cell enumeration method). Average live T-cell numbers measured at the experimental end point were normalized to the numbers obtained with either no coating (A) or with CCL21+ICAM1 coating (B).

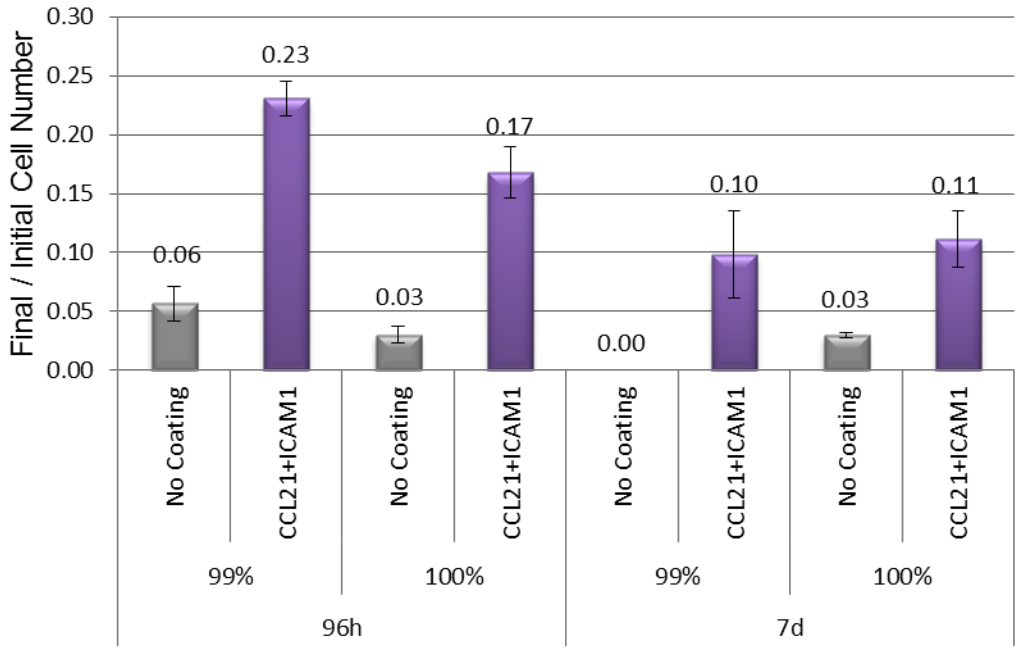

Figure S4 – The combined effect of CCL21 and ICAM1 coating on cell number is restricted to antigen-activated T-cells, and does not affect non-specific T-cells.

Bar graphs illustrating the ratio of viable non-ova T-cells to the originally seeded cell number as quantified by automated image analysis (Data are representative of 3 independent experiments with 6 replicates; 20 fields of view in each. Error bars represent standard error), in cultures with non-ova T-cells alone (marked as 100%) or with 1% OT-II T-cells expressing ubiquitin-GFP (marked as 99%). Unlike the enrichment of ova-specific T-cells (see Fig. 5, L), the ratio of the final number of non-specific cells to the originally seeded cell number was low (0.03-0.23), indicating a lack of significant proliferation, and massive cell death of the non-specific T-cells.

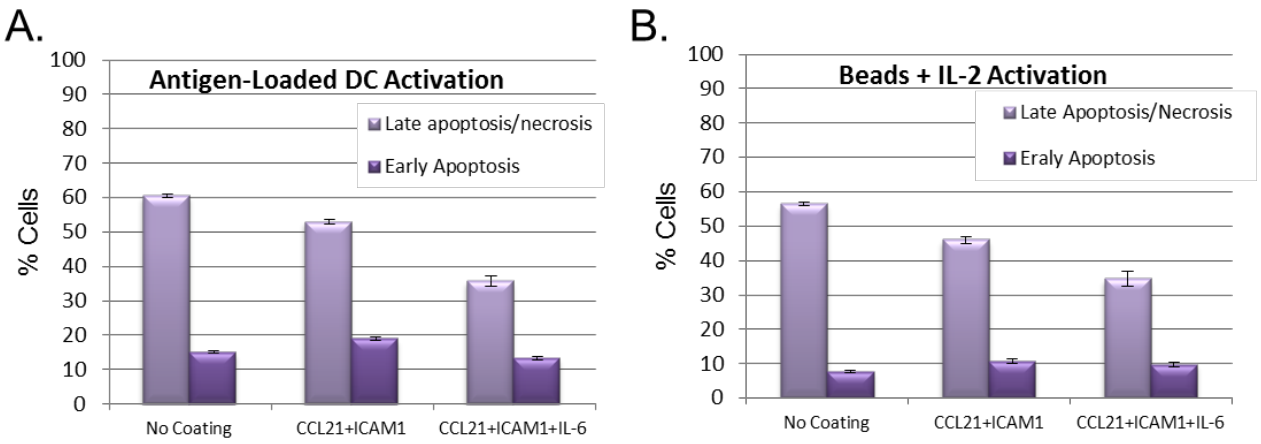

Figure S5 - IL-6 decreases the percentage of late apoptosis/necrosis

(A, B) Bar graphs illustrating the percentage of cells with early apoposis or late apoptosis/necrosis, following either antigen loaded DC activation (A), or beads and IL-2 activation (B); (Data are representative of 2 independent experiments with 4 replicates; Error bars represent standard error of the mean). IL-6 decreased the percentage of dead cells/late apoptosis/Necrosis by up to 20%, with no apparent effect on early apoptosis.

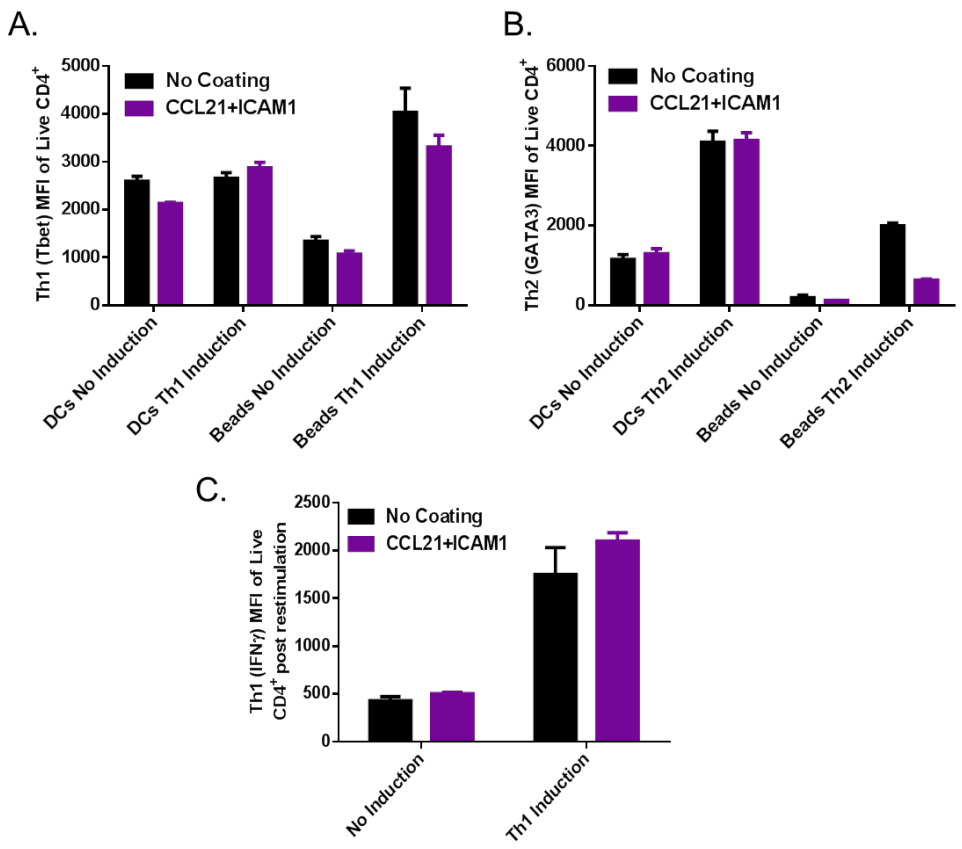

Figure S6 – CCL21 + ICAM1 hardly affect the sub-differentiation of CD4 T-cells

A-B – Bar graphs illustrating the proportions of Th1 (A), Th2 (B) with and without induction of sub-differentiation, following either antigen-loaded DCs or bead activation (Data are representative of 2 independent experiments with 3 replicates; Error bars represent standard error of the mean).

C – Bar graphs illustrating the mean fluorescent intensity of IFNγ staining of T-cells following restimulation. Cells were activated using antigen-loaded DCs and grown on uncoated substrate or substrate coated with CCL21+ICAM1, with or without induction of Th1 sub-differentiation (Data are representative of 2 independent experiments with 4 replicates; Error bars represent standard error of the mean).

Supplementary Videos Legends

Supplementary Video 1: T-cell clusters are highly dynamic

Time-lapse videos (A,B) and representative images (C-M) of OT-II T-cells (blue) and ovalbumin-loaded DCs (green) labeled prior to seeding with membrane permeable dyes and co-cultured with cell death marker (red) on a CCL21-coated surface. The time stamp in A, hh:mm; in B-M, hours. The movies show that clusters and single cells are able to join and leave existing clusters. Clusters marked with colored arrowheads (C-M), serial cluster merging is marked as follows: white + grey = red (C-D) + pink (D-F) = light green + dark green (F-I) = light orange + dark orange (I-J) = light purple + dark purple = blue (J-L). Scale bar: 50μm.

Supplementary Video 2: ICAM1 and CCL21 affect the morphology of individual T-cell

Time-lapse videos of OT-II T-cells co-cultured with ovalbumin-loaded DCs on an uncoated surface (A, E), or on surfaces coated with CCL21 (B, F) or ICAM1, either alone (C, G), or in combination (D, H). Time stamp is hh:mm:ss. The movie indicates that surface coating with ICAM1 affects non-clustered, single cell morphology. Specifically, individual cells on an uncoated surface are mostly round (A, E), while on a CCL21-coated surface, they develop an elongated morphology (B, F). ICAM1 coating, with or without CCL21, induced massive cell spreading on the surface and multiple cell-cell contacts (C, D, G, H). Scale bars: 50μm.

Supplementary Video 3: ICAM1, with or without CCL21, reduces the culture's height

Representative 3D animations of a single cluster of OT-II T-cells, co-cultured for 72 hours with ovalbumin-loaded DCs on different surface coatings. T-cell membranes are live-stained in green; cell nuclei are live-stained red. Culture height was greater on CCL21-coated surfaces (B), compared to that on an

uncoated surface (A). The cell layers on the ICAM1 or CCL21+ICAM1 coated surface were considerably thinner (C and D); Scale bars: 20μm.

Supplementary Video 4: The combined effects of CCL21 and ICAM1 on cell morphology are confined to the functionalized areas along the substrate

See experimental layout in Fig. 4A.

Time-lapse video of a stitched image of the surface with cultured T-cells, taken at 36 hours. Each area is marked underneath the horizontal line, down to the bottom of the image: no coating in yellow; ICAM1 in blue; CCL21 in green; and CCL21 + ICAM1 in purple. Magnified single fields are shown for each area in matching colored squares. T-cells are found mainly within the coated areas (blue, green and purple), which display a higher cell density than that of the uncoated area (yellow). The induction of large clusters by CCL21 coating (green) and the cell spreading and culture flattening induced by ICAM1 (blue, purple), are confined to the coated area, and do not affect the morphology of T-cells in other areas that share the same original cell pool and culture medium. Scale bar: 500μm.