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Supplemental Data

The Balance between T Cell Receptor Signaling and Degradation at the Center of the Immunological Synapse Is Determined by Antigen Quality

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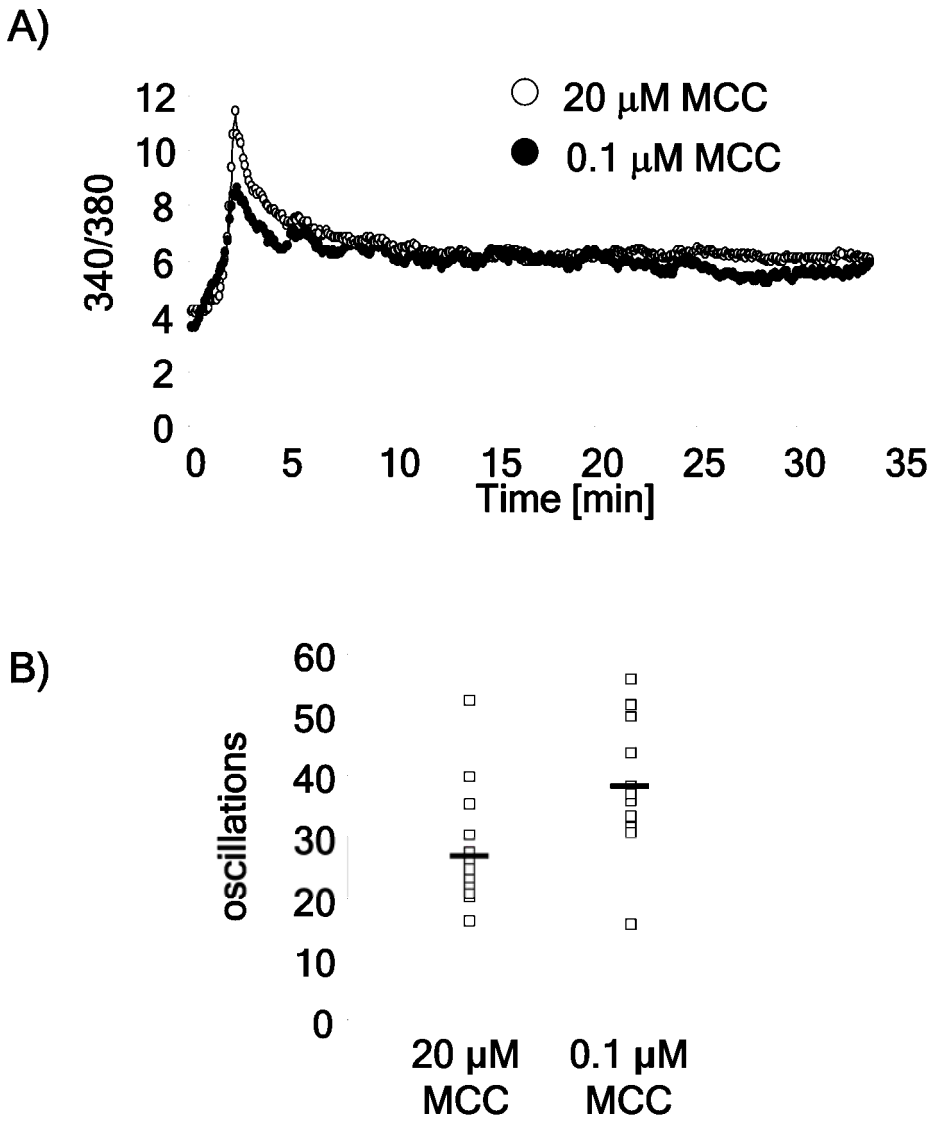
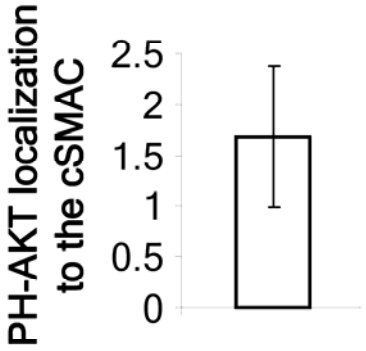

A)
20 μM MCC
0.1 μM MCC
340/380
12
10
8
6
4
2
0
0
5
10
15
20
25
30
35
Time [min]
B)
oscillations
60
50
40
30
20
10
0
20 μM MCC
0.1 μM MCC

Figure S1

A)


PH-AKT localization
to the cSMAC
2.5
2
1.5
1
0.5
0

B)

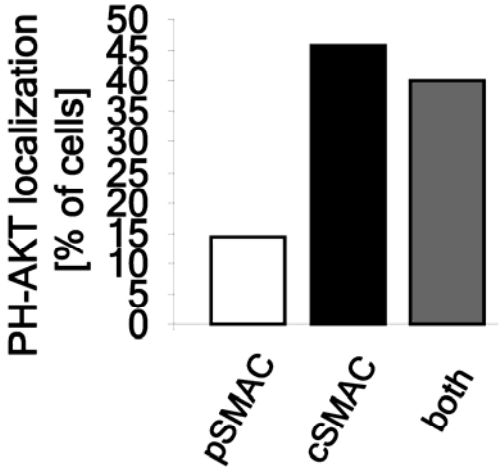

PH-AKT localization
[% of cells]
50
45
40
35
30
25
20
15
10
5
0
pSMAC
cSMAC
both

Figure S2

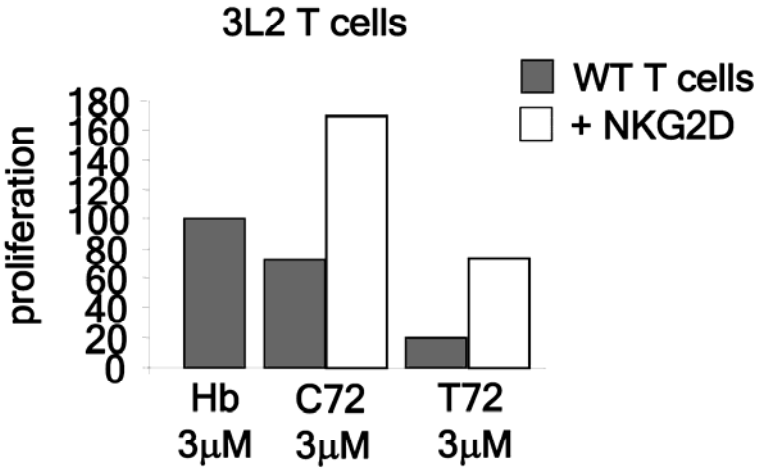

3L2 T cells
WT T cells
+ NKG2D
proliferation
180
160
140
120
100
80
60
40
20
0
Hb
3μM
C72
3μM
T72
3μM

Figure S3

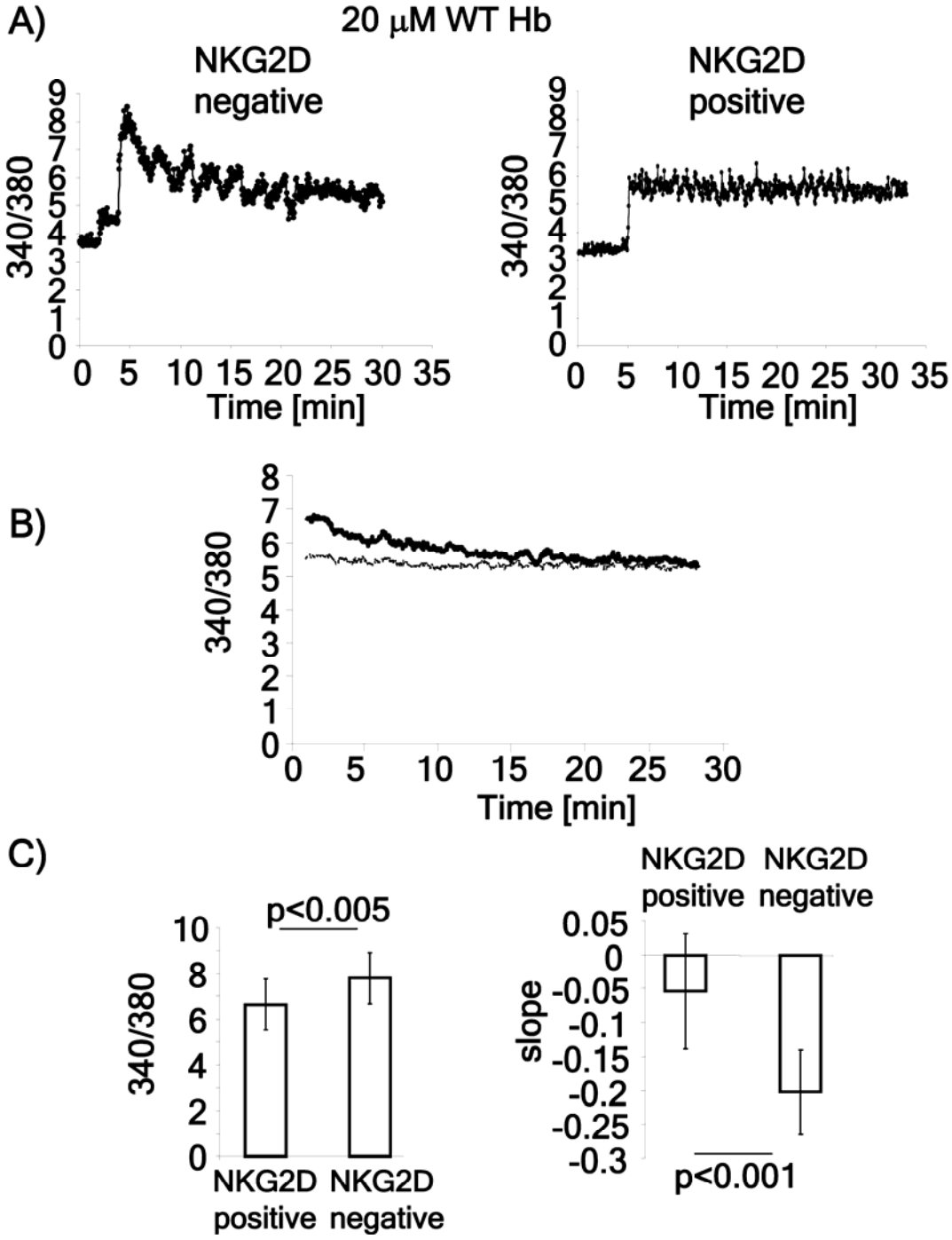

A)
20 μM WT Hb
NKG2D
negative
NKG2D
positive
340/380
Time [min]
B)
340/380
Time [min]
C)
p<0.005
340/380
NKG2D
positive
NKG2D
negative
slope
p<0.001

Figure S4

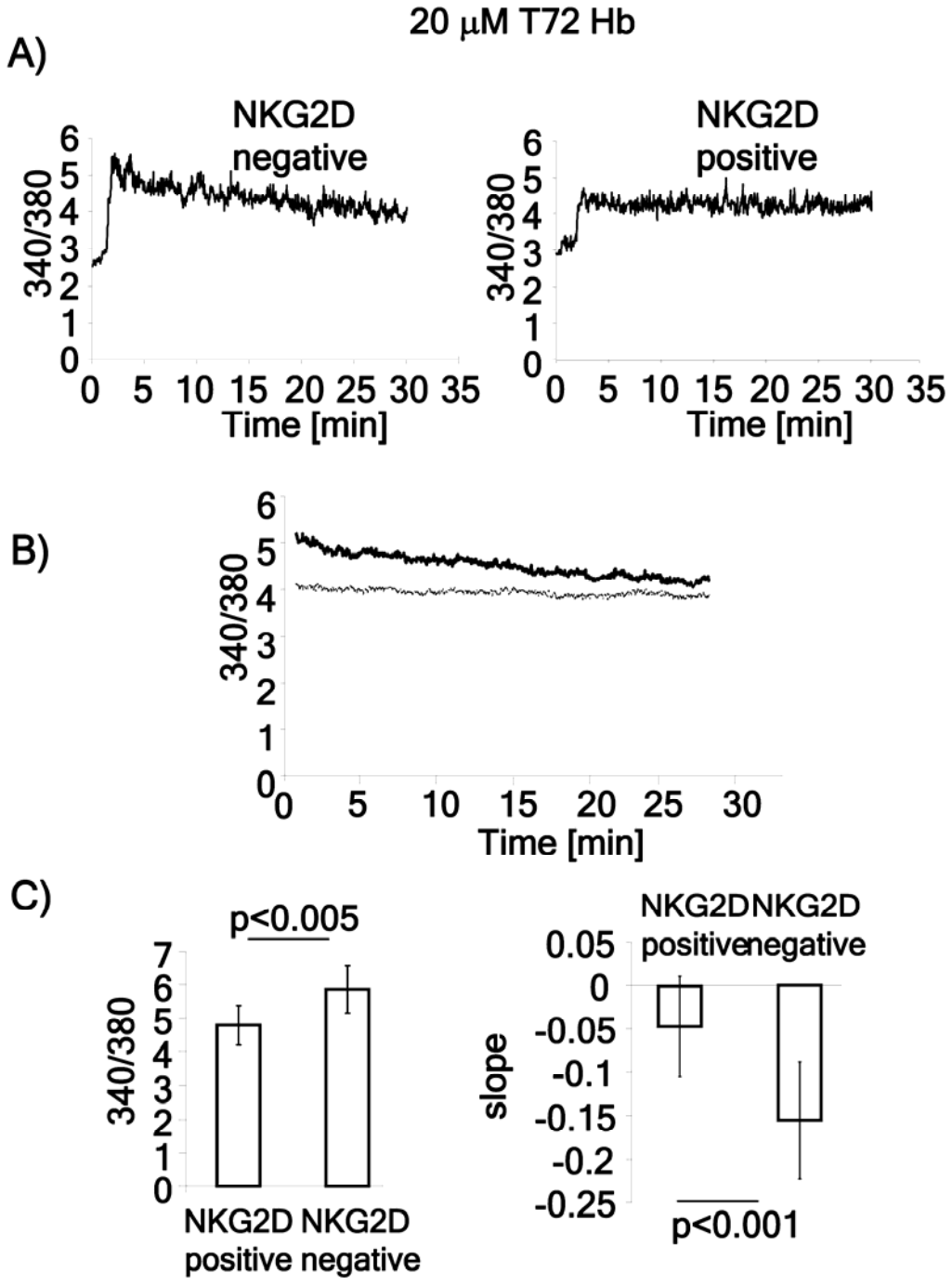

A)
20 μM T72 Hb
NKG2D negative
NKG2D positive
340/380
Time [min]
B)
340/380
Time [min]
C)
p<0.005
340/380
NKG2D positive
NKG2D negative
slope
p<0.001

Figure S5

Supplementary Figures:

Figure S1. CH27 cells were allowed to adhere to a-B220-coated 8-well chambers and loaded with 20 or 0.1 μM MCC.

AND T cells were loaded with the calcium indicator Fura-2 and stimulated by the immobilized, peptide-loaded CH27 cells. The 340/380 ration was measured during 40-60 minutes upon adding the T cells, taking images every 3 seconds. Representative calcium traces of AND T cells stimulated with the indicated doses of peptide are shown. B) Calcium oscillations during the sustained phase of calcium mobilization are evaluated by the average difference of the individual calcium trace from the linear trendline of the analyzed part of the curve. At least 12 cells were analyzed for each peptide dose.

Figure S2. Quantification of PH-AKT accumulation at the synapse.

A) The accumulation of PH-AKT in the pSMAC and cSMAC of the cells that fromed mature synases on bilayers in figure 6 was quantified using ImageJ. The graph shows the mean ± s.d of over 20 cells from two independent experiments. B) The accumulation of PH-AKT in the cSMAC and pSMAC of AND-CH27 conjugates that formed mature synapses (from figure 6) was quantified using ImageJ and expressed as the percentage of cells having PH-AKT pSMAC-enriched (empty bars), cSMAC enriched (black bars) or equally pSMAC and cSMAC enriched (grey bars). Over 30 cells from two independent experiments were scored

Figure S3. NKG2D-induced cSMAC formation enhances the recognition of weak peptides.

CD4+ T cells were purified from spleens of 3L2 mice retrovirally transduced with NKG2D and DAP10-YFP. Sorted NKG2D/DAP10 positive as well as negative cells were stimulated with CH27 cells transduced with Rae1ε loaded with the indicated peptides. Proliferative responses were assessed by [^{3}H]-thymidine incorporation during the last 16 hours of a 72 hour stimulation and expressed as percentage of control (proliferation in response to WT peptide). The results shown are representative of three experiments.

Figure S4. CH27 cells transduced with Rae-1ε were allowed to adhere to a-B220-coated 8-well chamber and loaded with 20 μM of WT Hb.

WT or NKG2D/DAP-10-positive 3L2 T cells were loaded with the calcium indicator Fura-2 and stimulated by the immobilized, peptide-loaded CH27 cells. The 340/380 ration was measured for 40-60 minutes after adding the T cells, taking images every 3 seconds. Representative calcium traces of WT and NKG2D/DAP10-positive T cells stimulated with the WT Hb peptide

are shown in A). The amplitude of the calcium response and the scope of the sustained phase of calcium mobilization were analyzed in over 20 cells obtained in two independent experiments and presented as: B) the average sustained calcium phase; C) the average amplitude of the calcium trace and the average slope of the sustained calcium phase. The graphs show the means ± s.d of over 20 cells from two independent experiments. P values were obtained using the Student's t test.

Figure S5. CH27 cells transduced with Rae-1ε were allowed to adhere to a-B220-coated 8-well chambers and loaded with 20 μM of T72 Hb.

WT or NKG2D/DAP-10-positive 3L2 T cells were loaded with the calcium indicator Fura-2 and stimulated by the immobilized, peptide-loaded CH27 cells. The 340/380 ration was measured for 40-60 minutes upon adding the T cells, taking images every 3 seconds. Representative calcium traces of WT and NKG2D/DAP10-positive T cells stimulated with the T72 Hb peptide

are shown in A). The amplitude of the calcium response and the scope of the sustained phase of calcium mobilization were analyzed in over 20 cells obtained in two independent experiments and presented as: B) the average sustained calcium phase: C) the average amplitude of the calcium trace and the average slope of the sustained calcium phase. The graphs show the means ± s.d of over 20 cells from two independent experiments. P values were obtained using the Student's t test.

Supplemental Experimental Procedures

Calcium imaging

WT and NKG2D/DAP-10-positive T cells were incubated with 1 μM fura 2-AM (Molecular Probes) for 30 min at 37°C in Ringers imaging solution (150 mM NaCl, 10 mM glucose, 5 mM HEPES, 5 mM KCl, 1 mM $MgCl_2$, and 2 mM $CaCl_2$), washed, and then incubated in Ringers solution for another 30 min at 37°C. After washing, fura-2-loaded T cells were pipetted onto 8-well chamber slides (Lab-Tek, Nalge Nunc International) with immobilized Rae-1e expressing CH27 cells pulsed for 4 hours with 20 mM of the indicated peptide. The location of the APCs and T cells was monitored by visualizing the cells with transmitted light every 3 s. Calcium imaging was performed at 37°C using a temperature-controlled environmental chamber on a Zeiss axiovert 200M microscope equipped with a xenon arc lamp. Fura 2-loaded cells were excited using 340 and 380 excitation filters (71000a set; Chroma Technology) and a polychroic mirror (73100bs; Chroma Technology). Fluorescence was passed through a 510 ± 40 wide band emission filter (Chroma Technology) and captured by a Cascade 512B camera (Roper Scientific). Ratio measurements (340/380) were recorded at 3-s intervals over a 50-min time period.

Data analysis

Fluorescent images were analyzed using MetaMorph (Molecular Devices), and the 340/380 ratio displayed on a pseudocolor scale, with calculations done on randomly selected cells. SDs were calculated vertical distances of the data points from the

regression line obtained in excell (GraphPad Prism; GraphPad). The linear regression line was fit for data points from 1 to 31 after the calcium. Statistical analysis were performed using standard student t-tests.