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Nanoscale ligand spacing influences receptor triggering in T cells and NK cells

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Abstract

Bioactive nanoscale arrays were constructed to ligate activating cell surface receptors on T cells (the CD3 component of the TCR complex) and NK cells (CD16). These arrays are formed from biofunctionalized gold nanospheres with controlled interparticle spacing in the range 25 – 104 nm. Responses to these nanoarrays were assessed using the extent of membrane-localized phosphotyrosine in T cells stimulated with CD3-binding nanoarrays, and the size of cell contact area for NK cells stimulated with CD16-binding nanoarrays. In both cases, the strength of response decreased with increasing spacing, falling to background levels by 69 nm in the T cell/anti-CD3 system and 104 nm for the NK cell/anti-CD16 system. These results demonstrate that immune receptor triggering can be influenced by the nanoscale spatial organization of receptor/ligand interactions.

Keywords

Nanopatterning; biofunctionalized nanoparticles; ligand density; T cell; NK cell

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Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Supporting Information

The following supporting information is provided: Experimental Details, Supplementary T cell results.

Immunological synapses, domains of close adhesive intercellular contact, play a crucial role in key decision-making processes in the immune system. By holding two cells stably together, they enable integration of signals from a complex range of ligand-receptor pairs on the cell surfaces. In the innate immune system, immunological synapses between Natural Killer (NK) cells and target cells determine the NK response^{1, 2}

Work over the last two decades has shown that the immunological synapse is complex not only in the large number of distinct cell surface molecules that are involved, but also in terms of other factors including the stiffness and fluidity of the antigen-presenting surface^{5, 6}, and spatial structure across micrometer and nanometer lengthscales. Both T cell and NK cell synapses have been divided into distinct signaling areas a few micrometers across, known as central, distal and peripheral Supramolecular Activating Clusters (c-, d-, p-SMAC)^{7, 8}. The spatial segregation of receptors has also been highlighted^{9–11}. More recently, advanced microscopy approaches including total internal reflection fluorescence microscopy (TIRF)¹², and super-resolution techniques such as PALM¹³ have revealed submicron clusters of cell surface receptors including the T cell antigen receptor (TCR)^{12, 13}, Lat^{13, 14}, ZAP-70^{7, 15, 16} and SLP-76^{7, 16}. Similarly, NK cell immunological synapses have revealed clusters of both activating (CD16^{17, 18} and NKG2D¹⁹) and inhibitory (KIR2DL1²⁰) receptors.

It is rapidly becoming apparent that this spatial structuring of immunological synapses can act as a driver for signaling, playing a major role in the integration of localized signals into a cell-level activation event. One class of evidence for this comes from microscopy studies that show downstream signaling molecules associated with receptor microclusters²¹. Such microscopic studies correlate synaptic structure with observed signaling. To actively control cellular signaling using spatial effects, biomimetic patterned surfaces can be constructed that mimic and aim to control the distribution of ligated receptors on cell surfaces^{22–31}. In the context of lymphocytes, substrates patterned with micrometer-scale patches of ligands have been used to change the shape of the T cell c-SMAC and p-SMAC, and to separate or colocalize different ligand-receptor pairs in T cells^{32–34} and NK cells³⁵. Additionally, patterned lipid bilayers can guide and limit TCR microcluster motion^{36, 37}, while patches of self-assembled monolayers ~750 nm in size have revealed the effect of antigen density on TCR-like chimeric antigen receptors³⁸. In contrast to these studies, which examine larger scale structure, there has been to our knowledge no use of nanopatterned substrates to drive lymphocyte signaling at sub-100 nm length-scales, despite the presence of important synaptic structure at these lengthscales.

In this paper we create artificial ligand nanopatterns with extremely small anchoring points that bind only one to a few receptors, varying the spacing between adjacent points in the range 25 – 104 nm. We use these surfaces to stimulate both T cells and NK cells, investigating how spacing affects signaling from two paradigmatic immune receptors: the TCR and the activating NK cell receptor CD16.

The two receptor systems studied here both initiate activation of their respective cell types, and signal using broadly similar mechanisms, via associated immunoreceptor tyrosine-based activation motifs (ITAMs)³⁹. The TCR is the prime receptor responsible for immunological

specificity in T cell activation, and thus controls many facets of the adaptive immune response including production of high affinity antibodies and immunological memory^{40, 41}. The CD16 receptor binds the Fc portion of antibodies, enabling NK cells to recognize and kill opsonized cells via Antibody Dependent Cellular Cytotoxicity (ADCC)⁴². This is a powerful mechanism linking antibody specificity with cellular cytotoxicity to target pathogen-infected cells. ADCC is also exploited therapeutically to target B cell mediated diseases including leukaemias and lymphomas by treatment with Rituximab^{43, 44}, a monoclonal antibody (mAb) that binds the CD20 motif on B cells, exposing its Fc portion to binding by CD16 on NK cells^{43, 44}.

Here, we construct immune cell stimulating nanoarrays by attaching T cell or NK cells stimulatory ligands to an array of evenly spaced gold nanoparticles generated by block copolymer micellar nanolithography⁴⁵. This self-assembly-based technique has proved its value in determining spatial effects on signaling in a variety of cellular systems^{22, 46–49}, founded on the ability to readily pattern large substrate areas with feature sizes as small as ~10 nm. Nanopattern formation is based on forming two-dimensional close-packed layers of block copolymer micelles whose cores are loaded with gold (III) chloride. Treatment with a hydrogen plasma removes the polymer leaving metallic gold nanoparticles, positioned in a hexagonal lattice whose spacing depends on the original polymer molecular weights. The gold nanoparticles can then be functionalized with peptide or protein molecules using the well-established thiol-gold chemistry^{46, 50}.

Coupling of immunoreceptor-stimulating antibodies to the nanoarrays was accomplished using strong bonds based on the near-covalent chemisorption of thiol and disulfide groups to gold, and on biotin-streptavidin bonding, promising a firmer attachment than is given by the alternative NTA-histidine system^{48, 51}. To produce TCR-binding nanoarrays, the gold nanoparticles were directly functionalized with F(ab')₂ fragments derived from the UCHT-1 antibody that bind the CD3 ϵ component of the TCR complex⁵². The hinge region of the F(ab')₂ fragment contains two disulphide bridges (-S-S-) interconnecting the heavy chains, which are expected to strongly chemisorb to gold surfaces⁵³, so that the F(ab')₂ fragments should be predominantly oriented with the base bound to the gold nanoparticles (Figure 1B right). In an alternative approach, NK cell activating nanoarrays were produced by functionalizing gold nanoparticles with a biotin alkanethiol, followed by streptavidin and CD16-binding antibodies (3G8 mAb and Rituximab) that were biotinylated on surface (-NH₂) sites using a short NHS-PEG-biotin linker (Figure 1B left). In all cases, to ensure ligand molecules are presented only on the gold nanospheres, the glass background was initially passivated prior to biofunctionalization by physisorbing a layer of the anti-fouling polymer poly(L-lysine) – *graft* – poly(ethylene glycol) (PLL-*g*-PEG)⁵⁴.

Importantly, in this setup each nanosphere can anchor only one to a few antibodies or F(ab')₂, due to the small nanoparticle size (8 – 17 nm), combined with steric repulsions between antibodies or F(ab')₂ and the effect of PEG chains from the background passivating layer (swollen height ~10 nm⁵⁵). Indeed a previous AFM study suggests that such nanopatterns can anchor as little as one single protein molecule per nanoparticle⁵¹. The exact number of bound molecules is not critical to this study, since the aim is simply to generate an anchoring

point small enough to bind only one of the smallest observed receptor nanoclusters present on the cells, c. 35 – 70 nm across¹³.

Since both the TCR and CD16 rely on the presence of other ligand-receptor pairs to enhance cell adhesion *in vivo*, nanopatterns were further modified to generate an adhesive background between the gold nanospheres. In T cell experiments, the integrin ligand ICAM-1 was added to the background, by biotin-streptavidin bonding using a PLL-g-PEG with incorporated biotin⁵⁶. For the NK cell-stimulating nanoarrays, the PLL-g-PEG was displaced by PLL in a final step after the nanosphere functionalization, giving a surface that stimulates cell adhesion.

Results

T cells

The early-stage response of T cells to anti-CD3 nanoarrays was assessed using the degree of tyrosine phosphorylation in the vicinity of the cell membrane, a good measure of overall signaling in the early stages of activation⁵⁷. These measurements were performed using TIRF immunofluorescence, 5 minutes after plating. It can be seen that the extent of tyrosine phosphorylation reduced substantially when the nanoarray interparticle spacing was increased from 25 nm to 104 nm (Figure 2A, see Fig S.1 for images of nanopatterns).

To confirm the effect, quantitative measurements of total phosphotyrosine intensity across multiple spacings and 4 donors were performed (Fig 2B; intensities normalized to 25 nm values for each donor to enable comparison). It can be seen that phosphotyrosine intensity in T cells stimulated with anti-CD3 nanoarrays decreased strongly when the spacing was increased from 25 nm, falling to not significantly greater than background by 69 nm. The significance of the decreasing trend is shown by a Spearman's rank correlation test ($p < 0.001$), as well as the pairwise comparisons shown (Figure 2B). Wells with <25 cells are not shown in Figure 2B but were included in the correlation test.

The number of cells adhered to the surface also decreased with increasing nanoarray spacing (Figure 2C). This provides complementary evidence that more closely-spaced anti-CD3 nanoarrays generate a stronger response, since one of the first consequences of signaling from the TCR complex is the inside-out activation of integrin LFA-1, leading to stronger ICAM-1 mediated adhesion.

The trend of decreasing phosphotyrosine intensity with increasing spacing was confirmed by experiments performed in the absence of an ICAM-1 background (Supplementary Information, Figure S.2; Spearman's rank correlation test $p < 0.001$). Note the sparseness of the data in this case (only 1 donor presented for 3 of the 4 spacings tested), due to the inevitably smaller number of adhered cells in the absence of ICAM-1.

The proportion of naïve and memory phenotypes in the adherent population at each nanoarray spacing was quantified using the expression of CD45RA as a marker for naïve cells (Figure 2D)⁵⁸. Interestingly, the results show that whilst the number of cells adhered decreases as spacing increases, the proportion of naïve and memory phenotypes within the

adherent population also changes. As spacing increases from 25 to 69 nm the proportion of naïve adherent cells is reduced, meaning that the population is enriched for memory cells. This is consistent with the fact that memory T cells express significantly more of the ICAM-1 binding integrin LFA-1 than naïve T cells.

NK cells

It is not straightforward to use phosphorylation of tyrosines as a surrogate marker for NK cell activation since these residues will be phosphorylated in both activating and inhibitory receptors. Instead, we assayed for the area of contact between the NK cell and the activating surface, (6 minutes after plating) as an indication of the cell's initial responsiveness. NK cells stimulated with CD16-binding nanoarrays were seen to be more spread at the closest spacing (25 nm), whereas widely-spaced (104 nm) nanoarrays showed no significant increase in spread area compared with a PLL control surface. The observed spacing dependence is the same for the B cell-depleting drug Rituximab (Figure 3B) as for a monoclonal anti-CD16 antibody (Figure 3A).

In summary, the T cell and NK cell experiments showed strikingly similar results, with both cell types showing a decrease in signalling with increasing nanoarray spacing, with the response of the T cell falling to background levels by 69 nm, and the NK cell by 104 nm.

Discussion

Our experiments show a strong dependence of immunoreceptor signaling on the spacing of ligand nanoarrays, with initial cellular responses dropping to background levels as the spacing is increased from 25 nm to 104 nm. A striking aspect of these results is that the lengthscales over which the signaling was observed to fall off are of the same order of magnitude as the size of the receptor islands or nanoclusters in which immune receptors have been observed to congregate. The size of such domains of TCR, in non-activated T cells, has been measured by PALM (35 – 70 nm¹³), TEM (40 – 300 nm¹³) and fluorescence correlation spectroscopy¹³. It must be recognized that the aggregation state of the TCR on the cell surface is controversial with measurements that suggest the TCR diffuses as a monomer or forms closely packed clusters, particularly on previously activated cells^{59–61}. However, weak interactions leading to nanodomain formation may allow for diffusion of individual TCR as has been observed for Ras and GPI anchored proteins in nanodomains^{62, 63}. In NK cells, there have to our knowledge been no super-resolution investigations of CD16 distribution, although micrometer-scale clusters have been observed¹⁷, however other NK cell receptor nanoclusters have been sized at 110 nm (KIR2DL1 measured by ground state depletion (GSD) and PALM²⁰).

The similarity between the length-scales of signaling fall-off and receptor nanocluster size can be explained in terms of previous observations in T cell activation, where the coming together of nanoclusters to form larger microclusters is regarded as an essential step in TCR signaling¹³. While it was speculated that this concatenation is integral to signaling there was no obvious linkage between the previously defined dimension of soluble TCR triggering ligands and nanocluster size⁶⁴. If each ligand-functionalized nanoparticle acts to anchor a single nanocluster, then the closely-spaced nanoarrays will bring nanoclusters into close

contact with one another, enabling such concatenation to occur. If the size of TCR nanoclusters is taken as 35 – 70 nm¹³, then the observed decrease in signaling from 25 nm to 34 nm to 69 nm spacing, with signaling barely above background at 104 nm is highly consistent with this model. A similar effect is plausible in CD16 signaling, given the importance of cluster formation¹⁷.

There are alternative interpretations as to how nanoarray spacing interacts with receptor nanoclusters. An internally consistent model is to suppose that each nanocluster needs to interact with a certain number of surface-anchored ligand molecules. Taking nanocluster size as ~ 70 nm, the most widely-spaced 104 nm array will only allow each nanocluster to interact with a single gold nanoparticle bearing one to a few ligands. In contrast, the 25 nm and 34 nm spacings will allow each nanocluster to interact with several anchoring points, with the 69 nm on the borderline, consistent with observations. This interpretation is internally consistent and plausible for the CD16 data. It is, however, difficult to reconcile in the case of TCR signaling with preexisting observations of T cell activation based on very few ligand molecules. For example, a study using ligand-functionalized lipid bilayers suggests that signaling can occur with no more than 1 ligand molecule per signaling focus^{36, 65}, while direct measurements of cell-cell contacts have even reported signaling from a single antigen-ligated TCR, although this may depend on the presence of additional TCR that bind self-antigens⁶⁶. In addition, in these continuous nanorarrays, the changes in inter-particle spacing are linked to changes in density of ligands. Therefore, it is possible that the threshold is based on ligand density, rather than spacing, but at 104 nm spacing the density is still ~115 particles/ μm^2 , which should be more than sufficient for triggering highly sensitive T cells or NK cells.

An intriguing feature of the NK cell results is the lack of difference between the effect of nanoarrays functionalized with Rituximab and the anti-CD16 mAb 3G8. This is despite the difference in valency: with each Rituximab molecule binding only one CD16 receptor via its Fc domain, in contrast to the bivalent 3G8⁶⁷. This observation is consistent with the concept that the effect of each functionalized gold nanoparticle is determined by its small size, e.g. relative to receptor nanoclusters, rather than the exact number of bound receptors, and also suggests that minor variations in the number of ligand molecules bound to each gold nanosphere are unlikely to be important.

The spatial dependences in receptor triggering described here open important directions for future work. One notable question is whether nanoarray spacing also influences immune effector functions of T and NK cells. Equally, it would be interesting to investigate the kinetics of signaling beyond these initial time-points. On a broader level, an exciting direction is the development of advanced bioengineered platforms that combine nanoscale patterning with other approaches such as varying the mechanical properties and 3D structure of the underlying substrate^{5, 68}.

Regardless of the underlying mechanisms, our results clearly demonstrate that spatial factors on length-scales of order 10 – 100 nm not only correlate with signaling, but can actually control it. This insight has importance for the design of future therapeutics, showing the benefit of moving beyond drugs that ligate single receptors or dimers towards larger

nanoscale therapeutics. Such therapeutics will mimic and manipulate synaptic spatial structures, for example driving nanocluster concatenation.

Conclusion

We have constructed biofunctionalized nanopatterns based on gold nanosphere arrays that stimulate T cell activation and CD16-mediated activation of NK cells. These are ideal for investigating nanoscale effects on immune cells, since the functionalized gold nanospheres provide receptor-binding points that are much smaller than the immunoreceptor nanoclusters that seem to constitute fundamental structures for signaling, and the spacing between adjacent nanospheres is of the same order as the nanocluster size. For both T cells and NK cells, the initial cellular response to stimulation decreased with increasing spacing, falling to background levels by the time spacing reached 69 nm for T cells and 104 nm for NK cells. The striking similarity between these results hints at a common principle whereby cells cease to respond to ligand stimulation as the spacing between stimulating features exceeds receptor nanocluster size. Our results are consistent with optimal triggering when ligands are spaced to effectively bridge nanoclusters, or alternatively engage multiple sites within one nanocluster. Our results affirm that nanoscale spatial structure can play a causative role in controlling immune cell activation, suggesting the need for nanoscale therapeutics in next generation immunotherapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Orange JS. *Nature Rev Immunol.* 2008; 8(9):713–25. [PubMed: 19172692]
2. Eissmann P, Davis DM. *Curr Top Microbiol Immunol.* 2010; 340:63–79. [PubMed: 19960309]
3. Xie J, Tato CM, Davis MM. *Immunol Rev.* 2013; 251(1):65–79. [PubMed: 23278741]
4. Kalos M, Levine BL, Porter DL, Katz S, Grupp SA, Bagg A, June CH. *Sci Transl Med.* 2011; 3(95): 95ra73.
5. O'Connor RS, Hao X, Shen K, Bashour K, Akimova T, Hancock WW, Kam LC, Milone MC. *J Immunol.* 2012; 189(3):1330–9. [PubMed: 22732590]
6. Platzman I, Janiesch J-W, Matic J, Spatz JP. *Israel Journal of Chemistry.* 2013 Published Online.
7. Grakoui A, Bromley SK, Sumen C, Davis MM, Shaw AS, Allen PM, Dustin ML. *Science.* 1999; 285(5425):221–7. [PubMed: 10398592]
8. Orange JS, Harris KE, Andzelm MM, Valter MM, Geha RS, Strominger JL. *Proc Natl Acad Sci USA.* 2003; 100(24):14151–6. [PubMed: 14612578]
9. Davis SJ, van der Merwe PA. *Nature Immunol.* 2006; 7(8):803–9. [PubMed: 16855606]
10. James JR, Vale RD. *Nature.* 2012; 487(7405):64–9. [PubMed: 22763440]

11. Kohler K, Xiong S, Brzostek J, Mehrabi M, Eissmann P, Harrison A, Cordoba SP, Oddos S, Miloserdov V, Gould K, Burroughs NJ, van der Merwe PA, Davis DM. *PloS one*. 2010; 5(11):e15374. [PubMed: 21179506]
12. Varma R, Campi G, Yokosuka T, Saito T, Dustin ML. *Immunity*. 2006; 25(1):117–27. [PubMed: 16860761]
13. Lillemeier BF, Mortelmaier MA, Forstner MB, Huppa JB, Groves JT, Davis MM. *Nature Immunol*. 2010; 11(1):90–6. [PubMed: 20010844]
14. Williamson DJ, Owen DM, Rossy J, Magenau A, Wehrmann M, Gooding JJ, Gaus K. *Nature Immunol*. 2011; 12(7):655–62. [PubMed: 21642986]
15. Campi G, Varma R, Dustin ML. *J Exp Med*. 2005; 202(8):1031–6. [PubMed: 16216891]
16. Sherman E, Barr V, Manley S, Patterson G, Balagopalan L, Akpan I, Regan CK, Merrill RK, Sommers CL, Lippincott-Schwartz J, Samelson LE. *Immunity*. 2011; 35(5):705–20. [PubMed: 22055681]
17. Liu D, Peterson ME, Long EO. *Immunity*. 2012; 36(4):600–11. [PubMed: 22464172]
18. Liu D, Bryceson YT, Meckel T, Vasiliver-Shamis G, Dustin ML, Long EO. *Immunity*. 2009; 31(1):99–109. [PubMed: 19592272]
19. Brown AC, Dobbie IM, Alakoskela JM, Davis I, Davis DM. *Blood*. 2012; 120(18):3729–40. [PubMed: 22966166]
20. Paegeon SV, Cordoba SP, Owen DM, Rothery SM, Oszmiana A, Davis DM. *Sci Signal*. 2013; 6(285):ra62. [PubMed: 23882121]
21. Yokosuka T, Sakata-Sogawa K, Kobayashi W, Hiroshima M, Hashimoto-Tane A, Tokunaga M, Dustin ML, Saito T. *Nature Immunol*. 2005; 6(12):1253–62. [PubMed: 16273097]
22. Cavalcanti-Adam EA, Micoulet A, Blummel J, Auernheimer J, Kessler H, Spatz JP. *Eur J Cell Biol*. 2006; 85(3–4):219–224. [PubMed: 16546564]
23. Kilian KA, Bugarija B, Lahn BT, Mrksich M. *Proc Natl Acad Sci USA*. 2010; 107(11):4872–7. [PubMed: 20194780]
24. Tang J, Peng R, Ding J. *Biomaterials*. 2010; 31(9):2470–6. [PubMed: 20022630]
25. Curran JM, Stokes R, Irvine E, Graham D, Amro NA, Sanedrin RG, Jamil H, Hunt JA. *Lab on a chip*. 2010; 10(13):1662–70. [PubMed: 20390207]
26. Senaratne W, Sengupta P, Jakubek V, Holowka D, Ober CK, Baird B. *J Amer Chem Soc*. 2006; 128(17):5594–5. [PubMed: 16637600]
27. Huang NF, Patlolla B, Abilez O, Sharma H, Rajadas J, Beygui RE, Zarins CK, Cooke JP. *Acta Biomater*. 2010; 6(12):4614–21. [PubMed: 20601236]
28. Dalby MJ, Gadegaard N, Tare R, Andar A, Riehle MO, Herzyk P, Wilkinson CD, Oreffo RO. *Nature Mater*. 2007; 6(12):997–1003. [PubMed: 17891143]
29. Taylor ZR, Keay JC, Sanchez ES, Johnson MB, Schmidtke DW. *Langmuir*. 2012; 28(25):9656–63. [PubMed: 22656270]
30. Li JR, Shi L, Deng Z, Lo SH, Liu GY. *Biochemistry*. 2012; 51(30):5876–93. [PubMed: 22783801]
31. Deng Z, Weng IC, Li JR, Chen HY, Liu FT, Liu GY. *ACS nano*. 2011; 5(11):8672–83. [PubMed: 21999491]
32. Doh J, Irvine DJ. *Proc Natl Acad Sci USA*. 2006; 103(15):5700–5705. [PubMed: 16585528]
33. Shen K, Thomas VK, Dustin ML, Kam LC. *Proc Natl Acad Sci USA*. 2008; 105(22):7791–6. [PubMed: 18505845]
34. Sekula S, Fuchs J, Weg-Remers S, Nagel P, Schuppler S, Fragala J, Theilacker N, Franzreb M, Wingren C, Ellmark P, Borrebaeck CA, Mirkin CA, Fuchs H, Lenhert S. *Small*. 2008; 4(10):1785–93. [PubMed: 18814174]
35. Culley FJ, Johnson M, Evans JH, Kumar S, Crilly R, Casasbuenas J, Schnyder T, Mehrabi M, Deonarain MP, Ushakov DS, Braud V, Roth G, Brock R, Kohler K, Davis DM. *PLoS Biol*. 2009; 7(7):e1000159. [PubMed: 19636352]
36. DeMond AL, Mossman KD, Starr T, Dustin ML, Groves JT. *Biophys J*. 2008; 94(8):3286–92. [PubMed: 18199675]
37. Mossman KD, Campi G, Groves JT, Dustin ML. *Science*. 2005; 310(5751):1191–3. [PubMed: 16293763]

38. Fishler R, Artzy-Schnirman A, Peer E, Wolchinsky R, Brener R, Waks T, Eshhar Z, Reiter Y, Sivan U. *Nano Lett.* 2012; 12(9):4992–6. [PubMed: 22900991]
39. Bezradica JS, Medzhitov R. *Curr Opin Immunol.* 2012; 24(1):58–66. [PubMed: 22240121]
40. Pulendran B, Ahmed R. *Cell.* 2006; 124(4):849–63. [PubMed: 16497593]
41. Victora GD, Schwickert TA, Fooksman DR, Kamphorst AO, Meyer-Hermann M, Dustin ML, Nussenzweig MC. *Cell.* 2010; 143(4):592–605. [PubMed: 21074050]
42. Lanier LL, Kipps TJ, Phillips JH. *J Exp Med.* 1985; 162(6):2089–106. [PubMed: 2415663]
43. Cartron G, Dacheux L, Salles G, Solal-Celigny P, Bardos P, Colombat P, Watier H. *Blood.* 2002; 99(3):754–8. [PubMed: 11806974]
44. Keating GM. *Drugs.* 2010; 70(11):1445–76. [PubMed: 20614951]
45. Spatz JP, Mossmer S, Hartmann C, Moller M, Herzog T, Krieger M, Boyen HG, Ziemann P, Kabius B. *Langmuir.* 2000; 16(2):407–415.
46. Cavalcanti-Adam EA, Bezler M, Tomakidi P, Spatz JP. *J Bone Miner Res.* 2004; 19:S64–S64.
47. Ranzinger J, Krippner-Heidenreich A, Haraszti T, Bock E, Tepperink J, Spatz JP, Scheurich P. *Nano Lett.* 2009; 9(12):4240–4245. [PubMed: 19772290]
48. Wolfram T, Spatz JP, Burgess RW. *Bmc Cell Biol.* 2008; 9:64. [PubMed: 19055842]
49. Muth CA, Steinel C, Klein G, Lee-Thedieck C. *PloS one.* 2013; 8(2)
50. Cherniavskaya O, Chen CJ, Heller E, Sun E, Provezano J, Kam L, Hone J, Sheetz MP, Wind SJ. *J Vac Sci Technol, B.* 2005; 23(6):2972–2978.
51. Wolfram T, Belz F, Schoen T, Spatz JP. *Biointerphases.* 2007; 2(1):44–48. [PubMed: 20408635]
52. Arnett KL, Harrison SC, Wiley DC. *Proc Natl Acad Sci USA.* 2004; 101(46):16268–73. [PubMed: 15534202]
53. Bain CD, Biebuyck HA, Whitesides GM. *Langmuir.* 1989; 5(3):723–727.
54. Huang NP, Michel R, Voros J, Textor M, Hofer R, Rossi A, Elbert DL, Hubbell JA, Spencer ND. *Langmuir.* 2001; 17(2):489–498.
55. Heuberger M, Drobek T, Spencer ND. *Biophys J.* 2005; 88(1):495–504. [PubMed: 15501935]
56. Huang NP, Voros J, De Paul SM, Textor M, Spencer ND. *Langmuir.* 2002; 18(1):220–230.
57. Depoil D, Zaru R, Guiraud M, Chauveau A, Harriague J, Bismuth G, Utzny C, Muller S, Valitutti S. *Immunity.* 2005; 22(2):185–94. [PubMed: 15723807]
58. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. *Nature.* 1999; 401(6754):708–12. [PubMed: 10537110]
59. James JR, White SS, Clarke RW, Johansen AM, Dunne PD, Sleep DL, Fitzgerald WJ, Davis SJ, Klenerman D. *Proc Natl Acad Sci USA.* 2007; 104(45):17662–7. [PubMed: 17971442]
60. Fahmy TM, Bieler JG, Edidin M, Schneck JP. *Immunity.* 2001; 14(2):135–43. [PubMed: 11239446]
61. Schamel WW, Arechaga I, Risueno RM, van Santen HM, Cabezas P, Risco C, Valpuesta JM, Alarcon B. *J Exp Med.* 2005; 202(4):493–503. [PubMed: 16087711]
62. Tian T, Plowman SJ, Parton RG, Kloog Y, Hancock JF. *Biophys J.* 2010; 99(2):534–43. [PubMed: 20643072]
63. Sharma P, Varma R, Sarasij RC, Ira, Gousset K, Krishnamoorthy G, Rao M, Mayor S. *Cell.* 2004; 116(4):577–89. [PubMed: 14980224]
64. Cochran JR, Cameron TO, Stone JD, Lubetsky JB, Stern LJ. *J Biol Chem.* 2001; 276(30):28068–28074. [PubMed: 11384988]
65. O'Donoghue GP, Pielak RM, Smoligovets AA, Lin JJ, Groves JT. *e-Life.* 2013; 2:e00778. [PubMed: 23840928]
66. Irvine DJ, Purbhoo MA, Krogsgaard M, Davis MM. *Nature.* 2002; 419(6909):845–9. [PubMed: 12397360]
67. Sun PD. *Immunol Res.* 2003; 27(2–3):539–48. [PubMed: 12857997]
68. Platzman I, Janiesch JW, Spatz JP. *J Am Chem Soc.* 2013; 135(9):3339–42. [PubMed: 23419177]

Figure 1. Experimental setup

(A) Manufacture of gold nanoparticle array by block copolymer micellar nanolithography. (B) Nanoarray biofunctionalization with NK cell and T cell-stimulating ligands. Protein structures and nanoparticles are to scale (for 25 nm-spaced array). (C) Scanning electron micrograph of gold nanosphere array (25 nm spacing, 3D representation of secondary electron image), with T cell-binding molecules on top (to scale in XY plane).

Figure 2. Nanoscale spacing of anti-CD3 ligand nanoarrays controls early stage T cell activation signalling

(A) Representative cells stimulated by nanoarrays presenting UCHT-1 F(ab')₂ fragments in the presence of background ICAM-1, with interparticle spacings 25 nm and 104 nm respectively: brightfield images (scale bar 5 μm) and TIRF-immunofluorescence of phosphotyrosine (3D representation). (B) Plots show intensity of TIRF-immunofluorescence of phosphotyrosine after 5 minutes stimulation, as a function of nanoarray spacing. (Left) The mean of the phosphotyrosine intensity normalized to the 25 nm intensity for each donor and then averaged across 4 donors. (Only conditions with > 25 cells were included. + No error bar as normalized to 1 by definition, ++ No error bar as only one donor with > 25 cells.) (Right) Individual cells shown as dots; central line shows the median, boxes the 25th to 75th percentile and whiskers the upper and lower inner fence values. *** = p<0.001, ** = p<0.01 (Wilcoxon rank sum test). (C) Number of adhered cells per unit area as a function of spacing. (D) Fraction of adhered cells that are naïve (CD45RA positive).

Figure 3. Nanoscale spacing of CD16-binding ligands controls NK cell responsiveness

Plots show NK cell contact area after 6 minutes of cells stimulated on nanoarrays functionalized with anti-CD16 mAb (A) and Rituximab (B). (Left) solid bars show the mean and error bars the standard error in the mean of cell spread area, averaged for each donor and then across donors. Data are normalized to PLL for each donor to enable comparison, with significance determined using a non-parametric Mann-Whitney test: *** $p < 0.001$. (Right) Donor by donor comparison of the cell-nanoarray contact area at 25 nm and 104 nm: lines indicate data points from the same donor.