Published in final edited form as:

J Mater Chem B Mater Biol Med. 2017 June 21; 5(23): 4410–4415. doi:10.1039/C7TB00150A.

Janus Nanoparticles for T Cell Activation: Clustering Ligands to Enhance Stimulation

Kwahun Lee and Yan Yu*

Department of Chemistry, Indiana University, Bloomington, IN

Abstract

The *in vitro* activation of T cells by synthetic particles is a promising technique for adoptive cancer immunotherapy. While it is known that cell-surface receptors form clusters during T cell activation, the use of clustered ligands on synthetic particles to modulate T cell response is a largely unexplored concept. Building upon our previous finding that T cells respond differently to various micro-sized patterns of ligands, we here investigate the effect of nano-sized ligand clusters on T cell activation. Two-faced Janus nanoparticles were fabricated to display ligands of different functions in spatially segregated clusters on single nanoparticles. Going beyond our earlier qualitative study, here we precisely quantified and controlled the surface density and the total amount of ligands on single nanoparticles. We show that nanoparticles with clustered ligands activate T cells to a greater level than ones uniformly coated with the same number of ligands. The enhanced effect is due to increased local surface density of ligands. The results demonstrate that the spatial arrangement of ligands on particles influences activation response of T cells and may be used as a new strategy to increase T cell stimulation in the presence of insufficient amount of stimuli. This fundamental study also represents an initial step in using nanoscale Janus particles for manipulating immune cell responses.

TOC image

Janus nanoparticles displaying clustered ligands enhance T cell activation by increasing local surface density of ligand stimuli

*Corresponding author: yy33@indiana.edu.

Notes

The authors declare no competing financial interest.

The design of synthetic particles for biomedical applications is often inspired by the ways that cells interact with their environment. A number of particle properties, including size,^{1, 2} shape,²⁻⁴ and mechanical stiffness,^{5, 6} have been used as design parameters to achieve particle-cell interactions that mimic those occurring in natural settings. As our fundamental understanding of cells grows, more features critical to cell function have been discovered, but many have not yet been incorporated into the design of therapeutic particles. The spatial presentation of ligands on cell membranes is one such feature. It is well known that the response of a T cell to external stimuli is determined by not only the individual ligand-receptor recognition, but also collective interactions between many simultaneous binding events. Thousands of ligand-receptor pairs form clusters on cell surfaces. These clustering interactions profoundly influence the multivalent binding kinetics and subsequent signal transduction.^{7, 8} The significance of using the spatial presentation of ligands on biomaterials to control cell behavior has been demonstrated by advances in tissue engineering, in which two-dimensional patterns of ligands are widely used to regulate cell adhesion, proliferation, and differentiation.^{9, 10} However, this approach is still largely absent in the design of nanoparticles for controlling cell functions. This is likely due to the technical challenge of fabricating and controlling clusters of biomolecules on particle surfaces.

Here we explore the role of ligand clustering in nanoparticle-induced T cell stimulation. T cells are a subtype of white blood cells that, in normal functions, are activated by “foreign” signals presented on infected cells. The activation of T cells triggers additional immune responses such as cytokine-mediated cell killing. In the vicinity of a cancerous tumor, T cell functions are suppressed. Thus, a promising approach for treating cancer is to re-activate the patient’s T cells in culture flasks and then re-infuse them back to the patient’s body to fight against tumors. Because of the difficulties of using natural cells for the *in vitro* T cell activation, synthetic particles displaying stimulatory signals have been developed as an alternative.^{11, 12} But, in many cases, particles that are randomly coated with ligands show limited effectiveness because they do not interact with T cells in the same way that natural cells do.¹³ To make particles better mimic cells, efforts have been devoted to presenting stimulatory ligands on their surfaces that resemble one or more aspects of natural cell surfaces. The surfaces investigated include lipid vesicles,¹⁴ ellipsoidal particles,¹⁵ carbon nanotubes,^{16, 17} and long-chain polymers.¹⁸ Those strategies have shown improved efficacy in T cell activation. Our group has recently reported a different approach in which stimulatory ligands were patterned on micron-sized particles to mimic the “bull’s eye” pattern of receptors formed on the surface of a T cell upon activation.¹⁹ We have shown that the activation response of T cells can be influenced by the ligand pattern they interact with. This promising initial study highlights the possibility of using clustered ligands on particles to modulate T cell activation.

In this study, we investigate how nano-sized particles with clustered ligands activate T cells. Unlike our previous work on microparticles, this study is focused on the effect of nanoscale clustering of ligands. More importantly, we quantitatively elucidate the working mechanism by which the spatial arrangement of ligands affects the response of T cells. To create nanoscale clusters of ligands on single particles, we use Janus nanoparticles. These nanoparticles have different surface functionalities on opposite hemispheres, allowing ligands of different functions to be tethered on spatially segregated patches.^{20, 21} By

precisely measuring and controlling the exact number and surface density of ligands on the nanoparticles, we show quantitatively that Janus nanoparticles with clustered ligands activate T cells more efficiently than particles coated uniformly with the same amount of ligands. Ligand clustering enhances T cell activation by increasing the local density of stimuli. This study demonstrates that by manipulating the spatial arrangement of ligands on particles, one can enhance T cell activation at ligand concentrations that would otherwise be so low to only weakly stimulate T cells.

In our experiments, we first sought to create 500-nm Janus nanoparticles that display clusters of two different types of ligands for T cell activation. The stimulatory ligand is anti-CD3 antibody, which crosslinks the CD3 subunits in T cell receptor complexes to initiate T cell stimulation signaling. Anti-CD28 antibody was used as a co-stimulatory ligand. It binds the CD28 co-receptors on T cell surfaces to provide an additional signal required for optimal T cell activation and proliferation. To create clusters of ligands on each Janus nanoparticle, both types of ligands were selectively attached onto spatially separated patches by using a combination of microcontact printing and a protect-and-conjugate strategy (Figure 1a). In the microcontact printing procedure, a thin piece of polydimethylsiloxane (PDMS) elastomer was “inked” with biotinylated bovine serum albumin (BSA) and then pressed against a monolayer of azide-functionalized silica nanoparticles to transfer biotin-BSA onto the particle surface. When the PDMS elastomer was lifted off, the nanoparticles remained partially embedded, leaving the exposed surface of the particles available for click chemistry with alkyne-tagged anti-CD28. The anti-CD3 antibodies were conjugated onto the patch of biotin-BSA via streptavidin linkage at the final step of fabrication. The final bifunctional Janus nanoparticles displayed the stimulatory ligand anti-CD3 and co-stimulatory ligand anti-CD28 in two spatially segregated domains, as shown in super-resolution fluorescence images obtained using structured illumination microscopy (SIM) (Figure 1b). Mixing of the two ligands was negligible, confirming that the PDMS elastomer effectively protected the embedded surface of particles from the click chemistry reaction.

Besides the clustered morphology of ligands, we also sought to control the patch size of anti-CD3. Previous studies have shown that the size of T cell receptor clusters on activated T cells ranges from tens of nanometers to about 500 nm.²² Therefore, we intended to render the cluster size of anti-CD3 on the Janus nanoparticles to be on the scale of 100–200 nm, comparable to that of clusters in natural T cells. This was achieved by tuning the stiffness of the PDMS elastomer and the stamping pressure – softer elastomer and higher stamping pressure lead to larger size of the BSA-biotin patch transferred from the PDMS elastomer to the silica nanoparticles. In this study, we chose a stamping pressure of 1.5×10^4 Pa and elastomers made from monolayers and crosslinking agent at 10:1 ratio. With those conditions, we created anti-CD3 clusters that were (347 ± 31) nm in arc diameter, corresponding to an average surface coverage of $(0.11 \pm 0.02) \mu\text{m}^2$ on the 500-nm nanoparticles (Figure 1c). We further measured the surface density of ligands on the Janus nanoparticles to be (180 ± 4) anti-CD3/ μm^2 and (224 ± 15) anti-CD28/ μm^2 , by using a quantitative fluorescence method (see SI Experimental Section).

We next investigated the ability of bifunctional anti-CD3/anti-CD28 Janus nanoparticles to activate Jurkat T cells. We used intracellular calcium elevation as a quantitative measure of T

cell activation (Figure 2a). It is known that T cell activation leads to rapid influx of calcium ions into the cell cytosol from both the endoplasmic reticulum (ER) and the extracellular solution.^{23, 24} The amplitude and duration of the calcium influx in T cell cytosol is a direct indicator of the strength and persistence of T cell activation; the more prominent and sustained the calcium elevation, the stronger is a T cell activation. We quantified the elevation of calcium ion concentrations in single T cells by using a calcium indicator, Fluo-4 acetoxymethyl ester (AM). The fluorescence emission of this dye increases dramatically upon binding to Ca^{2+} without shift in the emission wavelength, so its fluorescence intensity is directly proportional to the intracellular Ca^{2+} concentration. The calcium response of > 60 T cells upon their stimulation by the Janus nanoparticles is shown in Figure 2b. In this figure, each color-coded horizontal bar indicates how the calcium concentration in a single T cell fluctuates as a function of time. The calcium concentration in most T cells peaked within a few to ten minutes before gradually decaying to a lower level, which is characteristic of a typical T cell activation. It is also evident from the plot that the calcium response from each cell differs in many aspects, including the timing of the calcium peaks, peak amplitude, and duration of calcium response. Such cell-to-cell variation of calcium response is a known characteristic of T cell activation^{14,43} and may be further augmented here by the various time points when each T cell made contact with the Janus nanoparticles. It is important to point out that because the T cell activation might depend on the number of nanoparticles they interact with, we kept a low particle-to-cell number ratio in all T cell activation experiments, such that each T cell interacted with only one nanoparticle or none. Cells not in contact with any nanoparticles were not included in the calcium plot.

The chief question we sought to answer was: What is the effect of ligand clustering (the Janus geometry) on T cell response? In other words, how does the T cell stimulation by particles with clustered ligands compare with that by control particles with randomly mixed ligands? To answer this question, we created 500-nm particles that were uniformly coated with a mixture of anti-CD3 and anti-CD28 as the control. To facilitate direct comparison, we carefully controlled the surface density of both ligands such that the uniform particles had the same amount of both ligands as the Janus nanoparticles. Uniform particles are referred to as uniform-number particles (U#) for simplicity. The surface density of both ligands on those particles were (25 ± 2) anti-CD3/ μm^2 and (194 ± 18) anti-CD28/ μm^2 (Table 1, experimental details in SI). The calcium response plot shows that T cells stimulated by the Janus nanoparticles exhibited an overall higher level of activation compared to those stimulated by the uniform-number particles, as indicated by the greater number of T cells showing activation signals above the threshold as well as the higher intensities of calcium peaks (Figure 2b). We defined two parameters to quantify these differences in the calcium response, following a method reported previously.²⁵ One parameter is average fluorescence amplitude, defined as the time-average of normalized fluorescence intensities on the entire calcium plot for each T cell. It quantifies the average intensity of a calcium response. The other is response fraction. It was defined as the fraction of time when the normalized fluorescence intensity of a T cell is greater than the threshold and used to quantify the persistence of a calcium response. Results from both parameters reveal that T cell activation by Janus nanoparticles is overall stronger and more sustained than that by uniform-number particles (Figure 2c, $P < 0.01$ for analyses of both parameters).

We hypothesized that Janus nanoparticles enhance T cell activation by providing a higher local density of ligands. To test this hypothesis, we created 500-nm particles uniformly coated with anti-CD3 and anti-CD28 at the same surface density as on Janus nanoparticles (Table 1). These particles are referred to as “uniform-density” (Ud) particles for brevity. Comparing the calcium response of T cells stimulated by the uniform-density particles to that by Janus nanoparticles, we observed that both types of particles activate T cells to a similar level ($P > 0.4$) both in intensity (indicated by the average fluorescence amplitude) and in persistence (indicated by the response fraction) (Figure 2c). Both activate T cells better than the uniform-number particles that have lower surface density of ligands. The results demonstrate that surface density of ligands plays a more important role in T cell activation than the total number of ligands in this experimental system. By clustering ligands into segregated patches, Janus nanoparticles activate T cells as efficiently as uniform particles that have 7 times more ligands.

Because CD28 co-stimulation enhances signals from TCRs,²⁶ we next investigated the extent to which the presence of anti-CD28 affects the Janus nanoparticle-induced T cell activation. We first confirmed in control experiments that anti-CD28 alone does not effectively activate T cells, as Janus nanoparticles with only a cluster of anti-CD3 activated T cells to a significantly greater level than particles with only a cluster of anti-CD28 (Figure S1). We then compared T cell activation by three types of anti-CD3 particles without anti-CD28: Janus nanoparticles with only a patch of anti-CD3, particles uniformly coated with the same amount of anti-CD3, and particles uniformly coated with the same density of anti-CD3 (Figure 3). We observed that both Janus and uniform-density particles, which have the same ligand density, led to more sustained T cell activation than the uniform-number particles. However, all three types of particles, without anti-CD28 co-stimulation, activate T cells to the same intensity, as no difference in calcium peak amplitudes was observed. This agrees with previous findings that CD28 co-stimulation enhances the intensity but not duration of the calcium response.²⁷ The result shows that ligand clustering enhances T cell activation and this effect is more prominent in the presence of CD28 co-stimulation.

In conclusion, our results demonstrate that clustering ligands on nanoparticles enhances T cell activation by increasing local surface density of ligand stimuli. This effect is augmented by the presence of CD28 co-stimulation. Previous studies have reported contradictory conclusions regarding the roles of the ligand density and the number of ligands in T cell activation.^{28–30} Some study suggested that T cell activation depends on the density of ligands,²⁹ while others argued that the number of ligands presented is more important for T cell activation than local ligand density.^{28, 30} It is currently unclear what causes such discrepancy. Our results here show that T cell activation by synthetic particles is dependent on the local density of ligands. This study suggests that in the case of weak T cell activation by an insufficient amount of ligands, one can enhance the cell response by clustering ligands into concentrated domains on particles. We have reported previously that T cells respond differently to various micron-sized patterns of ligands. The underlying mechanism was that the spatial organization of ligands on particles changes the large-scale distribution of signaling proteins inside cells and thus affects the cell response. The seemingly different working mechanisms for micro- and nano-scaled particles in T cell activation might be due to the fact that the size of receptor clusters on T cell surfaces increases as T cell activation

proceeds for different functions. Receptors form nanoclusters in the beginning of T cell activation to initiate signals, but coalesce into microclusters at the end to terminate activation.³¹ Regardless of the different mechanisms, results from both studies evidently demonstrate that the spatial arrangement of ligands on synthetic particles, either nano- or micro-sized, influences activation response of T cells. This fundamental understanding provides the foundation for future work that will explore the development of nanoparticles with clustered ligands for *in vitro* T cell activation in more clinically relevant settings. While Janus nanoparticles have been developed for various biomedical applications,^{20, 21} this study represents a first step in using this unique particulate system for immune cell stimulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors gratefully thank Dr. Yi Yi at Indiana University for help with the surface functionalization of particles and helpful discussions, Dr. Stephen M. Anthony at Sandia National Laboratories for providing the Matlab algorithm for calcium analysis, and Dr. Jim Powers at the IUB Light Microscopy Imaging Center for assistance with instrument use. Fabrication and characterization of Janus particles were performed at the Nanoscale Characterization Facility at Indiana University. This study was supported by Indiana University, and the Indiana Clinical and Translational Sciences Institute, funded in part by grant #UL1 TR001108 from the National Institutes of Health, National Center for Advancing Translational Sciences, Clinical and Translational Sciences Award. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

1. Pacheco P, White D, Sulchek T. Plos One. 2013; 8:e60989. [PubMed: 23630577]
2. Doshi N, Mitragotri S. Plos One. 2010; 5:e10051. [PubMed: 20386614]
3. Herd H, Daum N, Jones AT, Huwer H, Ghandehari H, Lehr C-M. ACS Nano. 2013; 7:1961–1973. [PubMed: 23402533]
4. Champion JA, Mitragotri S. Proc Natl Acad Sci U S A. 2006; 103:4930–4934. [PubMed: 16549762]
5. Merkel TJ, Jones SW, Herlihy KP, Kersey FR, Shields AR, Napier M, Luft JC, Wu H, Zamboni WC, Wang AZ, Bear JE, DeSimone JM. Proc Natl Acad Sci U S A. 2011; 108:586–591. [PubMed: 21220299]
6. Zhang L, Cao Z, Li Y, Ella-Menye JR, Bai T, Jiang S. ACS Nano. 2012; 6:6681–6686. [PubMed: 22830983]
7. Irvine DJ, Hue KA, Mayes AM, Griffith LG. Biophys J. 2002; 82:120–132. [PubMed: 11751301]
8. Sharma P, Varma R, Sarasij R, Gousset K, Krishnamoorthy G, Rao M, Mayor S. Cell. 2004; 116:577–589. [PubMed: 14980224]
9. Lutolf M, Hubbell J. Nat Biotechnol. 2005; 23:47–55. [PubMed: 15637621]
10. Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE. Science. 1997; 276:1425–1428. [PubMed: 9162012]
11. van der Weijden J, Paulis LE, Verdoes M, van Hest JCM, Figdor CG. Chem Sci. 2014; 5:3355–3367.
12. Perica K, De León Medero A, Durai M, Chiu YL, Bieler JG, Sibener L, Niemöller M, Assenmacher M, Richter A, Edidin M, Oelke M, Schneck J. Nanomedicine. 2014; 10:119–129. [PubMed: 23891987]
13. Deeths MJ, Mescher MF. Eur J Immunol. 1997; 27:598–608. [PubMed: 9079798]
14. De La Peña H, Madrigal JA, Rusakiewicz S, Bencsik M, Cave GWV, Selman A, Rees RC, Travers PJ, Dodi IA. J Immunol Methods. 2009; 344:121–132. [PubMed: 19345222]

15. Sunshine JC, Perica K, Schneck JP, Green JJ. *Biomaterials*. 2014; 35:269–277. [PubMed: 24099710]
16. Fadel TR, Look M, Staffier PA, Haller GL, Pfefferle LD, Fahmy TM. *Langmuir*. 2009; 26:5645–5654.
17. Fadel TR, Sharp FA, Vudattu N, Ragheb R, Garyu J, Kim D, Hong E, Li N, Haller GL, Pfefferle LD, Justesen S, Herold KC, Fahmy TM. *Nat Nanotechnol*. 2014; 9:639–647. [PubMed: 25086604]
18. Mandal S, Eksteen-Akeroyd ZH, Jacobs MJ, Hammink R, Koepf M, Lambeck AJA, van Hest JCM, Wilson CJ, Blank K, Figdor CG, Rowan AE. *Chem Sci*. 2013; 4:4168–4174.
19. Chen B, Jia Y, Gao Y, Sanchez L, Anthony SM, Yu Y. *ACS Appl Mater Interfaces*. 2014; 6:18435–18439. [PubMed: 25343426]
20. Hu SH, Gao X. *J Am Chem Soc*. 2010; 132:7234–7237. [PubMed: 20459132]
21. Byeon JH, Park JH. *Sci Rep*. 2016; 6:35104. [PubMed: 27713519]
22. Sherman E, Barr V, Samelson LE. *Immunol Rev*. 2013; 251:21–35. [PubMed: 23278738]
23. Lewis RS. *Annu Rev Immunol*. 2001; 19:497–521. [PubMed: 11244045]
24. Feske S. *Nat Rev Immunol*. 2007; 7:690–702. [PubMed: 17703229]
25. Salles A, Billaudeau C, Sergé A, Bernard A-M, Phélipot M-C, Bertaux N, Fallet M, Grenot P, Marguet D, He H-T, Hamon Y. *PLoS Comput Biol*. 2013; 9:e1003245. [PubMed: 24086124]
26. Yokosuka T, Saito T. *Immunol Rev*. 2009; 229:27–40. [PubMed: 19426213]
27. Michel F, Attal-Bonnefoy G, Mangino G, Mise-Omata S, Acuto O. *Immunity*. 2001; 15:935–945. [PubMed: 11754815]
28. Deeg J, Axmann M, Matic J, Liapis A, Depoil D, Afrose J, Curado S, Dustin ML, Spatz JP. *Nano Lett*. 2013; 13:5090–5097. [PubMed: 24111628]
29. Matic J, Deeg J, Scheffold A, Goldstein I, Spatz JP. *Nano Lett*. 2013; 13:5090–5097. [PubMed: 24111628]
30. Patrick SM, Kim S, Braunstein NS, Thomas JL, Leonard EF. *J Immunol Methods*. 2000; 241:97–108. [PubMed: 10915852]
31. Dustin ML, Groves JT. *Annu Rev Biophys*. 2012; 41:543–556. [PubMed: 22404679]

Figure 1.

Fabrication and characterization of Janus nanoparticles. (a) Silica particles (500 nm in diameter) were first functionalized with azide groups. A piece of PDMS elastomer “inked” with biotin-BSA (shown in blue) was pressed against a monolayer of azide-functionalized silica particles to transfer a patch of biotin-BSA proteins onto the particle surface. After lifting off the PDMS elastomer, particles remained embedded and their exposed surface was functionalized with alkyne-tagged anti-CD28 antibodies via click chemistry. After click chemistry, particles were sonicated off the PDMS and functionalized with biotinylated anti-CD3 antibody using biotin-streptavidin (SA) conjugation. (b) Super-resolution fluorescence images from structured illumination microscopy (SIM) show the spatial segregation of the anti-CD3 (shown in red) and anti-CD28 (shown in green) clusters. Scale bar: 500 nm. (c) Histogram showing the distribution of the size of anti-CD3 clusters ($N = 28$). The average surface coverage of anti-CD3 is $0.11 \pm 0.02 \mu\text{m}^2$ per particle.

Figure 2.

(a) A schematic illustration of the calcium response of T cells upon stimulation by three different types of nanoparticles: Janus particle (JP), uniform particle with the same number of ligands (U#), and uniform particles with the same density of ligands (Ud). (b) Color-coded plots show the calcium response of T cells stimulated by different types of nanoparticles as indicated. Each horizontal line indicates the calcium response of a single T cell as a function of time. The calcium response, measured as the fluorescence intensity of the calcium indicator Fluo-4, was normalized and color-coded on the same scale. (c) Quantification of T cell activation. Response fraction, defined as the fraction of time when the normalized fluorescence intensity of a T cell is greater than the threshold, quantifies the persistence of a calcium response. Average fluorescence amplitude, defined as the time-average of normalized fluorescence intensities on the entire calcium plot for each T cell, measures the average intensity of a calcium response. Both parameters extracted from results shown in (b) are plotted in a scatter plot together with a box plot. Each box plot indicates the median and the interquartile range from 25% to 75% of the corresponding data set. Statistical significance is highlighted by p-values (student's t test) as indicated.

Figure 3.

Calcium response of T cells stimulated by three types of nanoparticles coated only with anti-CD3. (a) Color-coded plots show the calcium response of T cells stimulated by different types of nanoparticles as indicated. (b) Quantification of T cell activation. Two parameters, response fraction and average fluorescence amplitude, extracted from results shown in (a) are plotted in a scatter plot together with a box plot. Each box plot indicates the median and the interquartile range from 25% to 75% of the corresponding data set. Statistical significance is highlighted by P-values (student's t test) as indicated.

Table 1

Surface density of anti-CD3 and anti-CD28 on three types of particles.

Type of particles				Janus particles (JP)		Uniform particles with same number (U#)		Uniform particles with same density (Ud)	
Density of anti-CD3 (molecules/ μm^2)				180 $\pm$ 4		25 $\pm$ 2		180 $\pm$ 19	
Density of anti-CD28 (molecules/ μm^2)				224 $\pm$ 15		194 $\pm$ 18		224 $\pm$ 21	