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Perspective

Functional DNA as a Molecular Tool in Regulating Immunoreceptor−Ligand Interactions

Lele Sun,*,○ Fengyun Shen,○ Yanfei Qu, and Zhuang Liu*

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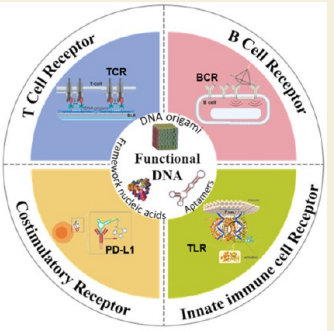
ABSTRACT: During immune responses, activating ligands would trigger dynamic spatiotemporal organization of immunoreceptors at the cell interface, governing the fate and effector functions of immune cells. To understand the biophysical mechanisms of immunoreceptor signaling, diverse tools, including DNA technologies, have been developed to manipulate receptor−ligand interactions during the immune activation process. With great capability in the controllable assembly of biomolecules, functional DNA-based precise arrangement of immune molecules at cell interfaces has provided a powerful means in revealing the principles of immunoreceptor triggering, even at the single-molecule level. In addition, precisely regulating immunoreceptor−ligand interactions with functional DNA has been applied in immunotherapies of major diseases. This Perspective will focus on the recent advances in exploring immunoreceptor signaling with functional DNA as the molecular tool as well as the applications of functional DNA mediated regulation of immunoreceptor activation. We also outline the challenges and opportunities of applying functional DNA in immune modulation and immunotherapy.



KEYWORDS: *DNA origami, Aptamer, T-cell receptor, B-cell receptor, Costimulatory receptor*

INTRODUCTION

Immunoreceptor−ligand interactions are crucial to the immunopathological process from pathogen recognition to target cell killing by immune effector cells. These interactions entail specific binding of immunoreceptors, such as T-cell receptors (TCRs), B-cell receptors (BCRs), and costimulatory receptors, with their corresponding ligands, such as peptide-major histocompatibility complexes (pMHCs), epitope peptides, and costimulatory ligands.[1−6] The dynamic spatiotemporal organization of immunoreceptors induced by their ligands is critical in modulating receptor-mediated signaling, thereby determining immune cell activation and differentiation pathways.[5,7−10] For instance, in the context of dendritic cell (DC) and T-cell interaction, immune synapse formation, which involves dynamic binding between pMHCs and TCRs, as well as costimulatory receptors and their ligands, is essential for signaling maintenance and T-cell activation.[11] Dysregulation of immunoreceptor signaling can result in various diseases such as cancer and autoimmune diseases.[12] Consequently, elucidating the biophysical mechanisms of immunoreceptor signaling and developing advanced tools to regulate immunoreceptor−ligand interactions are of paramount importance for developing immunotherapies for major diseases.

Artificially manipulating the interaction between receptors and ligands is necessary to reveal the biophysical mechanisms underlying immunoreceptor activation. In this regard, micropatterned or nanopatterned arrays of T-cell activating ligands based on microfabrication techniques have been used earlier to investigate the spatial parameters that affect receptor-mediated signaling on T-cells, including the spacing and distribution morphology of a single type of ligands, as well as the location of different types of ligands.[13−17] However, these microfabricated arrays on solid substrate cannot work on the soft live cell membrane, thus being limited in exploring the mechanism of receptor−ligand interaction at the real immune cell−cell interface and in the application of immunomodulatory therapies. Moreover, it is hard for homogeneous chemical modification on microfabricated surfaces to control the spatial organization of different types of ligands at single molecular level, but this might be valuable, because multiple receptor−ligand interactions working in tandem are required at the immune cell−cell interface.

DNA was initially considered solely as a genetic information carrier, however, in the last three decades, the great programmability of DNA has made it a multifaceted and manipulable molecular tool capable of executing intricate and exacting functions.[18−24] At present, there exist diverse approaches to endow DNA with functional capacity. In particular, the advent of structural DNA nanotechnology in

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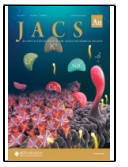

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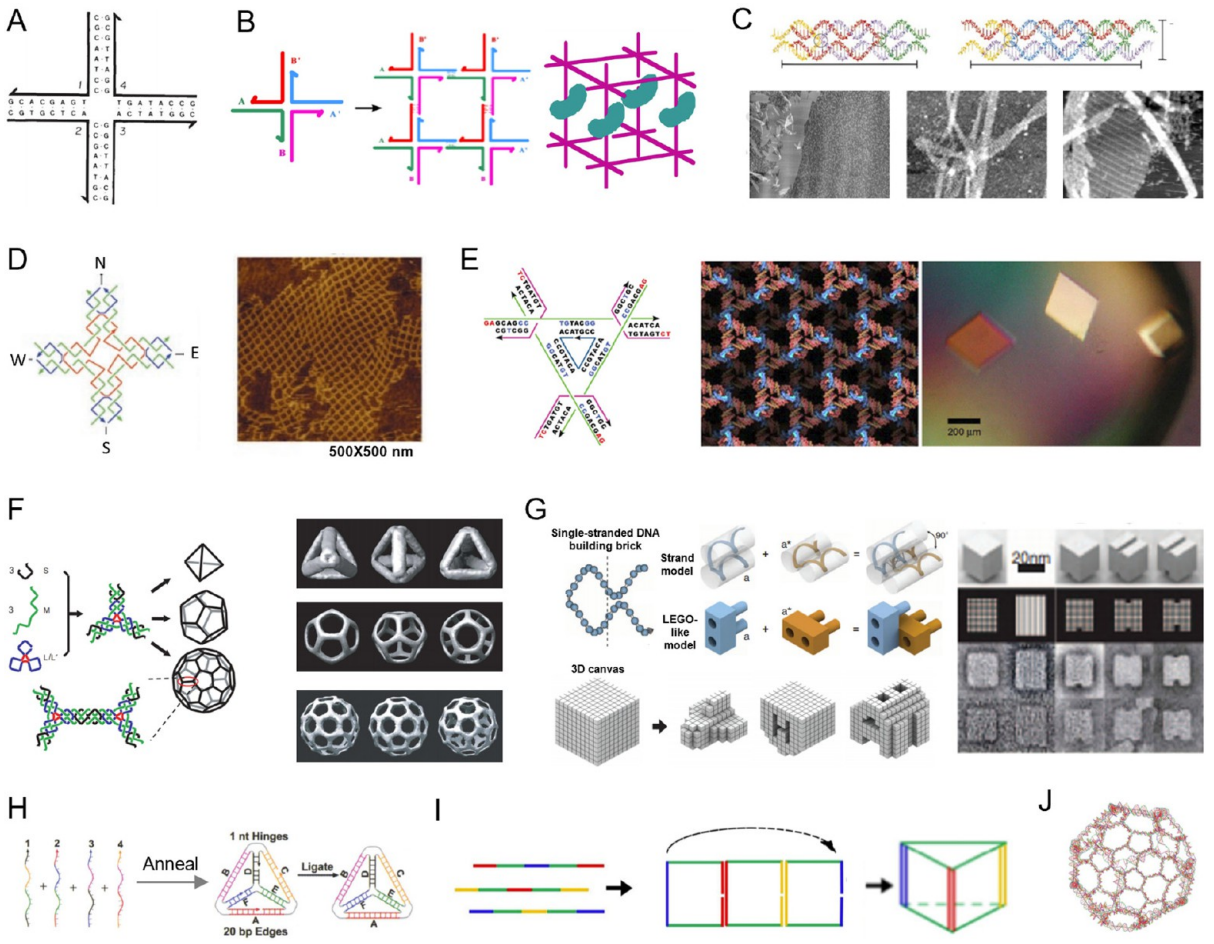

Figure 1. (A) Schematic of an immobile cross DNA junction. Reproduced with permission from ref 25. Copyright 1983 Springer Nature. (B) 2D DNA lattice self-assembled from immobile cross DNA junctions and 3D DNA scaffold (purple) for organizing macromolecules (green). Reproduced from ref 49. Copyright 2019 American Chemical Society. (C) 2D crystalline self-assembled from synthetic DNA double-crossover molecules. Reproduced with permission from ref 52. Copyright 1998 Springer Nature. (D) 2D DNA nanogrids self-assembled from four-arm tiles. Reproduced with permission from ref 53. Copyright 2003 The American Association for the Advancement of Science. (E) Schematic of a DNA tensegrity triangle and an image of crystals formed by the tensegrity triangle. Reproduced with permission from ref 54. Copyright 2009 Springer Nature. (F) Schematic of hierarchical symmetric DNA supramolecular polyhedral. Reproduced with permission from ref 55. Copyright 2008 Springer Nature. (G) Schematic of 3D structures self-assembled from DNA bricks. Reproduced with permission from ref 56. Copyright 2012 The American Association for the Advancement of Science. (H–J) Schematic of DNA tetrahedrons, DNA triangular prism, and DNA soccer ball. Reproduced with permission from refs 57, 59, and 60, respectively. Copyright 2005 The American Association for the Advancement of Science; Copyright 2018 American Chemical Society; Copyright 2009 Oxford University Press.

the 1980s facilitated the use of DNA to construct nanoscale objects with precise control of size and geometry,[25−31] which lays the structural foundation for the applications of DNA in many fields. In addition, the emergence of nucleic acid aptamer technology has endowed DNA with recognition attributes akin to antibodies, thus conferring upon it the capacity to selectively bind to small molecules, proteins, and even cells.[32−39] Furthermore, the burgeoning refinement of DNA synthesis and modification methodologies has further empowered DNA molecules to be conjugated with diverse functional moieties, which has been instrumental in facilitating their widespread application. For instance, the attachment of lipid molecules to one end of DNA expedites their facile integration into cellular membranes,[40−42] thereby ushering in a plethora of biological applications. The integration of the above approaches equips DNA with an unparalleled ability to precisely assemble molecules with stoichiometric accuracy and regulate the spatial distribution parameters of these molecules.[43−45] Consequently, functional DNA has engendered novel paradigms in the exploration of immunoreceptor−ligand interactions[46] and holds immense potential for immune engineering applications.

In this Perspective, we aim to highlight the latest progress in the field of functional DNA as a versatile molecular tool for regulating immune receptor−ligand interactions, including studies on the activation mechanisms of immunoreceptors as well as the applications of functional DNA-based regulation of immunoreceptor activation. Initially, we provide a concise overview of the evolution of functional DNA. Subsequently, we elucidate the intricate relationship between immunoreceptor signaling and major diseases and review recent reports on the cutting-edge applications of functional DNA in the study of immunoreceptor−ligand interactions and immunotherapies. Finally, we identify the challenges and opportunities for future research, outlining a roadmap toward the further development of this exciting field.

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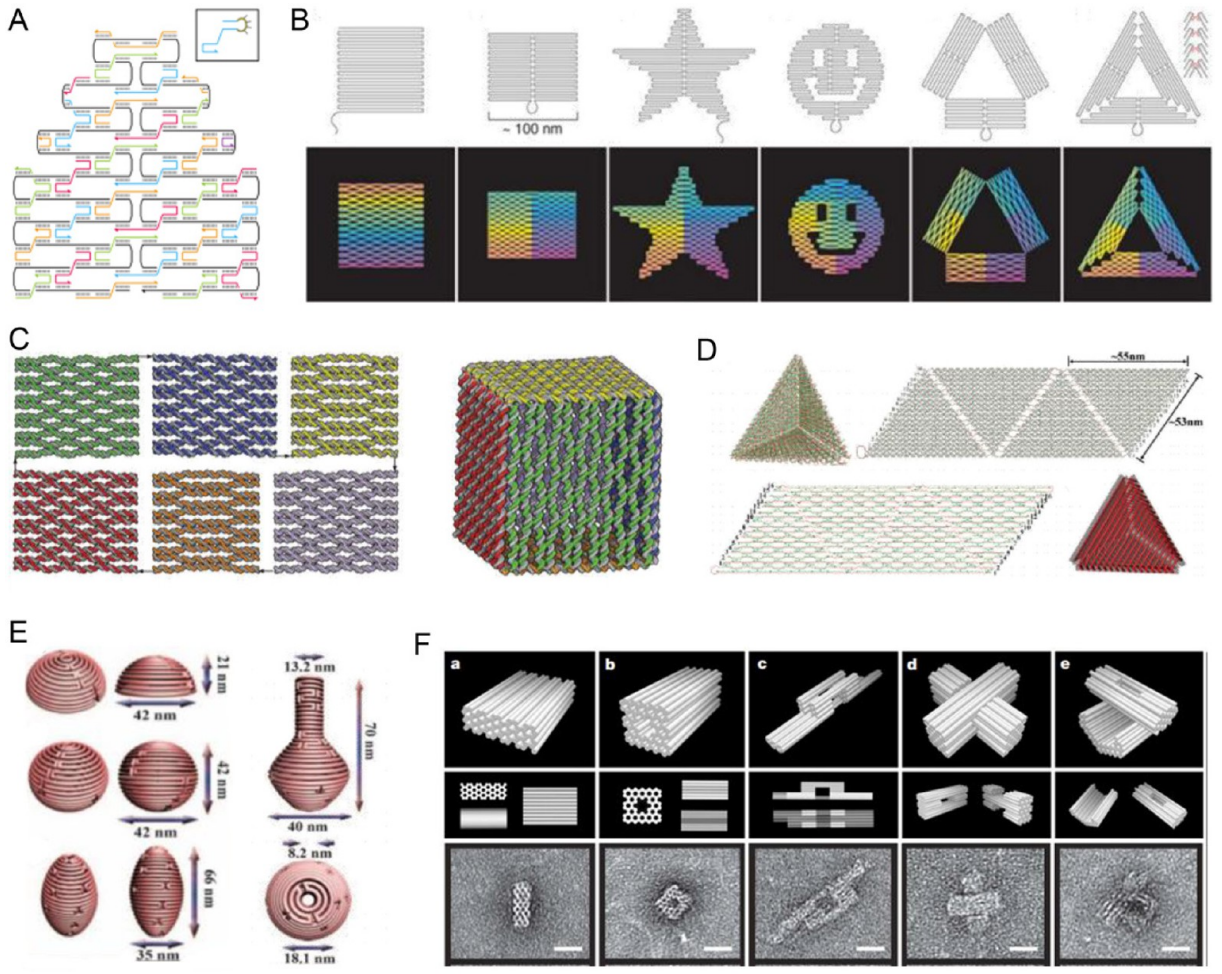

Figure 2. (A) Schematic of the principle of DNA origami. Reproduced with permission from ref 61. Copyright 2006 Springer Nature. (B) Schematic of DNA origami shapes. Reproduced with permission from ref 61. Copyright 2006 Springer Nature. (C) A DNA box with a controllable lid. Reproduced with permission from ref 62. Copyright 2009 Springer Nature. (D) A DNA origami tetrahedron. Reproduced from ref 63. Copyright 2009 American Chemical Society. (E) 3D DNA origami with complex curvatures. Reproduced with permission from ref 64. Copyright 2011 The American Association for the Advancement of Science. (F) 3D DNA nanomechanical devices. Reproduced with permission from ref 65. Copyright 2009 Springer Nature.

FUNCTIONAL DNA

DNA was initially discovered as the carrier of genetic information in life. In 1953, Watson and Crick revealed the structure of double-stranded DNA, which is formed by the complementary pairing of two single-stranded DNA chains through base pairing (A−T, C−G) to form a double helix.[47] This discovery not only opened the door to molecular biology but also laid the theoretical foundation for the use of DNA as a functional material in later stages. It is apparent that, in addition to the specificity of hybridization reactions that can give DNA recognition functions, thereby being used in areas such as gene detection, DNA can also acquire additional functions in various other ways.

Structural DNA Nanotechnology

Structural DNA nanotechnology allows DNA molecules to form specific structures through self-assembly, thus having certain functions.[31] This technology originated from pioneering research work by Professor Seeman at New York University in the 1980s.[25,48] It fully utilized the specificity of DNA hybridization and the programmability of DNA molecules to construct specific morphologies of DNA nanostructures. Initially, inspired by the symmetry of Holliday junctions, Professor Seeman constructed an immobile cross structure composed of four interlocking single-stranded DNA chains (Figure 1A).[25] This was the first DNA nanostructure, and it was speculated that such structures could be assembled into regular 3D structures through sticky end connections (Figure 1B).[48,49] From this, the concept of structural DNA nanotechnology gradually formed and matured, enabling the manufacture of a series of regular 2D DNA arrays or 3D DNA nanostructures.[50,51] For instance, by using small DNA unit structures, including DNA tiles proposed by Seeman and DNA bricks proposed by Yin et al., DNA structures with boundaries (Figure 1C−E) and without boundaries have been constructed (Figure 1F,G).[52−56] In addition, single-stranded DNA can also be used as a structural unit to construct various boundary polyhedron structures, including simple polyhedrons, such as DNA tetrahedrons (Figure 1H),[57] DNA cubes,[58] DNA triangular prism (Figure 1I),[59] and a DNA soccer ball structure (Figure 1J).[60] Periodic 2D and 3D DNA crystals are theoretically boundaryless and can reach sizes of hundreds of micrometers,[54] while DNA nanostructures with certain shapes are bounded and have sizes ranging from a few nanometers to several hundred nanometers.[56]

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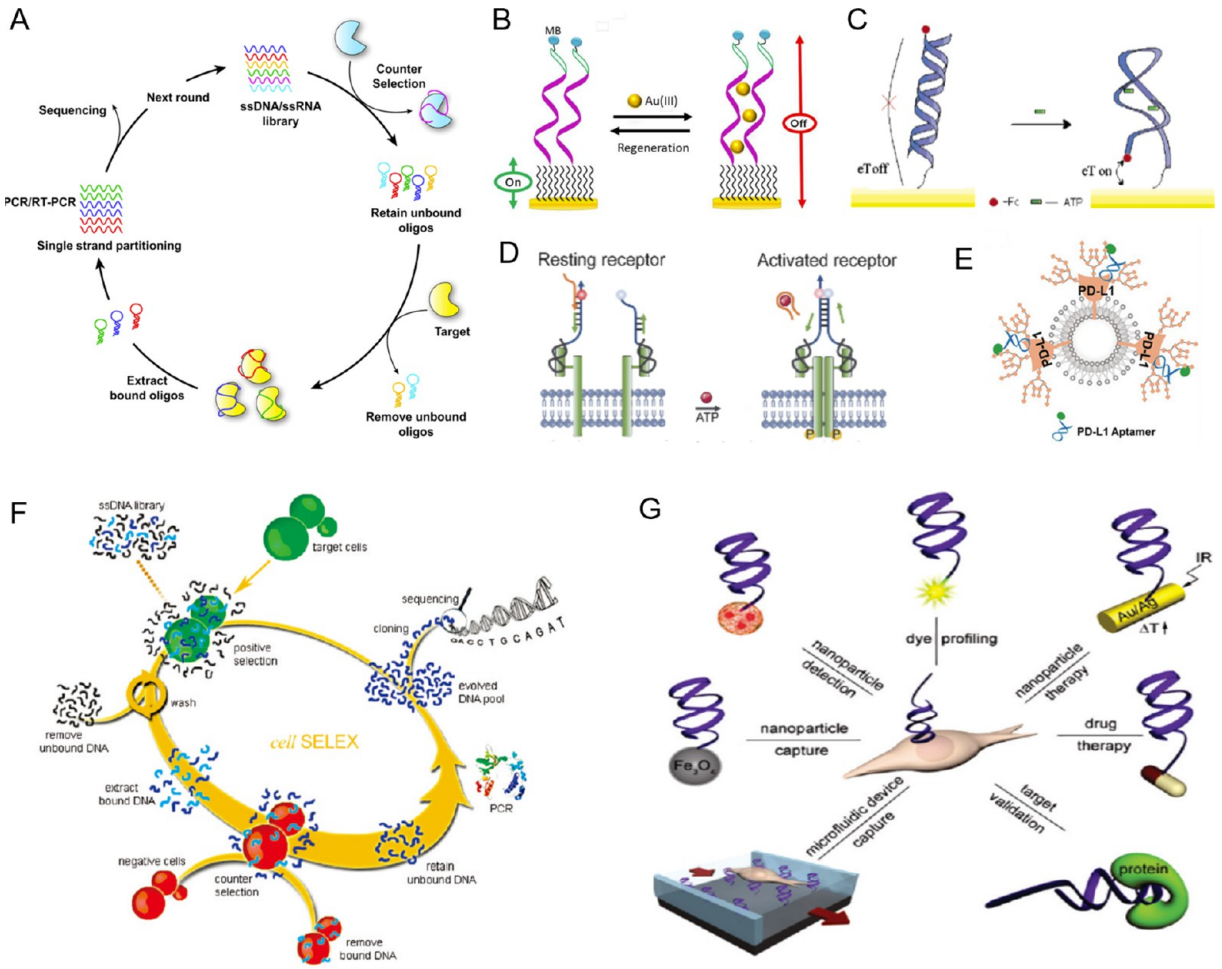

Figure 3. (A) Schematic of the process of SELEX. Reproduced from ref 70. Copyright 2021 American Chemical Society. (B–E) DNA aptamers which could recognize Au ions (B), ATP (C), and proteins (D, E). Reproduced with permission from refs 71–74, respectively. Copyright 2016 American Chemical Society; Copyright 2007 American Chemical Society; Copyright 2018 John Wiley and Sons. Copyright 2020 John Wiley and Sons. (F, G) Schematic of cell SELEX and the application of aptamers which can recognize cells. Reproduced from ref 33. Copyright 2010 American Chemical Society.

In 2006, Rothemund reported on DNA origami as another important development in structural DNA nanotechnology.[61] This technique involves the mixing of a long single-stranded DNA scaffold with approximately several hundred staple strands and then annealing them through thermal annealing to achieve high-yield DNA nanostructures (Figure 2A), including 2D triangular structure and 2D square structure as well as a plane five-pointed star structure (Figure 2B). The morphology of the DNA origami can be precisely controlled by designing the sequences of the staple strands. In addition to the above-mentioned 2D DNA origami, high-order 3D hollow boxes (Figure 2C),[62] hollow tetrahedron (Figure 2D),[63] and vases (Figure 2E)[64] as well as complex nanomechanical devices (Figure 2F)[65] have also been constructed, suggesting that DNA nanostructures of almost any shape can be fabricated.

Currently, DNA nanostructures with specific shapes and boundaries are more widely applied in practice. The addressability of DNA nanostructures is key to their functional applications. As each DNA strand that constitutes DNA nanostructures has a specific position, by extending one of these DNA strands with an overhand sequence, proteins, nanoparticles, and small molecules conjugated with DNA strands complementary to that overhand DNA can be precisely fixed to specific sites on the DNA nanostructures via DNA hybridizing. This feature can solve many problems that cannot be overcome by traditional physical and chemical methods, such as precise regulation of biosensing interfaces,[66,67] manipulation of molecular motion,[43] and controlling ligand spacing at single molecular level.[68]

Nucleic Acid Aptamer Technology

The nucleic acid aptamer technology, which originated in the 1990s,[32] is an alternative pathway for DNA to acquire functionality. Aptamers, also known as "chemical antibodies", are nucleotide fragments with recognition ability obtained by screening from a random library using the systematic evolution of ligands by exponential enrichment technology (SELEX) (Figure 3A).[32,69,70] Aptamers possess special secondary structures such as hairpins, stem-loops, pseudoknots, and G-quadruplexes, which enable them to form various tertiary structures that allow them to bind to target molecules with high affinity and specificity. Nowadays, DNA aptamers have been screened to recognize ions (Figure 3B),[71] small molecules (Figure 3C),[72] proteins (Figure 3D,E),[73,74] and even cells (Figure 3F,G).[33] This antibody-like function of DNA aptamers makes them widely used in analytical detection, targeted drug delivery, immunotherapy, and other fields.[70,75] For example, aptamers to programmed cell death 1 ligand (PD-L1) can be

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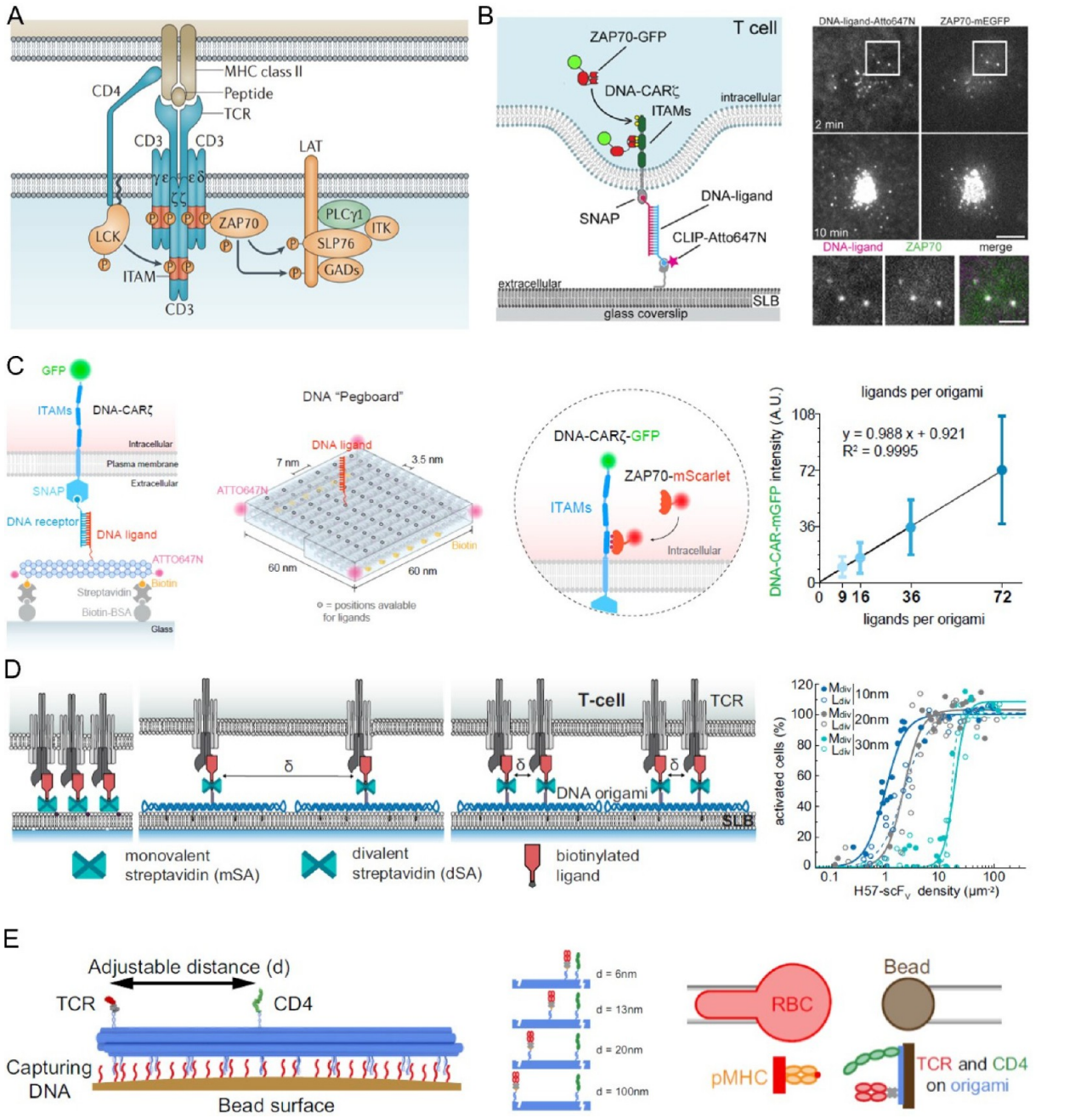

Figure 4. (A) Schematic of TCR priming. Reproduced with permission from ref 1. Copyright 2016 Springer Nature. (B) Schematic of the DNA-based TCR-ligand interaction system and microscopy images showing the formation of microclusters of ligand−receptor complexes. Reproduced with permission from ref 85. Copyright 2017 Elsevier. (C) Schematic of DNA origami patterning of synthetic T-cell signaling system and representative data showing the effect of ligand number per origami on TCR activation. Reproduced with permission from ref 88. Copyright 2021 Natl Acad Sciences. (D) Schematic of APC-mimicking biointerface by anchoring rectangular DNA origami functionalized with pMHCs on SLB and representative data showing the effect of ligand density on T-cell activation. Reproduced with permission from ref 90. Copyright 2021 Natl Acad Sciences. (E) Schematic of 10-helix bundle DNA origami nanorod to precisely control the arrangement and positioning of TCR and CD4 on the surface of beads. Reproduced with permission from ref 95. Copyright 2022 Springer Nature.

used in immunotherapy for cancer based on immune checkpoint blockade.[76]

Chemical Modification

By chemical modification at either the end or middle of the DNA strand, certain functions can also be conferred to DNA molecules. Thanks to the current mature DNA chemical synthesis and modification technology, many chemical groups can be conveniently linked to DNA, including chromophores, lipids, biotin, thiol, amino, azide (N3), diphenylbutadiyne (DBCO), and other cross-linkable chemical groups. Among them, the modification of chromophores is a necessary condition for DNA molecular probes to function.[77] In addition, by using the aforementioned chemical cross-linking groups, DNA molecules can be easily linked to protein molecules, nanoparticles, and drug molecules, enabling the precise spatial distribution of substances together with DNA nanostructures.[49] Furthermore, spherical nucleic acids are a

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type of functional DNA material formed by coupling DNA molecules to the surface of nanoparticles, such as gold nanoparticles and liposomes, at high density using chemical groups.[78] They have many applications in nucleic acid molecular imaging and drug delivery,[79] among others. It should also be emphasized that the coupling of lipid molecules with DNA enables DNA molecules to be easily integrated into the surface of living cells, thus facilitating cell surface engineering or regulation of cell interaction interfaces,[80] which is an increasing research direction in recent years.

REGULATING IMMUNORECEPTOR SIGNALING WITH FUNCTIONAL DNA

T-Cell Receptor

T-cell receptor (TCR) is an important component involved in adaptive immunity, playing a crucial role in recognizing and binding to specific antigenic peptides as well as eliminating pathogens or mutated cells. On one hand, dysregulated TCR signaling can result in autoimmunity, like multiple sclerosis, rheumatoid arthritis, and type 1 diabetes, where the immune system mistakenly attacks self-tissues.[81] On the other hand, enhancing TCR signaling can be exploited for cancer immunotherapy by redirecting T-cells to recognize and eliminate tumor cells.[82] However, the mechanism of TCR signaling remains controversial, and a deeper understanding of this undoubtedly has important implications for the development of immunotherapeutic strategies targeting T-cell-related major diseases.

The process of foreign antigen recognition by T-cells involves the binding of peptide-loaded MHC to the T-cell receptor (TCR), which leads to the phosphorylation of immunoreceptor tyrosine activation motifs (ITAMs) in the TCR zeta and associated CD3 chains by the Src family kinase Lck (Figure 4A).[1] TCR zeta associated protein kinase 70 kDa (ZAP70) is then recruited by phosphorylated ITAMs, which in turn phosphorylates the adaptor protein LAT (Linker for activation of T-cells). This initiates a cascade of downstream signaling pathways, including mitogen-activated protein kinases (MAPKs), actin polymerization, elevation of intracellular calcium, and changes in transcription. Although TCR shares similarities in signal transduction with many enzyme-linked receptors, TCR is unique in that it is presented with an immense number of different peptides loaded onto MHC molecules. To prevent an autoimmune response, mature T-cells must selectively activate in response to MHC loaded with higher-affinity foreign peptides. Previous studies have indicated that the lifetime of the TCR-pMHC interaction is a key determinant in activating versus nonactivating pMHC molecules,[83] and a theory of "kinetic proofreading" was developed to explain how relatively small differences in receptor-bound time might be discriminated.[84] However, compelling evidence for this theory is lacking.

In order to utilize a reductionist approach to reveal the mechanism by which T-cells discriminate antigens with different affinities, Vale and colleagues engineered a T-cell signaling system by replacing the extracellular domains of the TCR and pMHC with complementary strands of DNA, in which DNA hybridization acted as the receptor−ligand interaction (Figure 4B).[85] Because the length and nucleotide composition of DNA can be varied, the receptor−ligand affinity can be precisely tuned over a wide dynamic range. Furthermore, receptor−ligand interactions mimicked by DNA hybridization were not affected by other coreceptors or adhesion receptors. They demonstrated that T-cells could discriminate between DNA ligands differing by a single base pair. Using this DNA-based T-cell signaling system, they found that TCR signaling is initiated when single ligand-bound receptors are converted into clusters, a time-dependent process requiring ligands with longer bound times. The formation of receptor clusters is due to the proximity of pre-existing ligated receptors, where there is a significant increase in the ligand binding on-rate. In combination with mathematical modeling, it was revealed that spatial reorganization of TCRs plays an important role in ligand discrimination in T-cell signaling, enabling ligands with longer bound times to have disproportionally greater signaling outputs.

The above work by Vale and colleagues[85] suggested that the clustering of TCRs is crucial for initiating cellular activation. However, it has been challenging to comprehend how the spatial arrangement of pMHC-TCR interactions within clusters influences the signaling outcome. Previous studies had tried to manipulate the spatial arrangement of ligands through soluble cross-linkers, nanolithography, and two-dimensional protein arrays and provided valuable information regarding the spatial requirements for receptor−ligand engagement,[13,86,87] but these methods remain technically challenging and have limitations in terms of manipulating receptor−ligand reactions at single-molecular level.

To address these challenges, Douglas, Vale, and their colleagues designed a DNA origami-based system to achieve nanoscale manipulation of ligand patterns on the surface of glass slides (Figure 4C).[88] In this work, they combined DNA origami with the synthetic T-cell signaling system developed previously by themselves, allowing both manipulation of receptor−ligand affinity and control of receptor−ligand interactions at the single-molecule nanoscale. With this DNA origami-based system and live T-cell imaging, they found that the number and spacing of ligands within each DNA origami affected the triggering threshold and time course of the MAPK signaling response in T-cells. The spacing of ligands affects the initiation time of the MAPK response, while the size of the ligand cluster determines the duration of the MAPK signal. More interestingly, low-affinity ligands, when placed adjacent to stimulatory high-affinity ligands, can enhance the sensitivity of MAPK signaling. This may explain why a small amount of antigenic pMHC molecules on the surface of antigen-presenting cells can cause T-cell activation. The above findings imply that T-cells are capable of measure spatial arrangements of ligands via TCRs, interpreting that information to trigger different signaling dynamics, and offer valuable knowledge for engineering immunotherapies.

The above works suggested the preorganization of single ligand molecules had an important impact on T-cell activation by using high affinity ligands with long TCR dwell time. However, the physiological TCR-ligand pMHCs usually have short TCR dwell times[89] and whether T-cell activation in a physiological environment must require pMHC to have a certain spatial arrangement still needs to be studied. To test this, Hellmeier et al. devised an APC-mimicking biointerface by anchoring rectangular DNA origami functionalized with a certain number of pMHCs on the supported lipid bilayer (SLB) (Figure 4D).[90] In this design, DNA origami could on one hand generate defined exclusion zones around individual ligands to isolate them on a fluidic surface and, on the other hand, permit the preorganization of single ligand molecules at

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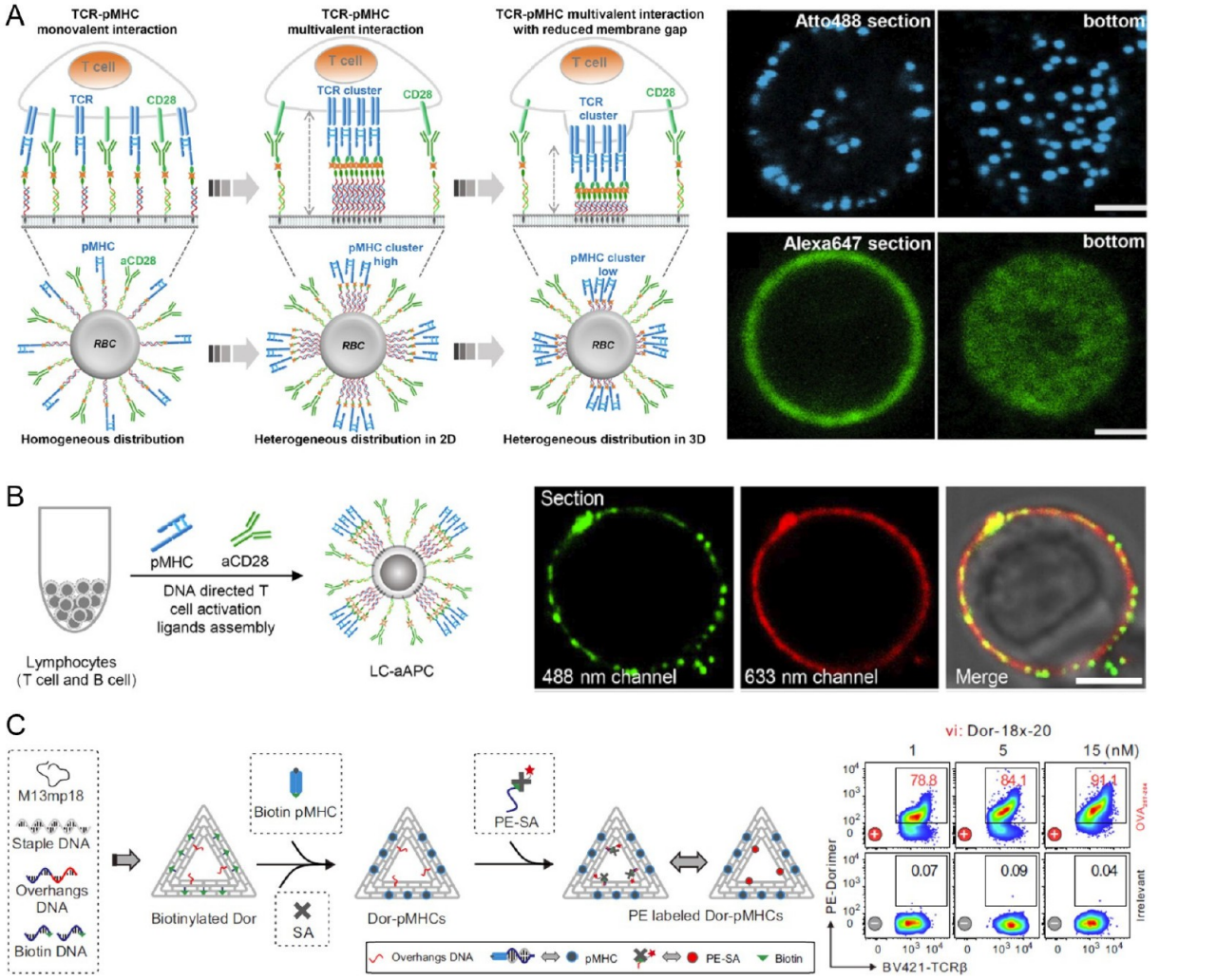

Figure 5. (A) Schematic of precise regulation of T-cell-activating ligands in both lateral and vertical manners on RBCs with lipid-DNA mediated bottom-up self-assembly and representative images showing the distribution of T-cell-activating ligands on RBCs. Reproduced with permission from ref 96. Copyright 2020 John Wiley and Sons. (B) Schematic of constructing lymphocyte-based aAPCs and representative images showing the distribution of T-cell-activating ligands on lymphocytes. Reproduced from ref 97. Copyright 2022 American Chemical Society. (C) Schematic illustrating the construction of pMHC multimers with triangular DNA origami and representative data showing staining of antigen-specific $CD8^+$ T-cells. Reproduced with permission from ref 98. Copyright 2022 Springer Nature.

nanoscale. With this platform, they could assemble one pMHC on DNA origami or two pMHCs with different spacing and controlled the density of DNA origami on SLB. By monitoring early events of T-cell activation, they found that single, well-isolated pMHC molecules could stimulate T-cells and spatial organization of the physiological ligand pMHC may have no influence on T-cell activation.

The results of the above study may be explained by a previous study of the dynamic interaction between pMHC and TCR based on single-molecule tracking, which indicated that both a single long-dwelling binding event and a sequence of spatially correlated short-dwelling binding events could induce T-cell activation.[91] However, this result is not comparable to that reported by Douglas et al.,[88] Hellmeier et al.[90] assembled up to two TCR ligands on DNA origami that performed two-dimensional motion, while Vale et al.[85] assembled 1−72 TCR ligands and the DNA origami was immobilized on a glass surface. Considering that pMHC forms clusters on the surface of natural antigen-presenting cells,[92] an influence of the spatial organization of pMHC on TCR activation cannot be ruled out.

On the surface of different T-cell subsets, the CD4 and CD8 coreceptors are believed to play a role in starting the intracellular signaling process by enhancing the stability of the interaction between TCR and pMHC and transporting its associated Lck to TCR.[93] However, in contrast to CD8, the extremely low CD4−MHC binding affinity has created challenges in fully comprehending the mechanisms that require CD4 binding to pMHC.[94] In a recent work, Ke and colleagues first demonstrated significant cooperative binding of CD4 to TCR prebound pMHC with two-dimensional (2D) mechanical-based assays, and by presenting TCR and CD4 on different surfaces to modulate molecular diffusion, they showed that the TCR−CD4 cooperativity was affected by the mobility of TCR and CD4.[95] They hypothesized that the effect of protein mobility could be due to TCR and CD4 needing to move around to come close enough to the same pMHC. To investigate this idea, they used a 10-helix bundle DNA origami nanorod with a length of 250 nm to precisely control the arrangement and positioning of TCR and CD4 on the surface of the magnetic beads (Figure 4E). They found that 7 nm

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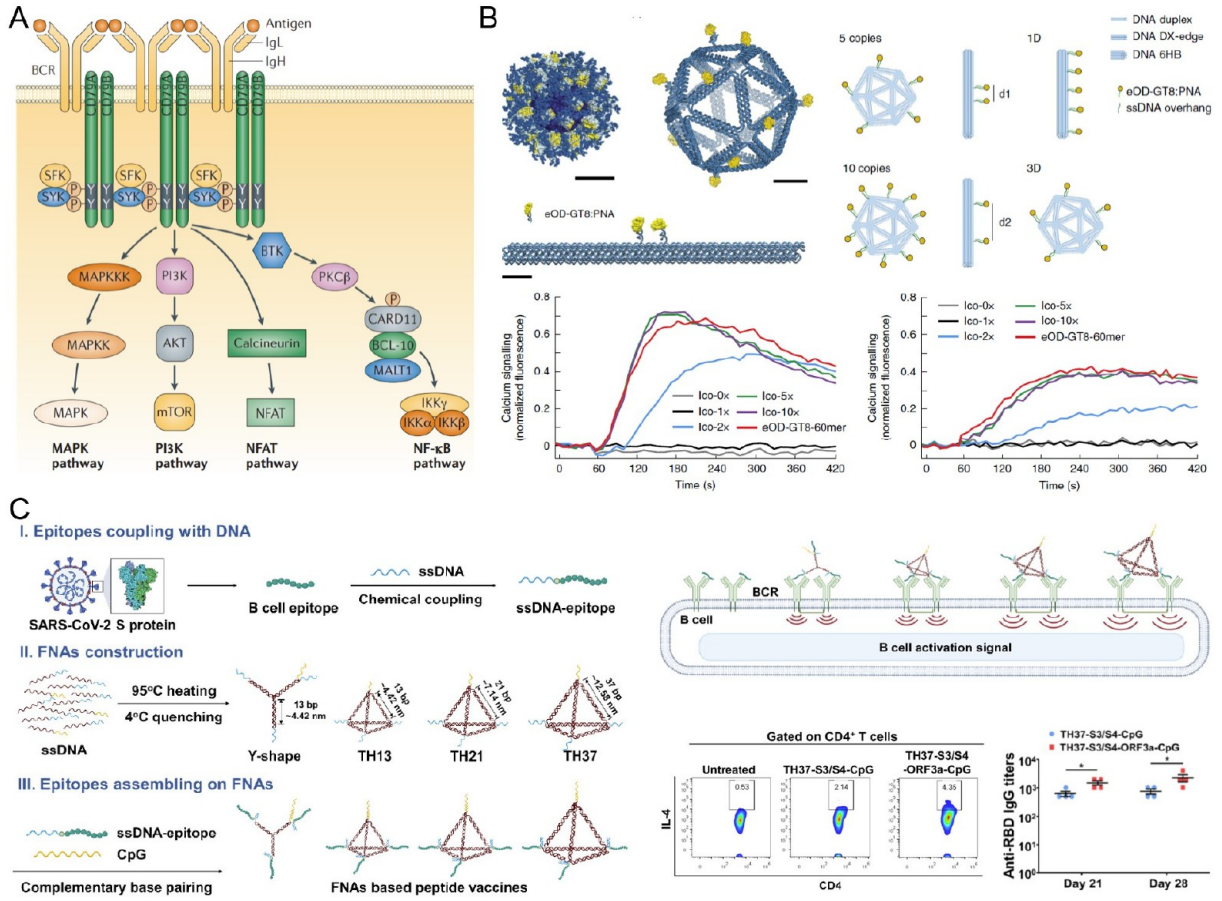

Figure 6. (A) Schematic of BCR signaling. Reproduced with permission from ref 3. Copyright 2013 Springer Nature. (B) Schematic of controlling nanoscale organization of HIV immunogens with DNA origami and representative data showing the effect of antigen copy number on BCR signaling. Reproduced with permission from ref 102. Copyright 2020 Springer Nature. (C) Schematic illustrating peptide vaccine construction with FNAs and representative data showing the immunological memory and IgG titer induced by FNA-based peptide vaccine. Reproduced with permission from ref 20. Copyright 2023 John Wiley and Sons.

proximity optimizes the TCR−pMHC−CD4 trimolecular bond formation, which is consistent with the crystal structure of the TCR−pMHC−CD4 trimolecule.

Apart from investigating the mechanisms of TCR signaling, modulating the interaction between TCR and their ligands with functional DNA can also be translated into immunotherapy strategies. Artificial APCs are indispensable for the expansion of therapeutic T-cells in vitro. In our previous work, using a bottom-up self-assembly based on cholesterol functionalized DNA, we could precisely control the distribution of pMHCs both laterally and vertically on the surface of artificial APCs based on red blood cells (RBCs) (Figure 5A).[96] By clustering the pMHC on the RBCs, which mimics natural APCs and regulating the vertical distance between pMHC and the RBC membrane, we were able to optimize the activation of T-cells, leading to more effective immune responses both in vitro and in vivo. As cholesterol can spontaneously incorporate into the phospholipid bilayer of cell membranes without affecting the function of surface proteins, we then proposed that the above ligand assembly strategy based on cholesterol-functionalized DNA could serve as a noninvasive method for engineering the surfaces of live cells and applied this method to construct artificial APCs based on lymphocytes (Figure 5B).[97] By using cholesterol-functionalized DNA to assemble pMHC and regulate its distribution on the surface of lymphocytes, we were able to mimic the distribution of pMHC on the surface of natural APCs without affecting the function of lymphocyte surface homing receptors. As a result, the lymphocyte-based artificial APCs were able to effectively target peripheral lymphoid organs and activate antigen-specific T-cells in vivo.

In addition, the detection of antigen-specific T-cells is required for the sorting of therapeutic T-cells and the evaluation of therapeutic effects. By precisely controlling the spacing and valency of pMHCs on a two-dimensional DNA origami, Pei et al. developed pMHC multimers (termed dorimers) for efficiently detecting antigen-specific T-cells (Figure 5C).[98] With DNA origami, the spacing of pMHCs could be set to ∼20−80 nm, and 3−18 pMHCs could be assembled on each DNA origami with a 20 nm spacing. They demonstrated that dorimers with relatively shorter pMHC spacing (20 nm) and higher pMHC valency (18) show enhanced affinity to TCR, thus being incapable of detecting more antigen specific T-cells in mouse $CD8^+$ T-cells compared with the equivalent tetramers and dextramers as well as T-cells that express low-affinity TCRs.

B-Cell Receptor

The B-cell antigen receptor (BCR) is a membrane-bound immunoglobulin that plays a critical role in both the immune response to antigens and B-cell development.[3] The BCR associates with the transmembrane molecules Ig-alpha (CD79a) and Ig-beta (CD79b) through noncovalent binding

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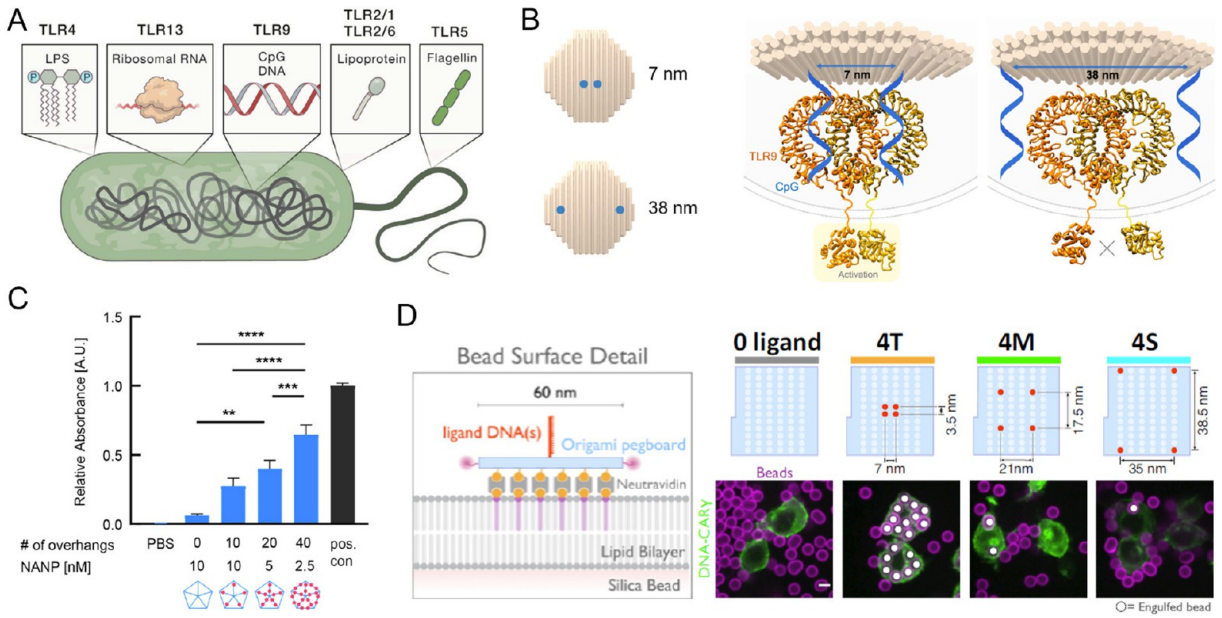

Figure 7. (A) Schematic of TLR family members. Reproduced with permission from ref 108. Copyright 2020 Elsevier. (B) Schematic of controlling the spacing of CpG-ODNs with DNA origami and the effect of CpG spacing on TLR-9 signaling. Reproduced from ref 109. Copyright 2022 American Chemical Society. (C) 3D wireframe DNA origami was used to reveal the influence of CpG-ODN valency on TLR9 activation. Reproduced from ref 111. Copyright 2022 American Chemical Society. (D) DNA origami-based engulfment system that allows precise nanoscale control of the number and spacing of DNA ligands that mimick the Fc of IgG and representative data showing the influence of ligands spacing on engulfment. Reproduced with permission from ref 113. Copyright 2021 eLIFE Sciences Publications Ltd.

to form a complex that recognizes antigens and initiates signal transduction (Figure 6A).[3] Upon binding to antigens, the BCRs will aggregate and cause the associated Src family protein tyrosine kinases (PTKs) to cluster and mutually phosphorylate, thereby activating Src PTKs followed by initiating of downstream signaling cascade. B-cells are the main effector cells of humoral immune responses; defects in BCR signaling can lead to immunodeficiencies and an increased risk of autoimmunity.[99] Regulating BCR signaling holds promise for developing more effective vaccines and therapies for autoimmune diseases.

Efficient activation of B-cells is a central goal in developing new vaccines. Compared to inactivated vaccines, protein subunit vaccines can induce antibodies against key functional epitopes of the pathogen, such as the receptor binding domain (RBD) of the spike protein of SARS-CoV-2, which can induce highly effective blocking antibodies against viral entry into host cells.[100] However, monovalent protein antigens often have lower immunogenicity because they are simpler than complete pathogens and difficult to cross-link BCRs. Research has shown that strongly initiating early B-cell signaling can be achieved by coupling multiple antigens on a polymer or displaying them on the surface of a virus-like nanoparticle (NP).[101] However, exploring the independent effects of antigen spatial arrangement and other design parameters, including antigen copy number, affinity, and the stiffness and dimensions of the scaffold presented, remains a challenge in initiating efficient B-cell receptor (BCR) signaling.

To address this issue, Veneziano et al. leveraged the addressability of DNA origami and assembled HIV antigens (engineered outer domain of the HIV-1 glycoprotein-120) at the scale of single virus-like DNA origami nanoparticles and systematically regulated the antigen copy number and spacing to independently investigate the effect of antigen valency and spacing on BCR triggering (Figure 6B).[102] It was observed that increasing the antigen spacing up to approximately 25−30 nm resulted in a gradual increase in B-cell activation, and B-cell signaling can be maximized by assembling as few as five antigens on the surface of a 40 nm viral-like DNA origami nanoparticle with maximum spacing between them. In addition, they also found that scaffold rigidity was essential for robust BCR triggering, and antigen assembly on rigid DNA origami stimulated BCR more strongly than coupling on flexible single-stranded DNA linkers and PEG molecules. This study suggests that optimizing the spatial arrangement of antigen molecules to enhance B-cell receptor (BCR) activation holds great potential in improving the immunogenicity of subunit vaccines.

Followed by the above work, Wamhoff et al. presented icosahedral DNA origami as a vaccine platform to display the receptor binding domain (RBD) of SARS-CoV-2 and precisely control the antigen valency.[103] It was found that the icosahedral DNA-based vaccine elicited RBD valency-dependent BCR signaling in vitro with corresponding increasement of RBD-specific antibody responses in mice. Of note, unlike protein scaffolds (virus-like particles, VLPs), the DNA-based scaffolds themselves, as thymus-independent antigens, induce only extrafollicular B-cell responses, thus avoiding scaffold-directed immunological memory that results in antibody dependent clearance of the vaccine platform. Almost at the same time, Oktay et al. also demonstrated that precisely patterning ten copies of a reconstituted trimer of the RBD of SARS-CoV-2 along with CpG adjuvants on DNA origami was able to elicit a robust protective immunity against SARS-CoV-2 in mice.[104] These works illustrated the potential of DNA nanotechnology for the construction of novel and highly effective subunit vaccines.

Peptide vaccines are a type of subunit vaccine that are prepared using chemical methods based on the amino acid sequences of known or predicted epitopes on the pathogen's

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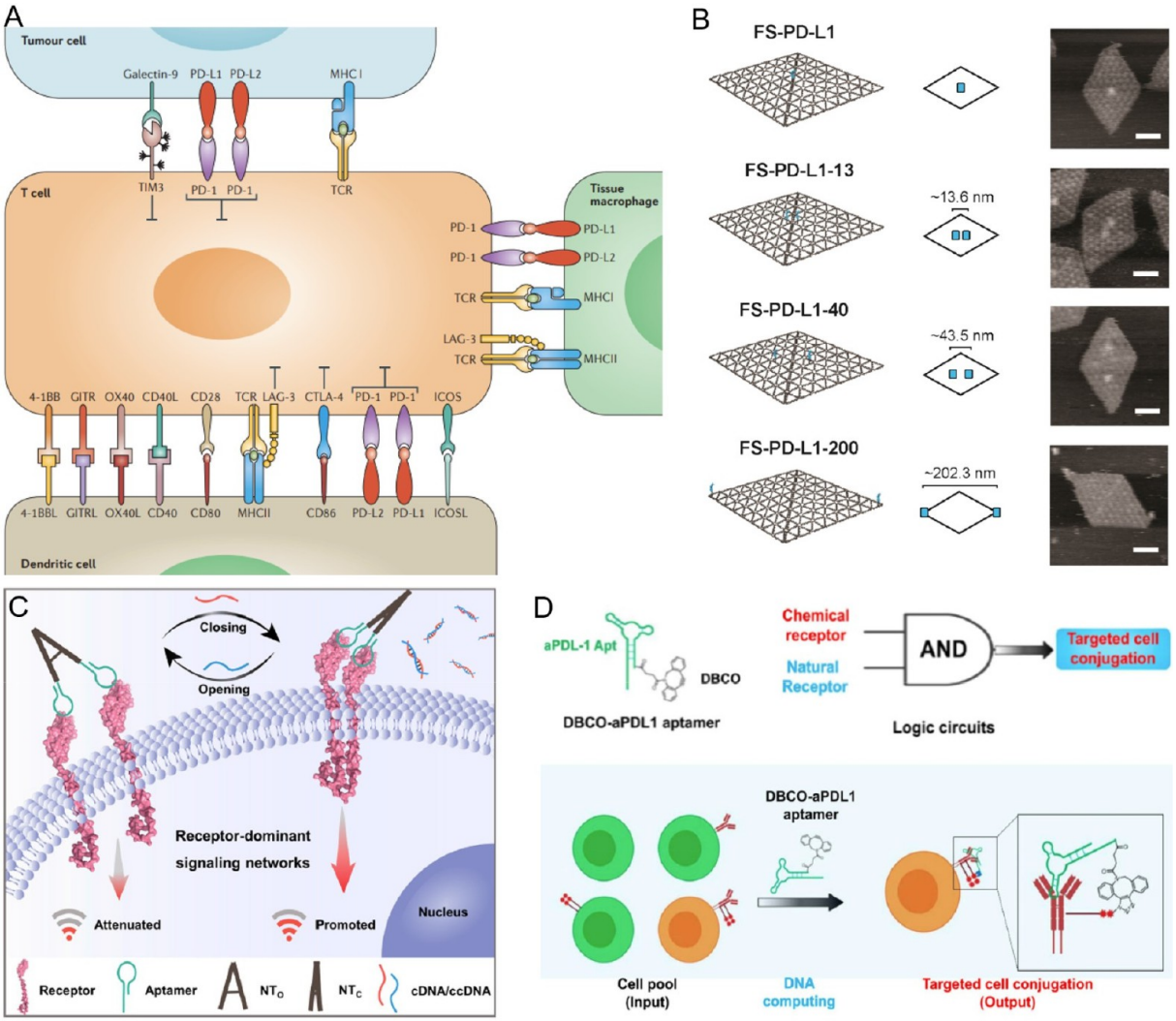

Figure 8. (A) Schematic of immune-checkpoint molecules involved in regulating antitumor immune response. Reproduced with permission from ref 2. Copyright 2017 Springer Nature. (B) Schematic designs of DNA flat sheets functionalized with PD-L1−oligo conjugates at different positions. Reproduced from ref 117. Copyright 2021 American Chemical Society. (C) Schematic aptamer-based tweezer-like DNA nanodevice to dynamically control the spacing of CD28. Reproduced from ref 119. Copyright 2022 American Chemical Society. (D) A logic computing reaction to achieve precise and sustained immune checkpoint blockade therapy with anti-PD-L1 aptamers. Reproduced from ref 76. Copyright 2021 American Chemical Society.

antigen proteins.[105] With the advent of mature epitope prediction technology, these vaccines hold immense potential in rapidly responding to emergent malignant virus outbreaks and virus mutations. However, the efficacy of peptide vaccines is hindered by the poor immunogenicity of epitopes. Although coupling multiple epitopes onto the surface of a nanocarrier can enhance the immunogenicity of peptide vaccines,[106] the effects of epitope spatial arrangement on vaccine efficacy remain unclear.

In our recent work, we have proposed using low-cost framework nucleic acids (FNAs) as self-adjuvant carriers to precisely assemble B-cell epitopes for systematically exploring the effect of epitope spacing and carrier rigidity on B-cell activation in vitro and humoral immunity in vivo (Figure 6C).[20] Utilizing a series of FNAs with varying sizes and rigidities to organize epitopes, we discovered that epitopes assembled on rigid tetrahedral FNAs can activate B-cells more strongly and that B-cell activation is enhanced as the distance between epitopes increases within the range of ∼4 to ∼13 nm. Based on this finding, we constructed a peptide vaccine prototype that can induce the production of high-titer IgG against the RBD of SARS-CoV-2 in mice by setting the distance between B-cell epitopes from the RBD at approximately 12.58 nm in a tetrahedral FNA with a side length of 37 base pairs. Our work, as well as that of Wamhoff et al.[103] highlights the importance of precise control of antigen molecular spatial arrangement to enhance BCR signaling for the development of highly effective subunit vaccines.

Innate Immune Cell-Related Receptors

Pattern recognition receptors (PRRs) are germline-encoded receptors that play a vital role in detecting microbial infections by innate immune cells.[107] Examples of host PRRs include Toll-like receptors (TLRs), nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and some C-type lectin receptors (CLRs). TLRs can sense microbial products such as bacterial cell wall components or viral proteins at the level of the cell surface or microbial nucleic acids exposed within vesicular compartments such as endo-

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somes and/or phagosomes (Figure 7A).[108] RLRs and NLRs serve as sensors for intracellular microbial invasion, and CLRs are directly involved in innate immune recognition of microbial products for the innate immune system.[107] Innate immune cells serve as the first line of defense against invading pathogens, and some of them, such as DCs, act as a bridge between innate and adaptive immunity. Therefore, a thorough understanding of the mechanisms underlying the interaction between innate immune receptors and their ligands is crucial for developing effective immunotherapy against microbial infections and even other diseases, such as cancer.

Toll-like receptor 9 (TLR9) is a widely expressed TLR in the innate immune system.[108] It primarily locates in the endoplasmic reticulum and endosomal system and can recognize unmethylated CpG dinucleotides in bacterial and viral DNA, triggering immune responses. TLR9 agonists (CpG-ODNs) can be used for tumor immunotherapy and vaccine research; however, the impact of the spatial organization of CpG-ODNs on TLR9 activation remains unclear. To address this problem, Comberlato et al. created DNA origami nanoparticles that presented CpG-ODNs at defined spacing at nanoscale to bind TLR9 in RAW 264.7 macrophages (Figure 7B).[109] It was demonstrated that two CpG-ODNs with a spacing of 7 nm achieved stronger immune activation than that with a spacing of 38 nm, which matched the active dimer structure of TLR9. Following that, Zeng et al. assembled 18 CpG-ODNs on a "square block" DNA origami platform and set the spacing between CpG-ODNs at 2.5 to 7 nm to investigate the influence of specific spatial arrangements of CpG-ODNs with narrower spacings on the Th1-polarized immune response.[110] Through in vitro cell-culture studies and in vivo tumor-treatment models, they found that the spacing of 3.5 nm between CpG-ODNs could induce a stronger Th1 immune response compared to larger or smaller spacings. Almost at the same time, a work by Du et al. further demonstrated the contribution of CpG-ODN copy number and spatial organization to the magnitude of TLR9 signaling by systematically organizing CpG-ODNs on a 3D wireframe DNA origami (Figure 7C).[111] These works highlight the importance of a spatially controlled presentation of therapeutics to increase efficacy of immune-modulating nanomaterials where multiple receptor−ligand interactions are involved.

Fc receptors are also commonly expressed on the surface of innate immune cells. Fc receptors are classified into two main types: IgG receptors and non-IgG receptors. IgG receptors include FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16), which recognize and bind to the Fc portion of IgG antibodies.[112] Non-IgG receptors include FcεRI (CD23) and FcαRI (CD89), which bind to the Fc portions of IgE and IgA antibodies, respectively.[112] They mediate antibody-dependent cell-mediated cytotoxicity (ADCC) as well as antibody-dependent cellular phagocytosis (ADCP). Both ADCC and ADCP rely on the recognition of the Fc of antibodies bound to targets by Fc receptors. For example, activation of multiple FcγRs by IgG is required for macrophages to engulf 3D targets via phagocytosis.[112]

To explore the mechanism controlling the threshold for FcγRs mediated phagocytosis, Kern et al. developed a DNA origami-based engulfment system that allows precise nanoscale control of the number and spacing of DNA ligands that mimick the Fc of IgG (Figure 7D).[113] When the spacing between ligands is reduced from 17.5 to 7 nm while keeping the number of ligands constant, it greatly enhances the process of engulfment in cells. This is mainly due to an increase in the efficiency of the initiation process of engulfment, which is facilitated by tighter clustering of the ligands. The tight clustering of the ligands leads to an increase in receptor phosphorylation and downstream signals. However, simply increasing the number of signaling domains on a single ligand−receptor complex was not enough to produce the same effect, indicating that clustering of multiple receptors is necessary. These findings suggest that macrophages can sense and respond to changes in local ligand densities, which has significant implications for antibody-mediated phagocytosis and the development of immunotherapies.

Costimulatory Receptors

Costimulatory receptors play a crucial role in T-cell activation by coordinating with the T-cell receptor (TCR) to interpret antigen recognition correctly as a response to a dangerous infection, rather than a harmless antigen. Examples of costimulatory receptors include CD28, PD-1 (programmed death receptor-1), and CTLA-4 (cytotoxic T-lymphocyte-associated protein 4, also called CD152), among others (Figure 8A).[2] CD28 is the most effective T-cell costimulatory receptor discovered to date, which promotes T-cell activation via binding with B7.1/7.2 (CD80/86),[2] while CTLA-4 functions as a negative regulator of T-cell activation by binding to B7.1/7.2 with higher avidity than CD28.[2] PD-1 expressed in antigen-stimulated T-cells also acts as a negative regulator of T-cell activation by binding to its ligands PD-L1 or PD-L2 overexpressed in cancer cells.[2] Downregulation of T-cell activation by CTLA-4 and PD-1 maintains self-tolerance and helps avoid collateral damage during physiological immune responses; therefore, CTLA-4 and PD-1 are also called immune checkpoints.[2] These costimulatory receptor−ligand interplays occur at different stages of T-cell activation and function, and targeting these interactions has become a therapeutic approach in the treatment of cancer and autoimmune diseases.

Although blocking the binding of PD-1 and PD-L1/PD-L2 with antibodies has shown success in some cancer treatments, there are still many patients who do not benefit from this therapy.[114] Additionally, blocking PD-1 can led to autoimmune adverse events.[115] To improve the efficacy of this therapy, it is necessary to better understand the mechanisms behind PD-1-mediated inhibition of T-cell signaling. A previous study has shown that, upon binding to its ligand PD-L1, PD-1 undergoes spatial reorganization and forms microclusters on the T-cell membrane.[116] However, it is challenging to elucidate how the spatial organization of PD-L1 influences PD-1 clustering and T-cell signaling. To this end, Fang et al. used DNA origami flat sheets to assemble PD-L1 at defined nanoscale distances (13, 40, and 200 nm) and investigate the regulation of T-cell signaling by PD-L1 in vitro (Figure 8B).[117] It was found that, when PD-L1 ligands were presented on flat sheets separated by approximately 200 nm, they caused a significant inhibition of T-cell signaling, whereas flat sheets presenting PD-L1 ligands at closer distances did not have the same effect. In addition, the researchers found that PD-L1 presented on flat sheets separated by 200 nm induced the formation of smaller PD-1 nanoclusters and resulted in a greater reduction in IL-2 expression compared to those separated by closer distances. These findings suggest that the spatial organization of PD-L1 is critical in regulating T-cell signaling and may have implications for optimizing immune checkpoint therapies in the future.

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Dimerization of enzyme-linked receptors on the cell surface is key to transmit extracellular recognition events across the plasma membrane to initiate signaling cascades inside cells. Although natural or synthetic ligands that can cause uncontrolled receptor dimerization have been used clinically, such as IL2, safety concerns have limited their application.[118] Therefore, regulating the signaling amplitude of membrane receptors in a user-defined manner might provide opportunities for precise immunotherapies. In a recent work by Tan, Qiu, and co-workers, the aptamer of costimulatory receptor CD28 was integrated with a tweezer-like DNA nanodevice to dynamically modulate the behavior of T-cells by controlling the spacing of CD28 (Figure 8C).[119] By controlling the opening and closing, this nanodevice realized excellent dynamic regulation on CD28 receptor-mediated T-cell immunity. Of note, this DNA nanotweezer mediated regulation of receptor dimerization do not rely on complicated protein structural analysis.

In addition, a variety of aptamer-based costimulatory receptor signaling regulation strategies have been used in cancer therapy research.[120,121] For example, aiming at the problems of off-target and poor efficacy of current antibody-based immune checkpoint blockade therapies, Yang et al. propose a logic computing reaction to achieve precise and sustained immune checkpoint blockade therapy with anti-PD-L1 aptamers (Figure 8D).[76] This innovative strategy introduces "And" logic gate by integrating the selectivity of aptamer with the specificity of azide/cyclooctyne-based bioorthogonal chemistry to achieve targeted modification of anti-PDL1 aptamers on cancer cells, leading to improved therapeutic efficacy and precision in clinical cancer therapy. This work provides a promising approach for aptamer-based cancer immunotherapies.

CONCLUSION AND OUTLOOK

In summary, we provide a brief overview of the recent progress in the field of functional DNA as a versatile molecular tool for regulating immunoreceptor signaling in this Perspective. A deeper understanding of the mechanisms of immunoreceptor–ligand interactions is crucial for developing corresponding regulatory strategies to treat many major diseases, as immunoreceptor signaling is closely associated with their onset. The integration of diverse approaches has equipped DNA with an unparalleled ability to precisely assemble molecules with stoichiometric accuracy and regulate the spatial distribution parameters of these molecules. Obviously, functional DNA has engendered novel paradigms in exploring the interplay of immunoreceptors and their ligands at the interface of cells and holds immense potential for future immune engineering applications.

Although the future is promising, the field of functional DNA for regulating immune receptor–ligand interactions is still in its nascent stages. Currently, functional DNA is only used to study a limited number of immunoreceptor–ligand interactions with a predominant focus on T-cell receptors. Moreover, immune cell activation is often the result of dynamic interactions among multiple immunoreceptors, rather than a single receptor. Therefore, one future direction for this field is to employ functional DNA to uncover more immunoreceptor signaling mechanisms and explore the collaborative mechanisms among different immunoreceptors. This requires a more convenient and efficient way to achieve precise and dynamic spatial arrangement of biomacromolecules using functional DNA. In addition, the chemical properties and conception of the ligands are critical to their interaction with the receptors, and more DNA labeling methods that have little impact on the properties of the protein–ligand need to be developed in the future. Although facing challenges, we anticipate that this exciting field will lead to new discoveries, improve our understanding of immunoreceptor signaling, and ultimately lead to new treatment strategies for major diseases.

AUTHOR INFORMATION

Corresponding Authors

Zhuang Liu − *Institute of Functional Nano & Soft Materials (FUNSOM), Jiangsu Key Lab Carbon Based Functional Materials and Devices, Soochow University, Suzhou, Jiangsu 215123, China;* orcid.org/0000-0002-1629-1039; Email: zliu@suda.edu.cn

Lele Sun − *Institute of Materiobiology, Department of Chemistry, College of Science, Shanghai University, Shanghai 200444, China;* orcid.org/0000-0002-8052-9451; Email: sunlele@shu.edu.cn

Authors

Fengyun Shen − *School of Chemistry and Chemical Engineering, Shanghai Jiao Tong University, Shanghai 201240, China*

Yanfei Qu − *Institute of Materiobiology, Department of Chemistry, College of Science, Shanghai University, Shanghai 200444, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/jacsau.3c00291

Author Contributions

○L.S. and F.S. contributed equally. CRediT: **Lele Sun** investigation, supervision, writing-original draft, writing-review & editing; **Fengyun Shen** writing-original draft; **Yanfei Qu** writing-original draft; **Zhuang Liu** supervision, writing-original draft, writing-review & editing.

Notes

The authors declare no competing financial interest.

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