Carbohydrate ligand engagement with CD11b enhances differentiation of tumor-associated myeloid cells for immunotherapy of solid cancers

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ABSTRACT

Background Efforts to modulate the function of tumor-associated myeloid cell are underway to overcome the challenges in immunotherapy and find a cure. One potential therapeutic target is integrin CD11b, which can be used to modulate the myeloid-derived cells and induce tumor-reactive T-cell responses. However, CD11b can bind to multiple different ligands, leading to various myeloid cell functions such as adhesion, migration, phagocytosis, and proliferation. This has created a major challenge in understanding how CD11b converts the differences in the receptor-ligand binding into subsequent signaling responses and using this information for therapeutic development.

Methods This study aimed to investigate the antitumor effect of a carbohydrate ligand, named BG34-200, which modulates the CD11b+ cells. We have applied peptide microarrays, multiparameter FACS (fluorescence-activated cell analysis) analysis, cellular/molecular immunological technology, advanced microscopic imaging, and transgenic mouse models of solid cancers, to study the interaction between BG34-200 carbohydrate ligand and CD11b protein and the resulting immunological changes in the context of solid cancers, including osteosarcoma, advanced melanoma, and pancreatic ductal adenocarcinoma (PDAC).

Results Our results show that BG34-200 can bind directly to the activated CD11b on its I (or A) domain, at previously unreported peptide residues, in a multisite and multivalent manner. This engagement significantly impacts the biological function of tumor-associated inflammatory monocytes (TAIMs) in osteosarcoma, advanced melanoma, and PDAC backgrounds. Importantly, we observed that the BG34-200-CD11b engagement triggered endocytosis of the binding complexes in TAIMs, which induced intracellular F-actin cytoskeletal rearrangement, effective phagocytosis, and intrinsic ICAM-1 (intercellular adhesion molecule I) clustering. These structural biological changes resulted in the differentiation in TAIMs into monocyte-derived dendritic cells, which play a crucial role in T-cell activation in the tumor microenvironment.

Conclusions Our research has advanced the understanding of the molecular basis of CD11b activation in solid cancers, revealing how it converts the differences in BG34 carbohydrate ligands into immune signaling responses. These findings could pave the way for the development of safe and novel BG34-200-based therapies that modulate myeloid-derived cell functions, thereby enhancing immunotherapy for solid cancers.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Previous research has highlighted the potential of the integrin CD11b to modulate tumor-associated myeloid cells and stimulate tumor-reactive T-cell responses. However, the diverse-ligand binding properties of CD11b and their impact on myeloid cell functions, including adhesion, migration, phagocytosis, and proliferation, pose a significant hurdle in deciphering the downstream signaling responses and leveraging this knowledge for therapeutic advancement. Consequently, elucidating the mechanisms by which CD11b translates receptor-ligand interactions into signaling cascades remains a critical area of investigation for therapeutic development in cancer.

WHAT THIS STUDY ADDS

⇒ In this study, we have identified a novel ‘differentiation’ epitope within the I domain of the CD11b receptor on inflammatory monocytes. Additionally, we have discovered that a carbohydrate glucan named BG34-200 can specifically bind to this epitope, leading to the differentiation of inflammatory monocytes to monocyte-derived dendritic cells (mo-DCs) in solid cancers. This finding unveils a previously unreported sequence and conformation of the ‘differentiation’ epitope and introduces a unique carbohydrate ligand system for CD11b-induced mo-DC differentiation, which has not been documented elsewhere.

Despite significant efforts over the past three decades to optimize the intensification of combination chemotherapy, radiation therapy, and surgery, the management of the advanced solid cancers remain complex and limited.1,2 Recent progress in immune-mediated approaches, including immune checkpoint blockade therapies, has renewed
In this study, we aimed to explore the molecular interaction between CD11b and the carbohydrate ligands BG34s and evaluate the resulting biological effects in solid cancer backgrounds. Specifically, we investigated whether the engagement of the carbohydrate ligand BG34-200 with CD11b is direct and specific, and whether it can alter myeloid cell function for the purpose of immunotherapy in solid cancers.

RESULTS
Administration of the carbohydrate ligand (BG34-200) via intravenous injection can induce antitumor effects in solid cancers by modulating CD11b+ cells
We recently developed a carbohydrate molecule that is safe and isolated from oats. This molecule has an unusual capacity to modulate myeloid-derived cells.15 16 Through our research, we found that the β-(1,3)-(1,4)-glucans (BG34) derived from oats with a weight average molecular weight (Mw) of ~200 kDa (BG34-200) can induce antitumor immune responses by modulating the surface marker CD11b (CR3; CD11b/CD18; Mac-1) of myeloid cells.15-17 In previous studies, we optimized the administration route, dosage, and schedule of BG34-200 and evaluated its antitumor effects using mouse models of melanoma.15 18

Building on these findings, we expanded our research to assess the in vivo effects of BG34-200 in multiple murine tumor models using the optimized treatment conditions. These models included metastatic melanoma (B16F10, figure 1A), metastatic osteosarcoma (K7M2, figure 1B), transgenic tamoxifen-inducible KPC pancreatic ductal adenocarcinoma (PDAC) (KRASLSL.G12D/++; TP53.LSL.R172H/++; Pdx1-Cre) (figure 1C–E), and an orthotopic KPC model (figure 1F). We chose these models because they allow solid tumors to grow in immunocompetent mice, which mimic key aspects of human diseases.16-22 Our results showed that the intravenous administration of BG34-200 at 50 mg/kg effectively induced antitumor effects in all of the models tested (figure 1A–F). However, when we tested CD11b knockout mice (B6.129S4-Itgamtm1Myd/J, CD11b−/−) with the same tumor burdens, we did not observe any antitumor effects (figure 1A and F).

These data, combined with our previous findings,15-18 suggest that BG34-200 induces antitumor effects in multiple solid cancers by modulating CD11b+ cells. BG34-200 mediates direct, multisite, and multivalent binding to CD11b
In order to understand how BG34-200 interacts with integrin CD11b, we conducted overlapping peptide microarrays to determine which peptide motifs are involved in their binding (figure 2A). Both linear and conformational overlapping peptides were used, and microarrays were performed on BG34 of 50, 200, and 500 kDa for comparison, as BG34-200 can induce antitumor effects but the BG34-50 and BG34-500 cannot.15 16 After incubating with

hopes for managing multiple refractory and advanced solid cancers. However, while this approach has shown initial success, a significant number of patients do not respond, and the promising impact on solid cancer has yet to be fully realized.3 4

Solid tumors, such as sarcomas, carcinomas, and lymphomas, have the organ-like structures that are composed of heterogeneous malignant cells in a complex tumor microenvironment (TME). Due to a lack of understanding of how the TME suppresses the immune system and how to overcome this challenge, the effectiveness of immunotherapy in treating solid cancers is limited.1 Research from clinical and experimental studies has revealed that a high infiltration of myeloid derived cells is a characteristic of the TME in most advanced cancers.3 6 Furthermore, evidence suggests that these tumor-associated myeloid cells can suppress antitumor immune responses, promote tumor metastasis, and drive resistance to virtually all types of therapies.7 Therefore, researchers are actively pursuing methods to disrupt the function of tumor-associated myeloid cells to overcome these challenges in immunotherapy and ultimately find a cure.

Integrin CD11b has emerged as a promising therapeutic target for modulating the function of myeloid derived cells and inducing tumor-reactive T-cell responses.8 As a C-type lectin receptor, CD11b is capable of sensing and responding to pathogen-associated molecular pattern (PAMP) molecules, thereby initiating innate immune responses.9 PAMPs are highly conserved carbohydrate structures that are uniquely present in bacterial, viral, plant, and fungal pathogens but absent in the human tissues. This makes them an attractive option for modulating the CD11b+ myeloid cells.10 11

However, the binding and recognition of carbohydrate ligands by CD11b can be affected by multiple factors including various external stimuli, leading to conformational changes that trigger a range of myeloid cell functions, including adhesion, migration, phagocytosis, and proliferation.12-14 This poses a significant challenge in understanding the molecular basis of how CD11b converts the receptor-ligand binding differences into the subsequent immune signaling responses and using this knowledge for therapeutic development.
biotin-conjugated BG34s or non-targeted glucan control, the peptides were stained with a streptavidin DyLight680 and control antibody, which generated heat maps of all the peptides (online supplemental figure S1A,B). The microarrays were also performed at three different concentrations of BG34 molecules (Online supplemental figure s1C), allowing us to eliminate noise signals and determine dose-dependent signals representing BG34 bound peptides.

Based on these data, we identified five peptides with different binding strengths to BG34s (figure 2B). Three of the five peptide species (S158-K170, V173-K182, and E238-247) shared a primary sequence of C-terminal K and were mapped to the consensus motifs located at the CD11b I (or A) ectodomain. The other two peptides (L380-K386 and Q485-C494) were located outside of the I domain, as determined by examining their crystallographic structure (PDB: 1IDO) using the computational simulation method (AlphaFold: AF-P11215-F1) (figure 2C). The database and simulation methods also allowed us to generate secondary structures of these peptides, which revealed that all five peptides were all located at the solvent-exposed surface of CD11b.

To verify the accuracy of these peptide sequences, we used peptide substitution microarrays. In this method, we replaced each amino acid (AA) in each of the five wild type (WT) peptides with all 20 AAs. The WT peptides and their variants were then synthesized and cyclized onto microarray chips that eventually contained 1300 different peptides printed in triplicates, framed by 134 control peptides (HA, YPYDVPDYAG). After the biotin-tagged BG34 molecules and controls were incubated...

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**Figure 1**  BG34-200 intravenous administration induces antitumor effects in multiple solid cancer models. Kaplan-Meier overall survival curves of mice bearing (A) B16F10 melanoma, (B) K7M2 osteosarcoma, (C–E) spontaneous KPC tumors and (F) orthotopic KPCY tumors. For A–C, n=9. (D) Cross sections of T2-weighted MR image of the spontaneous KPC mice. The red lines indicate tracing of the region of interest (ROI) for the tumor volume measurement. Four out of 20 slices of MR scans were presented as examples. (E) The areas of ROIs were summed to calculate volumes of spontaneous KPC tumors. n=5. (F) Mean fluorescence intensity (MFI) of KPCY tumors in wild type (WT) mice or CD11b−/− mice. n=9. For E and F, **, p<0.01; ***, p<0.001; ns, not significant. PBS, Phosphate-Buffered Saline.
with the peptide microarrays, they were stained with streptavidin DyLight680 and control antibody to measure the intensity of each peptide spot. This generated intensity plots and heat maps, enabling us to identify the mutated (MT) peptide that interacted with the BG34 molecules (online supplemental figure 2). Using this information, we produced AA plots for each WT peptide, which reflected the AA preferences at each given position, helping us determine the conserved and variable AA positions.

Figure 2  BG34-mediated multisite and multivalent binding to CD11b. (A) Schematics of CD11b overlapping peptides for microarrays. (B) Mean fluorescence intensity (MFI) of BG34 samples that bind to the peptides, as determined by microarray scanning and PepSlide analyzer. The conformational peptide microarrays were prestained with streptavidin DyLight680 for background control, then incubated with biotin-conjugated BG34-50, BG34-200, or BG34-500 at 100 µg/mL, followed by staining with streptavidin DyLight680. The MFI of DyLight680 signals were measured, calculated, and presented as the binding strength between BG34 and peptides. (C) Three-dimensional structures of CD11b (in teal) and the five peptides (in yellow), as determined by computational simulation method (AlphaFold: AF-P11215-F1), in combination with the crystallographic structure (PDB: 1IDO in magenta); the serine and threonine residues (in hot pink), the transmembrane region (in orange), followed by the intracellular domain (in red). (D) Fold increase or decrease of the binding strength between BG34 samples and the mutated (MT) peptides, as determined by amino acid substitution microarray assays. The binding strength between BG34-200 samples and wild type (WT) peptides were normalized to be 100%. Amino acid substitution of three peptides (158S-K170, 173V-K182, and 380L-K386) are shown. Every amino acid in these sequences was substituted by all 20 amino acids. The fold changes are quantified by measuring the increase or decrease of binding affinity to MT peptide relative to the binding affinity to the WT peptides (in red). The essential conserved sequences are framed by purple rectangles. BG34-200 showed general preference for amino acids K and R (in green), and general aversion to aromatic amino acids W and Y (in blue). (E) Amino acid sequence alignment of human (black) and murine (red) CD11b peptide residues (101Q-L300). BG34 bound peptide sequences were underlined black for human and red for mice. (F) Binding affinity plots of BG34 samples at different concentrations. Plots of three different molecular weights (Mws) are shown. (G) Binding affinity plots of BG34 samples at different Mws. BG34 samples at 0.2 µM are shown. For F and G, the binding affinities were determined through the overlapping peptide microarrays which measured the MFI of BG34 samples that binds to the peptides. Slope=MFI/(concentration×Mw).
Based on the data analyzed, we have observed that the AA positions of L380-K386 and Q485-C494 display variable characteristics, indicating that they may be non-specific sequences (data not shown). On the other hand, the 158S-K160, 173V-K170, and 238E-K247 peptides contain AA positions that are essential and highly conserved, such as the 163F-K170, Q174-K181, and 244N-K247 (indicated by purple rectangle frames). Substituting any of these AAs with others resulted in partial or complete loss of BG34-200 binding in these three sequences. Of particular interest is the fact that all AA positions in these sequences have a strong preference for K or R (indicated in green), and almost all the AA positions show an aversion to aromatic AAs Y and W (indicated in blue) (figure 2D).

In the 165F-K170 region, K170 showed significant sequence conservation as even substituting it with the strongest basic AA R led to a complete loss of binding. R168 and R169 appeared to be less conserved than K170, as substituting them with K resulted in slightly lower spot intensities and substituting them with any other AAs resulted in a 60% loss of binding. F165 and M168 were more tolerant of conservative exchange with K and R, as all other substitutions resulted in approximately 50% loss of binding.

In the 174Q-K182 region, Q174, K181, and K182 were highly conserved, as substitutions with all other AAs led to a ~20–50% loss of binding. However, L175 appeared to be variable. In the 244N-K247 region, all the AA positions were tolerant of substitutions with K and R, but substitutions with any other AAs resulted in 50–100% loss of binding. Taken together, these results suggest that BG34 is capable of directly engaging multiple sites in the I domain of CD11b at specific peptide epitopes.

Moreover, we conducted a comparison between the human and mouse CD11b. These two proteins have a high level of similarity, with a 78.7% DNA sequence similarity and 74.8% direct AA identity.25 By analyzing the three peptide regions in both human and mouse CD11b, we found that the BG34 bound regions retained a high degree of homology (figure 2E). Using substitution microarray analysis, we discovered that BG34 molecules had a lower affinity for mouse CD11b at 166FQKMK210 and 244NGARE247 compared with the corresponding human CD11b at the same position. This is because the replacement of 166RR (human) with 166QQ (mouse) resulted in a decrease of mean fluorescence intensity (MFI) by ~20%, and the substitution of 244K (human) with 244E (mouse) resulted in a decrease of MFI by ~30%.

However, BG34 molecules had a higher affinity for mouse CD11b at 159QFKK182 than for the corresponding human residues 179QLKK182, as the substitution of 180L (human) with 180F (mouse) led to an increase of MFI by ~60%. Despite the differences in BG34 binding affinities to the human and mouse peptides, replacing these AA residues (166RR167166KQ170244K247244E180L180F) with other AAs all resulted in a significant loss of binding (figure 2D). These findings suggest the three peptides are highly conserved in their sequences with respect to BG34 binding, and that the interaction between BG34 and CD11b occurs in a specific manner.

Furthermore, we conducted an analysis to understand how the Mw of BG34 affects its binding to CD11b using peptide substitution microarray data. Our results showed that all variations of BG34 could bind to the three peptides in a concentration-dependent manner, but with differing binding strength (figure 2F). Specifically, BG34-500 exhibited significantly stronger binding compared with BG34-50 and BG34-200.

Polysaccharides, or carbohydrate polymers, contain multiple copies of ligands or binding units that interact with their target, resulting in a multivalent interaction that enhances affinity. This phenomenon is seen in endocytic receptors such as CD11b and other molecules that play a crucial role in the first line of defense.24 This natural effect is known as the multivalent interaction through glycoside cluster effect, in which the clustering of lectin binding sites and carbohydrate recognition sites enhances affinity. This effect can be assessed by examining if the affinity enhancement effect by increasing Mw is greater than the affinity enhancement effect by increasing concentrations.24 25

Based on this knowledge, we used the microarray assay data and generated the binding affinity plots against Mw at a molar concentration of 0.2 µM (figure 2G). This concentration was selected to avoid any unwanted chain overlap or interpenetration caused by entanglement concentrations in BG34.26 27 Our results revealed that the affinity enhancement effect through the increase of Mw at the non-entanglement concentration is substantially greater than the affinity enhancement effect through an increase in concentration. This enhancement is indicated by the slopes shown in figure 2G. Therefore, these results suggest that BG34-CD11b engagement occurs through multivalent binding.

The engagement of BG34-200-CD11b occurs on the activated I domain, specifically at peptide residues that are distinct from other known epitopes

Research has demonstrated that the functional capacity of CD11b in myeloid and myeloid derived cells is influenced by a conformational change induced by environmental stimuli, rather than by upregulation or downregulation.28 29 When responding to stimuli, CD11b can shift from a low-affinity state to a high-affinity state. In contrast to the ‘closed’ conformation of the low-affinity state, the ‘open’ conformation of the high-affinity state is characterized by an unfolded I ectodomain (128S-L321), which is also known as the A domain (figure 3A). Although there is limited data linking the structure and function of the I domain, experimental results suggest that the I domain contains multiple functional epitopes that can interact with various ligands, triggering multiple cell functions.12–14

In figure 3A, the CBRM1/5 epitope is shown to be one of the well-known epitopes found on the activated I domain. This binding epitope has been characterized as...
**Figure 3** BG34-200-CD11b engagement occurs in the activated I domain at the distinguished peptide residues and mediated distinguished function. (A) Illustration of the CD11b ‘close’ and ‘open’ conformation in the I domain, highlighting functional epitopes that engage with corresponding ligands to induce cellular responses. (B) FACS analysis of surface expression of WT.1 and VIM12 in THP-1 cells treated with PBS or PMA. (C) (I) Frequency of cells displaying positive stain to BG34-200-AF647 after pretreatment with PBS or anti-CBRM1/5-FITC (CBRM1/5) antibodies; and (2) Frequency of cells displaying positive stain to CBRM1/5-FITC after pretreatment with PBS or BG34-200, as determined by FACS. (D) Cytokine production in cells receiving different treatments, as determined by ELISA assays. (E) Intracellular ROS production in cells receiving different treatments, as determined by ROS assays. For C, D, and E, PMA-treated THP-1 cells were used; *, p<0.5; **, p<0.01; ns, not significant. Unstained, singled stained, and non-specifically stained cells served as controls to establish positive versus negative gating in (B and C). CTL, control; FACS, fluorescence-activated cell analysis; ICAM-1, intercellular adhesion molecule 1; IL, interleukin; PBS, phosphate-buffered Saline; PMA, phorbol 12-myristate-13-acetate; ROS, reactive oxidative species; TNF, tumor necrosis factor; WT, wild type.
the $^{147}\text{P}^{149}\text{H}^{151}\text{R}$ in the loop before and at the beginning of the $\alpha$-helix 1, as well as $^{200}\text{K}^{205}\text{T}^{206}\text{L}$ in the loop connecting $\alpha$-helices 3 and 4. These six residues are adjacent in the structure of the I domain and have side chains that are well-exposed to the solvent.\textsuperscript{30} The engagement of CBRM1/5 with the ICAM-1 (intercellular adhesion molecule 1) or fibrinogen ligands plays a critical role in monocyte adhesion.\textsuperscript{30,31} An ADH503 compound, also known as GB1275, was reported to be able to stabilize the CBRM1/5–ICAM-1 engagement by binding to the allosteric pocket (F-G) in the I domain. This results in the upregulation of pro-inflammatory cytokines interleukin (IL)-1$\beta$, IL-6, and tumor necrosis factor (TNF)-$\alpha$.\textsuperscript{31} The linear peptide residues at $^{232}\text{RA}^{245}$ are also of the well-known epitopes on the I domain. Its engagement with iC3b-opsonized cells or targets plays an important role in mediating intracellular reactive oxidative species (ROS) responses in monocytes and neutrophils, inducing a cytotoxic effect on target cells.\textsuperscript{32} Zymosan, a yeast-derived $\beta$-glucan, enhances the (F-G)–iC3b engagement and engagement-triggered ROS activity by binding to the non-membrane-activation related AA residues.\textsuperscript{33}

To investigate the potential impact of BG34-200-CD11b engagement on the function of known epitopes, we used a human monocyte model: THP-1 cells treated with PMA (phorbol 12-myristate-13-acetate) for in vitro assays. At low concentrations (<20 ng/mL), PMA treatment can polarize THP-1 cells towards pro-inflammatory activation, as evidenced by the high-level expression of pro-IL-1$\beta$, without the need for additional stimulation by cytokines or bacterial components.\textsuperscript{34} After PMA treatment, THP-1 cells were positive for both the FITC-tagged anti-CD11b-VIM12 (VIM12-FITC) and the PE-tagged anti-CD11b-WT.1 (WT.1-PE) stains. Since the VIM12 clones bind to non-membrane-activation-related epitopes and the WT.1 clones bind to the epitopes located at the I domain,\textsuperscript{35} these results suggested that PMA treatment leads to the generation of cells with an activated CD11b in the ‘open’ conformation (figure 3B).

After examining the cells, we observed that both WT.1-PE$^+$ cells that were prestained anti-CBRM1/5-FITC monoclonal antibodies (CBRM1/5-FITC) and those that were not showed the same level of positivity when further stained by AF647-tagged BG34-200 (BG34-200-AF647) (figure 3CJ). Our previous studies characterized BG34-200-AF647, which demonstrated high stability against different pH levels and various enzymes.\textsuperscript{15,17} In addition, when we prestained the WT.1-PE$^+$ cells with or without BG34-200-AF647, we observed the same level of positivity when further stained by CBRM1/5-FITC (figure 3CI).

Moreover, we found that the cells treated with ADH503 in the presence of ICAM-1-coated beads exhibited enhanced upregulation of IL-1$\beta$ and IL-6, compared with the cells receiving only ICAM-1 beads. In contrast, cells treated with BG34-200 in the presence or absence of ICAM-1-coated beads showed an upregulation in the TNF-$\alpha$ production (figure 3D).

Furthermore, we discovered that those which were subjected to zymosan treatment while exposed to iC3b-coated beads displayed a heightened intracellular activity in ROS. This was evidenced by the measurement of hydroxyl, peroxyl, and other ROS within the cells. On the other hand, cells that underwent BG34-200 treatment and were stimulated with iC3b beads exhibited a slight decrease in ROS activity, as depicted in figure 3E.

Based on the data we collected, it appears that the engagement of BG34-200 with CD11b did not have an impact on the I domain engagement at the allosteric pocket or the CBRM1/5 epitope. This was supported by the observation that the BG34-200 treatment did not affect the availability of peptide residues to interact with their correspondent ligands. Moreover, BG34-200 engagement had the potential to induce distinct cellular functions, as it resulted in TNF-$\alpha$ upregulation. This contrasted with the ADH503-stabilized CBRM1/5 engagement with its ligands, which resulted in IL-1$\beta$ and IL-6 upregulation. Of particular interest, our findings suggest that BG34-200 engagement exhibited higher binding affinity than I domain engagement with the iC3b targets at the epitope $^{232}\text{RA}^{245}$. This was implied by the slight reduction in the iC3b molecule-triggered ROS activity in cells following BG34-200 treatment.

Taken together, our findings suggest that BG34-200 acts as a specific carbohydrate ligand that engages with activated CD11b at the previously unreported peptide residues, which mediate different functions from the other known ligands.

The intravenous administration of BG34-200-AF647 had a direct impact on the tumor-associated inflammatory monocyte present in the peripheral blood

After demonstrating that BG34-200 could specifically engage with ‘open’ CD11b, we aimed to determine whether this engagement could impact any myeloid derived cell function in a specific and/or selective manner, following its intravenous administration. To investigate this, we conducted a fluorescence tracking study using BG34-200-AF647 in an xenograft KPC mouse model, which enabled us to track and monitor the kinetics, migration, and distribution of this compound.

Our findings revealed that the BG34-200-AF647 compound was completely cleared from the plasma in approximately 24 hours, as evidenced by the time-dependent decrease of AF647 fluorescence signals in the plasma samples (figure 4I). The BG34-200-AF647$^+$ cells were detected in the circulating white blood cells (WBCs) around 4 hours, peaked around 10–72 hours, and returned to baseline levels after 72 hours (figure 4AII). However, in tumor-free mice, we did not observe the plasma clearance of BG34-200-AF647 compound (figure 4AIII) nor the presence of the BG34-200-AF647$^+$ cells in the WBCs throughout the experiment within 72 hours (figure 4AIV). These results suggest that the
Figure 4  BG34-200-AF647 intravenous administration shows a direct impact on the tumor-associated inflammatory monocytes (TAIMs) in the peripheral blood. (A) Kinetic distribution of BG34-200-AF647 in the peripheral blood of KPC tumor-bearing mice (I and II) and tumor-free mice (III and IV). (I) and (III): MFI of BG34-200-AF647 in plasma samples of mice, as determined by colorimetric analysis. Tumor-free plasma sample spiked with BG34-200-AF647 (0.1 mg/mL) served as positive control. MFI of plasma sample from individual mouse was graphed as mean±SD. **, p < 0.01. (II) and (IV): Frequency of BG34-200-AF647 + cells in the WBCs, as determined by FACS analysis. (B) Surface expression of Ly6C and Ly6G by the CD11b +BG34-200-AF647 + cells in the peripheral blood in the KPC tumor-bearing mice at 10 hours after BG34-200-AF647 intravenous administration, as determined by FACS. (C) Frequency of TAIMs and the BG34-200-AF647 + TAIMs in the peripheral blood in the KPC, B16F10, K7M2 tumor-bearing and tumor-free mice. (D) Plasma CCL2 concentrations in mice bearing different solid tumors with PBS or BG34-200-AF647 treatment, as determined by ELISA assays. Tumor-free mouse plasma served as controls. (E) MFI of BG34-200-AF647 in tumors, tumor draining lymph nodes (TdLNs), and different organs, as determined by colorimetric analysis. MFI of samples from individual mouse were graphed as mean±SD. Two of the three data points represent pooled samples from two mice and one data point represents sample from one mouse. (F and G) Surface expression of CD11c, MHC II, and CCR2 by the KPC tumor-infiltrating CD11b +BG34-200-AF647 + cells (F) and circulating CD11b +BG34-200-AF647 + cells (G), as determined by FACS. For A, at 2, 10, 24 and 72 hours after BG34-200-AF647 intravenous injection, blood samples were collected and processed to collect white blood cells. The cells were then analyzed by FACS to determine the frequency of the BG34-200-AF647 + cells. Frequency of cells from individual mouse were graphed as mean±SD. For B, C, D, and G, the plasma and WBCs were collected at 10 hours after BG34-200-AF647 intravenous administration. For F, the tumor-infiltrating cells were collected at the 24 hours after BG34-200-AF647 intravenous administration. Mice bearing day-5 KPC tumors were intravenously administered with PBS or AF647-tagged BG34-200 (BG34-200-AF647) at 50 mg/kg. At 20 min, 2, 4, 10, 24 and 72 hours after BG34-200-AF647 injection, mice were sacrificed to harvest blood, tissue, organs, and tumor samples. n=5. BM, bone marrow; CTL, control; FACS, fluorescence-activated cell analysis; IV, intravenous; MFI, mean fluorescence intensity; PBS, Phosphate-Buffered Saline; WBCs, white blood cells.
tumor-associated cells were responsible for mediating the plasma clearance of BG34-200.

Based on these kinetic data, we decided to investigate the BG34-200-AF647 cells at 10 hours after intravenous administration in tumor-bearing mice. The BG34-200-AF647 cells were found to be positive for CD11b-AF405 and Ly6C-AF488, but negative for Ly6G-PE stain (figure 4B). These findings suggest that the tumor-associated cells responsible for mediating the plasma clearance of BG34-200 were likely monocytic rather than granulocytic CD11b+ cells, as they were Ly6C+Ly6G- in phenotype (figure 4B).

To better understand the tumor-associated CD11b+ monocytic cells, we implemented a complete separation strategy of CD11b+ cells. We analyzed the cells 10 hours after the intravenous administration of BG34-200-AF647 to the KPC, B16F10, and K7M2 mice. This approach divided the Lin (CD3, CD19, CD20, CD56)−CD11b+ cells into Ly6G+ and Ly6G− cells and then analyze the cell expression of CD62L and Ly6C. As a result, we could distinguish granulocytic-Myeloid-Derived Suppressor Cells (MDSCs, Lin'CD11b'Ly6G'CD62L'Ly6C') and monocytic MDSCs (Lin'CD11b'Ly6G'Ly6C'CD62L'CCR2'). Moreover, positive CCR2 expression by the Lin'CD11b'Ly6G'Ly6C'CD62L' cells allowed us to identify inflammatory monocytes (Lin'CD11b'Ly6G'Ly6C'CD62L'CCR2') (online supplemental figure s3A,B). Since murine anti-CD11b antibodies targeting different peptide residues within the I domain were not available, in this study we could only use anti-CD11b antibodies that bound to non-membrane-activation related sites. Based on these analyses, we found that tumor-associated inflammatory monocytes (TAIMs) directly interacted with the BG34-200-AF647 in the peripheral blood in the three mouse models of solid cancers, as almost all the TAIMs were BG34-200-AF647+ in the tumor-bearing mice (figure 4C).

According to literature, healthy individuals typically have only 1–5% of activated CD11b in the ‘open’ conformation in their peripheral blood, whereas patients with tumor burdens may have 10–30%. Based on this information, we believe that our findings are supported, demonstrating that BG34-200 can directly, effectively, and specifically target tumor-associated CD11b+ inflammatory monocytes by engaging with the activated CD11b in the open conformation I domain.

Since the tumor stroma could produce CCL2 chemokines that attract CCR2+ inflammatory monocytes to the TME, we characterized the CCL2 concentrations in the plasma and found a positive correlation between the frequencies of TAIMs and the CCL2 plasma concentration in the three mouse models (figure 4C and D). Next, we wanted to track and analyze the tumor-infiltrating BG34-200-AF647+ cells. Based on the kinetic biodistribution data, we determined to investigate the tumor-infiltrating cells 24 hours after the intravenous administration (figure 4E). Interestingly, the tumor-infiltrating BG34-200-AF647+ cells expressed CD11b, CD11c and MHC II but were negative for CCR2, suggesting a phenotype of monocyte-derived dendritic cells (mo-DCs) (figure 4F).

To investigate the correlation between the antitumor response and the impact of BG34-200 impact on TAIMs, we used a multiparameter fluorescence-activated cell analysis (FACS) analysis to characterize myeloid cell subsets in blood and tumor samples from untreated and treated groups. We applied a validated 15-parameter flow panel to characterize the cells, and statistical t-distributed stochastic neighbor embedding (t-SNE) analysis to identify distinct cell clusters within the samples.

In our multiparameter FACS setting, we opted to use 15 markers due to their expression across different subsets of myeloid cells. These markers are valuable in characterizing cell phenotype, function, and tissue localization, making them a useful tool for understanding cell subsets within a heterogeneous sample (figure 5A). However, defining specific cell subsets based on cellular markers alone can be challenging. To overcome this challenge, we employed statistical t-SNE analysis to identify distinct cell clusters within our samples by integrating the FACS data with tumor size data to provide additional context for cell identity and function (figure 5B–E).

In our study, we administered a high dose of BG34-200-AF647 (150mg/kg) B16F10 tumor-bearing mice intravenously, and collected blood (B) and tumor samples (T) from untreated PBS (Phosphate-Buffered Saline) and treated (BG34-200) groups at various time points following the intravenous administration of either PBS or BG34-200 to the B16F10 mice or tumor-free mice (figure 5B).

Using our 15-parameter flow panel, we stained both the blood and tumor samples and performed t-SNE analysis, which enabled us to identify and characterize a total of 27 distinct cell subsets within all the samples (figure 5C). The t-SNE analysis allowed us to generate a heatmap that visualizes the expression patterns of multiple markers, which helped to highlight the subsets of cells that exhibit unique expression profiles (figure 5D).

To illustrate our strategy of analysis, we presented the t-SNE analysis of blood samples B3 (PBS group, day 3) and B8 (BG34-200, day 3), as well as the tumor samples T3 (PBS, day 3) and T8 (BG34-200, day 3) (figure 5E and F). From these graphs, we identified the subsets of cells that differed in frequency between the PBS and BG34-200 groups and plotted the frequency of each subset in each sample against tumor size. Specifically, we selected cluster 3, 9, 11, 12, 13, 14, 18, 19, 23, 25 and 26 from the blood samples, as well as cluster 9, 13, 16, 17, 18, 20, 25 and 27 from the tumor samples (orange dash contour lines), which together accounted for over 90% of the cells in...
Figure 5 Multiparameter FACS and t-SNE analysis reveals a correlation between the direct impact of BG34-200 on circulating TAIMs and the tumor-infiltrating mo-DCs, and the observed antitumor effects. (A) Description of 15 markers used to analyze myeloid derived cells. (B) Illustration of experimental design for the schedule, sample collection, and labeling. Each sample label represents an average of three data points obtained from three individual mouse. (C) PhenoGraph analysis of all the samples identified 27 distinct cell clusters that can be spatially organized by t-SNE 1 and 2. (D) X-shift analysis of the 27 cell clusters (X-axis), characterized by phenotype color bar code to define the average expression of fluorescent markers (Y-axis). (E) Grayscale PhenoGraph analyses of the cell clusters in blood sample B3 (PBS group) and B8 (BG34-200 group), as well as tumor sample T3 (PBS group) and T8 (BG34-200 group). The selected clusters are highlighted by orange contour lines. (F) Illustration of plotting the frequency of a specific cell cluster in both the PBS and BG34-200 groups against tumor size on the same graph. (G) Cell frequency of each selected cluster in blood or tumor samples from both the PBS and BG34-200 groups against tumor size. ns, not significant; **, p<0.01. FACS, fluorescence-activated cell analysis; IV, intravenous; mo-DCs, monocyte-derived dendritic cells; TAIMs, tumor-associated inflammatory monocytes; t-SNE, t-distributed stochastic neighbor embedding.
these samples (E). Next, we analyzed the cell frequencies of each of these clusters in each sample and plotted the frequencies of both the PBS and BG34-200 groups on the same graph to determine whether and how the frequency of the subsets correlated with tumor size (F). Our analysis revealed that only cluster 3 in the blood samples and cluster 27 in the tumor samples were affected by BG34-200 treatment and exhibited a significant correlation with tumor reduction (figure 5G).

Based on the phenotype analysis, it was observed that cluster 3 exhibited a phenotype of CD11b^CCR2^CX-CR3^Ly6C^Ly6G^Siglec1^low^, suggesting that it displays a phenotype of inflammatory monocytes. Importantly, this cluster 3 was found to be absent in the blood of tumor-free mice, suggesting that it is a tumor-associated cell subset (online supplemental figure s4). The analysis of cluster 27 revealed that it exhibited a phenotype of F4/80^CD68^ Arg^CD86^IDO^MHC II^Siglec 1^CD11c^, suggesting that it is a macrophage-like DC subset or mo-DCs. Since our fluorescence tracking studies have identified a similar tumor-infiltrating subset (CD11b^CD11c^MHC II^CCR2^) referred as mo-DCs (figure 4), we preferred to refer to this F4/80^CD68^ Arg^CD86^IDO^MHC II^Siglec 1^CD11c subset as mo-DCs instead of macrophage-like DCs. It is important to note that DCs derived from monocytes have similar morphology and phagocytic activity as macrophages; however, they also possess the ability to present antigens to T cells, which is a hallmark function of DCs. Therefore, we studied the function of these cells in the following sections.

Although the results presented in figure 5 did not directly support the cluster 3 differentiation towards cluster 27, phenotypes of these two clusters demonstrated consistency to our findings in figure 4. Our findings in figure 4 regarding the correlation between the BG34-200-AF647^- CD11b^-CD11c^-Ly6C^-Ly6G^-Siglec 1^-infected cells in peripheral blood and tumor tissue suggested the potential impact of BG34-200 on monocyte differentiation towards DCs, which was further investigated in the following section. Here, considering the results presented in figure 5, as well as those obtained from figure 4, it could be inferred that BG34-200 exerted a direct effect on TAIMs, leading to an increase in mo-DCs within the TME.

**The intravenous administration of BG34-200 can promote the differentiation of TAIMs into mo-DCs, resulting in an increased capacity for antigen presentation**

To investigate whether the direct interaction of BG34-200 with TAIMs could impact the differentiation of cells into mo-DCs, ex vivo assays were performed to examine cell function and differentiation. Initially, the CD11b^high^CCR2^high^ cells sorted from the blood of PBS-treated mice showed significantly lower messenger RNA (mRNA) expression in PU.1 (transcriptional factor regulating cell differentiation into DCs) and MafB (transcriptional factor regulating cell differentiation into macrophages) compared with bone marrow (BM) derived inflammatory monocytes (figure 6A), indicating that TAIMs may have limited differentiation capabilities. In contrast, TAIMs sorted from the blood of BG34-200-treated mice exhibited significantly higher mRNA expression in PU.1 (transcriptional factor regulating cell differentiation into DCs) compared with cells from PBS-treated mice (figure 6B), suggesting that BG34-200 treatment could potentially convert the untreated TAIMs, the CD11b^high^CCR2^high^PU.1^low^ subsets, into the CD11b^high^CCR2^high^PU.1^high^ subsets in peripheral blood. Notably, the impact of BG34-200 treatment on PU.1 expression in TAIMs was observed in all three mouse models of solid cancers.

Next, we discovered that culturing the cells with a DC differentiation medium supplemented with Granulocyte macrophage Colony-Stimulating Factor (GM-CSF)/IL-4 and stimulating cytokines (TNF-α) led to the generation of approximately 18.9% CD11c^+^ cells. This was a significant increase compared with the CD11c^+^ cells (~7.3%) generated from the PBS group, as shown in figure 6C. This in vitro condition was employed based on established method for culturing DCs and was supported by our published results,10 which indicated elevated concentrations of the cytokines GM-CSF, IL-4 and TNF-α in tumor tissues following systemic administration of BG34-200.

Moreover, when we pulsed the cells derived from B16F10 mouse blood with irradiated B16F10-OVA cells and co-cultured them with CD8^+^ T cells from OT I mouse spleen, we observed a substantial increase in T-cell proliferation in the cultures from the BG34-200-treated groups compared with the cultures from the PBS group (figure 6D).

Taken together, these findings in figures 4–6 suggest that the intravenous administration of BG34-200 could potentiate the untreated TAIMs, the CD11b^high^CCR2^high^PU.1^low^ cells, leading to their transformation into the CD11b^high^-Ly6C^low^CCR2^high^PU.1^high^ cells in the peripheral blood. These cells exhibited a greater capability to differentiate into mo-DCs, which in turn displayed an improved ability to present antigens and stimulate antigen specific T-cell proliferation.

It is noted that in this study, we did not conduct experiment involving the injection of BG34-200-treated cells into tumor-bearing mice and investigate the secondary lymphoid organs, where the activation of the tumor specific immune response occurs. However, these studies have been conducted and are documented in our published paper.18 In that study, we successfully observed the presence of BG34-200^+^ DCs in both the tumor tissues and tumor-draining lymph nodes. Moreover, we performed injections of BG34-200-treated lymph node-derived T cells into tumor-bearing mice, which resulted in a significant improvement in anti-tumor efficacy.18
Intravenous administration of BG34-200 induces changes in the internalization rate of binding complexes, phagocytosis rate and intrinsic ICAM-1 status

To examine the phenotype of CD11c+ cells generated from groups treated with PBS and BG34-200, we gated on CD11b+CD11c+ mature DCs derived from mouse blood treated with PBS or BG34-200, and analyzed the surface expression levels of MHC I (kβ), MHC II (I-Aβ), CD86 (B7.2 co-stimulatory molecules), CD80 (B7.1), CD40, CD11b, and CD54 (intercellular adhesion molecule I, that is,
ICAM-1) using corresponding antibodies at saturation concentrations (figure 7A). The two groups showed no significant difference in the percentage of surface expression of these biomarkers. However, a subset of the CD11b+ cells from the BG34-200-treated group exhibited lower fluorescence intensity of CD11b compared with the cells from the PBS-treated group (figure 6A, red rectangle).

Previous studies have shown that certain ligand-mediated ICAM-1 using corresponding antibodies at saturation concentrations (figure 7A). The two groups showed no significant difference in the percentage of surface expression of these biomarkers. However, a subset of the CD11b+ cells from the BG34-200-treated group exhibited lower fluorescence intensity of CD11b compared with the cells from the PBS-treated group (figure 6A, red rectangle). Previous studies have shown that certain ligand-mediated interactions between ICAM-1 and its ligands (e.g., LFA-1) are crucial for the activation and migration of immune cells. In our study, the BG34-200 treatment modulated the function of the TAIMs-derived DCs through triggering internalization of binding complexes, phagocytosis, F-actin cytoskeletal rearrangement, and intrinsic ICAM-1 surface aggregation. (A) The per cent expression of DC-related biomarkers by matured CD11c+ cells derived from KPC tumor-bearing mice treated with PBS or BG34-200 treatment, as determined by FACS. Within the CD11b+ cells, a fraction of cells of the BG34-200-treated group exhibited lower fluorescence intensity than the cells of the PBS-treated group (red rectangles). Within CD54+ cells, a fraction of cells of the BG34-200-treated group showed higher fluorescence intensity than the cells of the PBS-treated group (green rectangle frame).

(B) Schematics of the ex vivo experimental setting to study the internalization rates of binding complexes and the phagocytosis rates. KPC-derived cell line TB32048 was directly injected into pancreas to create the KPC-tumor-bearing mice. For both assays, the TAIM-derived immature DCs were co-cultured with BG34 samples for 30, 60, 90, 120, 150 and 180 min. (C) Averaged MFI of the single CD11c+CD11b-AF488 ‘BG34-200 AF647’ cells at different co-culture time, as determined by FACS. (D) Average MFI of the single CD11c+PE‘E coli’-AF488 at different co-culture time, as determined by FACS. For C and D, the averaged MFI of the single cells = (Total MFI of the positive cells) / (total number of the positive cells); cells of PBS, BG34-50, BG34-200, and BG34-500 groups were examined in triplicates; Data expressed as mean±SD. (E) Stimulated emission depletion (STED) microscopic imaging (100×) of mature DCs from PBS and BG34 groups. The DCs were stained with ICAM-1-AF488 and F-actin-CF568 and fixed on non-functional glass surface. For statistical analysis, the STED imaging was performed on 50 randomly picked cells of each group. Image analysis of the four groups of 50 cells resulted in the identification of four different status (I, II, III, and IV) (left). The numbers of cells adopting each status were recorded for PBS, BG34-50, BG34-200, and BG34-500 groups to assess the effect of different treatments on ICAM-1 status and F-actin cytoskeletal structure (right). Abs, antibodies; DCs, dendritic cells; E. coli; Escherichia coli; FACS, fluorescence-activated cell analysis; GM-CSF, Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF); ICAM-I, intercellular adhesion molecule I; IV, intravenous; MFI, mean fluorescence intensity; PBS, Phosphate-Buffered Saline; ROI, region of interest; TAIMs, tumor-associated inflammatory monocytes.

Figure 7  BG34-200 treatment modulates function of the TAIMs-derived DCs through triggering internalization of binding complexes, phagocytosis, F-actin cytoskeletal rearrangement, and intrinsic ICAM-1 surface aggregation. (A) The per cent expression of DC-related biomarkers by matured CD11c+ cells derived from KPC tumor-bearing mice treated with PBS or BG34-200 treatment, as determined by FACS. Within the CD11b+ cells, a fraction of cells of the BG34-200-treated group exhibited lower fluorescence intensity than the cells of the PBS-treated group (red rectangles). Within CD54+ cells, a fraction of cells of the BG34-200-treated group showed higher fluorescence intensity than the cells of the PBS-treated group (green rectangle frame). (B) Schematics of the ex vivo experimental setting to study the internalization rates of binding complexes and the phagocytosis rates. KPC-derived cell line TB32048 was directly injected into pancreas to create the KPC-tumor-bearing mice. For both assays, the TAIM-derived immature DCs were co-cultured with BG34 samples for 30, 60, 90, 120, 150 and 180 min. (C) Averaged MFI of the single CD11c+CD11b-AF488 ‘BG34-200 AF647’ cells at different co-culture time, as determined by FACS. (D) Average MFI of the single CD11c+PE‘E coli’-AF488 at different co-culture time, as determined by FACS. For C and D, the averaged MFI of the single cells = (Total MFI of the positive cells) / (total number of the positive cells); cells of PBS, BG34-50, BG34-200, and BG34-500 groups were examined in triplicates; Data expressed as mean±SD. (E) Stimulated emission depletion (STED) microscopic imaging (100×) of mature DCs from PBS and BG34 groups. The DCs were stained with ICAM-1-AF488 and F-actin-CF568 and fixed on non-functional glass surface. For statistical analysis, the STED imaging was performed on 50 randomly picked cells of each group. Image analysis of the four groups of 50 cells resulted in the identification of four different status (I, II, III, and IV) (left). The numbers of cells adopting each status were recorded for PBS, BG34-50, BG34-200, and BG34-500 groups to assess the effect of different treatments on ICAM-1 status and F-actin cytoskeletal structure (right). Abs, antibodies; DCs, dendritic cells; E. coli; Escherichia coli; FACS, fluorescence-activated cell analysis; GM-CSF, Granulocyte Macrophage Colony-Stimulating Factor (GM-CSF); ICAM-I, intercellular adhesion molecule I; IV, intravenous; MFI, mean fluorescence intensity; PBS, Phosphate-Buffered Saline; ROI, region of interest; TAIMs, tumor-associated inflammatory monocytes.
binding to CD11b can trigger internalization of the binding complex, leading to a reduction in CD11b cell surface expression.\(^{40}\)

In addition, we observed that a subset of CD54\(^+\) cells from the BG34-200-treated group exhibited higher fluorescence intensity of CD54 (ICAM-1) compared with the cells from the PBS-treated group (figure 6A, green rectangle). Interestingly, DCs’ intrinsic ICAM-1, which is a co-stimulatory ligand that binds to leukocyte function-associated antigen 1 (LFA-1) of T cells to induce cell action, is known to exist in two statuses on the plasma membrane.\(^{41,42}\) The first status is a high lateral mobility status that is incapable of T-cell priming. This is supported by findings that DCs expressing a high mobility ICAM-1 mutant were unable to induce LFA-1 affinity maturation in T cells, resulting in diminished antigen-dependent conjugate formation with T cells. The second status is a constrained clustering status that is important for antigen presentation capability. These two statuses are regulated by DC maturation-induced changes, including phagocytosis and F-actin cytoskeletal rearrangement.

Based on this information, we sought to investigate whether treatment with BG34-200 could trigger these changes. To assess the CD11b-mediated internalization of binding complexes, we co-cultured immature DCs with AF647-tagged anti-CD11b antibodies (clone 1C4) or AF647-tagged BG34 samples for varying periods of time, stained by AF488-tagged anti-CD11b antibodies, and measured the averaged MFI of CD11c\(^+\) cells that potentially internalized the CD11b-AF488(BG34)-AF647\(^+\) complexes (figure 6B). The anti-CD11b-AF647 antibodies (1C4 clones) served as a control since they could induce internalization of the target,\(^{34}\) and we used them to monitor the baseline internalization of CD11b. By plotting the average MFI of the CD11c\(^+\)CD11b-AF488(BG34)-AF647\(^+\) cells versus co-culture time, we observed that BG34-200 treatment could induce immature DCs to internalize the binding complexes at a significantly higher rate than BG34 of 50 and 500 kDa, as well as the antibody control (figure 6C).

To assess the phagocytosis rate, we co-cultured immature DCs with AF488-tagged Escherichia coli microparticles for varying periods of time and then examined the average MFI of CD11c\(^+\) cells that phagocytosed the AF488\(^+\) E. coli (figure 6B). By plotting the average MFI of CD11c\(^+\)E. coli-AF488\(^+\) cells versus co-culture time, we observed that BG34-200 treatment could induce immature DCs to phagocytose E. coli particles at a significantly higher rate than the PBS, BG34-50 or BG34-500 treatment (figure 6D). Notably, although the BG34-500 could mediate a higher binding affinity to the same peptide residues (figure 2F), it did not appear to induce the internalization of the binding complex and phagocytosis.

To investigate the impact of BG34 treatment on the arrangement of the F-actin cytoskeleton, we generated mature DCs from four groups: PBS, BG34-50, BG34-200 and BG34-500. These DCs were cultured on non-coated (non-functional) coverslips in untreated tissue culture plates, fixed and stained with anti-ICAM-1-PE and CF568 Phalloidin. The coverslips were then mounted on premium frosted microscope slides for two-dimensional Stimulated Emission Depletion Microscopy (STED) imaging. We randomly selected 50 cells from each group for statistical analysis. The STED imaging generated stacks of images of ICAM-1 and the actin filament network at various axial positions. The resulting images revealed novel, distinct structures of ICAM-1 and filamentous actin, which could be categorized into four distinct types (figure 6E). Cells of type I and II displayed evenly distributed ICAM-1 on the cell membrane, while cells of type III and IV displayed aggregated ICAM-1.

In terms of actin cytoskeletal structures, cells of status I are distinguished by a ring-shaped structure and actin that forms ruffles at the cell perimeter. Cells of status II exhibited a dense network of long filaments at the cell perimeter, whereas cells of status III display densely organized short filaments that are evenly distributed in the cytoplasm space and on the cell surface, pointing towards the extracellular space. Finally, cells of status IV possess both a dense network of long filaments and densely organized short filaments in the cellular and intracellular spaces. Among 50 DC morphologies that we examined, the cell frequencies of status I–IV were significantly different among the four groups. While the visualization of actin dynamics in T cells during immune activation is well-established, little is known about actin dynamics in DCs. As a result, we are unable to predict DC functions based on their status I–IV. Nonetheless, our data demonstrate that BG34-200 treatment can induce a unique F-actin filament network that differs from those of the non-treated, BG34-50-treated and BG34-500-treated groups.

Taken together, these data indicate that treatment with BG34-200 can stimulate immature DCs to internalize the CD11d binding complex and phagocytose foreign particles at a different rate than the PBS control, BG34-50 and BG34-500 groups. Furthermore, the BG34-200 treatment-induced rearrangement of the F-actin cytoskeleton in mature DCs, which was accompanied by intrinsic ICAM-1 surface aggregation and an enhanced cross-antigen presentation capability. These effects were not observed in mature DCs derived from the PBS, BG34-50, and BG34-500 groups.

**DISCUSSION**

β-glucans have been shown to induce an immune response in solid cancers. Most of the clinical experience in humans has involved the use of fungi-derived or yeast-derived β-glucans in combination with tumor-targeting antibodies, tumor vaccines or immune checkpoint inhibitors (eg. NCT01829373, NCT00682032, NCT00289003, NCT01492167, NCT00689258 and NCT02981303). Some of these glucans have been found to modulate innate immune responses by interacting with toll-like receptors. Our study results demonstrate that the β-glucan ligand BG34-200 can modulate immune responses by interacting...
with a c-type integrin CD11b, which has shown promising antitumor effect in mouse model of solid cancers such as pancreatic ductal adenocarcinoma, metastatic osteosarcoma, and advanced melanoma.

Our findings reveal that the BG34 carbohydrate ligands can effectively interact with the activated CD11b on the I (or A) domain, specifically targeting peptide residues that have not been previously reported. These peptides are distinguishable from those used in the development of CD11b-targeting antibodies or agents, including ADH503, a recently developed agonist targeting allosteric epitopes of CD11b I domain and undergoing early clinical trials for pancreatic cancer treatment.

The interactions between BG34-200 and CD11b are predominantly ionic, given that the three peptide residues all contain basic AAs such as lysine or arginine. These interactions are also highly specific, as substituting non-lysine amino acids with WT AAs results in significant binding affinity loss. Prior to our study, it was widely believed that carbohydrate-lectin binding was only ionic and not specific, as aldehyde groups in carbohydrates were thought to interact with basic AAs such as lysine, arginine, or histidine through ionic binding. However, this view was based mostly on structure-based predictions in mimicked physiological environments, rather than on experimental observations. Our study provides compelling evidence that the molecular nature of carbohydrate-lectin interaction can be both ionic and specific.

Additionally, the peptide residues bound by BG34 exhibited varying sequences, indicating that the BG34 molecules may contain structurally diverse binding units. Previously, it was believed that the interaction between C-type lectin CD11b and carbohydrates occurred through a small binding site architecture capable of accommodating only a single monosaccharide unit, such as a glucose-related, galactose-related, or mannose-related carbohydrate structure. However, the results of this study suggest that the interaction between BG34 and CD11b is not a simple ionic binding mediated by recognition of a single monosaccharide unit.

The primary structure of BG34 comprises an oligosaccharide consisting of 3–5 glucose connected by 1–3 glycosidic linkages, with the oligosaccharide units are further connected through 1–4 glycosidic linkages. Unlike other branched or charged polysaccharides, BG34 displayed a relatively ordered structure with no branch or charged groups in the main chain. At present, we lack additional structural data to determine if and how BG34’s ordered structure can form secondary structural units in physiologically solvents to bind to different peptide epitopes. This investigation would require co-crystallization of BG34 with specific peptides followed by Nuclear Magnetic Resonance (NMR)/X-ray analysis or cryo-electron microscopic imaging. Nonetheless, our results provided robust experimental evidence to support the previous hypothesis that some carbohydrate polymer ligands can form secondary structures in physiological conditions, likely with different densities or spatial arrangement, which play a crucial functional role in lectin binding and immune recognition.

Moreover, although BG34 of 50, 200 and 500 kDa all have the ability to bind to the peptides, their binding strengths differ. Only BG34-200 was found to induce beneficial biological changes in the context of tumors, suggesting that $MW$ plays a crucial role in determining biologically relevant binding strength. The extended carbohydrate polymer structure contains multiple copies of binding units, allowing it to form multivalent interactions and enhance binding affinity by clustering lectin binding sites and carbohydrate recognition units.

Based on these findings, we believe that BG34 of 50 kDa may lack sufficient copies of binding units, thus unable to mediate binding at a biologically relevant strength to induce biological changes. On the other hand, BG34 of 200 kDa contains enough copies to induce favorable binding strength and residence time, resulting in biological activity depending on the environment. BG34 of 500 kDa contains substantially excessive copies, which may cause aggregation or even precipitation of target molecules, rendering it unable to trigger any biological activity.

It should be noted that carbohydrate-lectin multivalent bindings have traditionally been considered weak interactions and involve dissociation or diffusion of the binding complexes. This poses a significant challenge to the quantitative measurement of these interactions and the study of this complex biological process. Our data has demonstrated that the specific carbohydrate-CD11b multivalent engagement can be accurately measured by determining the BG34 binding affinity to all three peptides. This discovery can be used to develop a potency assay to screen BG34 of various $MW$ and carbohydrates with different structures. The assays can help identify the structural parameter(s) that define carbohydrates capable of mediating biologically relevant binding strength to the lectin CD11b. The potency assay can also be used to test whether the beneficial biological activity induced by BG34-200 can be achieved using interfering peptides or antibodies that mimic biologically relevant bindings. Therefore, these studies significantly advance our current understanding of lectin engagement in innate immune recognition and enable us to use this knowledge to design and develop innovative immune modulators.

Furthermore, our studies revealed that the untreated TAIMs may have immunosuppressive properties in solid cancers. While the CCL2-CCR2 (chemokine-chemokine receptor) axis has been linked to aggressive phenotype and disease progression in some solid cancers, such as inflammatory breast cancers and pancreatic cancers, its role remains uncertain. In our study, we found that the untreated TAIMs in metastatic melanoma, osteosarcoma, and PDAC could be identified as CD11b$^{high}$Ly-6C$^{high}$CCR2$^{+}$PU.1$^{low}$ cells. Exposure to the plant-derived cell wall polysaccharide BG34-200 resulted in a significant increase in PU.1 expression in these cells, indicating their potential to differentiate into mo-DCs. While it is known...
that plant-derived and microorganism-derived substances can trigger the differentiation of inflammatory monocytes into microbicidal macrophages or mo-DCs, the environmental cues that determine the fate of monocytes are still unclear. Our fluorescence tracking studies suggest that BG34-200 may function as an external plant-derived stimulus, potentiating the TAIMs into the CD11b<sup>high</sup>Ly6C<sup>high</sup>CCR2<sup>−/−</sup>Pu.1<sup>high</sup> subsets, and differentiate them into mo-DCs in the tumor microenvironment.

In our previous studies, we identified upregulation of several proinflammatory cytokines, including GM-CSF, IL-4, TNF-α, and interferon-γ in the TME following intravenous administration of BG34-200. We believed that this upregulation was due to the BG34-200-induced downregulation of M2 macrophages and upregulation of TILs. While these factors were beneficial in creating an immunogenic tumor microenvironment, it remained unclear whether these cytokines, at their current type and concentration, were sufficient to support mo-DCs differentiation in the TME. In our current study, we discovered that BG34-200 treatment triggers CD11b-mediated internalization of the BG34-200-CD11b binding complexes, leading to a significant improvement in cells’ phagocytosis rate. Interestingly, we did not observe these biological changes in cells treated with BG34-50 or BG34-500. Cells with a higher phagocytosis rate exhibited F-actin cytoskeletal rearrangement and changes in intrinsic cell surface ICAM-1 status, critical structural changes necessary for mo-DCs antigen presentation capability. These findings suggest that BG34-200 treatment could induce beneficial antitumor immune responses by directly promoting mo-DC differentiation within the TME.

The direct and immediate biological changes triggered by carbohydrate-lectin binding have been poorly understood due to the lack of experimental data. Our data suggests that we can use the uptake rate of binding complexes, the internalization coefficient, and the phagocytosis rate as on/off parameters for further investigation. Importantly, we have conducted additional computational modeling analysis which demonstrates that Ca<sup>2+</sup> binding to CD11b I domain overlaps with the BG34-CD11b binding peptides<sup>236</sup>E-K<sup>247</sup> (figure 8A). This finding suggests a potential association between cellular calcium-related metabolite networks and dynamic cell uptake rates and coefficients. We believe that the quantifiable binding affinity measurement and the biological changes defined in this study will be essential in determining metabolite networks, on/off internalization and phagocytosis rates that are important for mo-DC cell differentiation and antigen presentation capability in the context of solid cancers (figure 8A,B and C).

Finally, the results of this study raise a crucial question: do the untreated TAIMs identified in solid cancers contain any CD11b variants, and how might this impact the anti-tumor efficacy in BG34-200? Our research in protein atlas data sets showed that a CD11b variant, linked with aggressive phenotypes of solid cancers, is defined by a single AA mutant (D157) that occurs next to occurred next to the BG34-200 bound peptide (158S-K<sup>170</sup>). Substituting this AA with lysine or arginine resulted in a 4–5-fold increase in BG34-200 binding strength (figure 2F – left panel). These results suggest that BG34-200 may be capable of influencing specific CD11b variants to elicit selectively biological activity.

While the connection between CD11b variants and disease progression is limited, we believe that this study’s findings highlight the importance of investigating CD11b variants in tumor-associated myeloid cells. Doing so will enhance our understanding of carbohydrate-lectin binding in the context of diseases and help develop personalized clinical biomarkers for precision medicine.

CONCLUSIONS

CD11b has been identified as a potential therapeutic target to modulate suppressive myeloid derived cells and induce tumor-reactive T-cell responses in TCGA (The Cancer Genome Atlas) data sets. However, CD11b’s ability to bind to multiple extracellular carbohydrate ligands has created a challenge in understanding how it converts differences in receptor-lectin binding into subsequent signaling responses that could be used for therapeutic development.

The results of this study suggest that BG34-200 could be used as a ‘differentiation’ ligand tool to trigger CD11b-induced responses that are essential for mo-DC differentiation and maturation in the context of solid cancers. These findings could lead to the identification and establishment of a new paradigm of nature inspired ligand-binding repertoire of CD11b important for monocyte differentiation as well as the development of antigen presentation capability. This study’s experimental data greatly advance our understanding of innate immune recognition and activation, leading to potential development of innovative immune oncology agents that can effectively modulate tumor-associated myeloid cells for future cancer immunotherapy.

MATERIALS AND METHODS

**Animals, cell lines, chemicals, and antibodies**

The C57BL/6J mice (H-2b), BALB/c mice (H-2d), CD11b<sup>−/−</sup> mice (B6.129S4-Itgamtm1Myd/J), tamoxifen-inducible KPC mice of PDAC (KRASLSL.G12D/+; TP53LSL.R172H/+; Pdxf1-Cre) were purchased from Jackson Laboratory. Tamoxifen and ADH503 were acquired from Millipore Sigma (Sigma-Aldrich, St. Louis, Missouri, USA). For the orthotopic injected KPC mouse model, we received the KPC tumor-derived pancreatic tumor cell line (TB32048) from Dr Stanley Huang’s laboratory at Case Western Reserve University. We also purchased the YFP-tagged KPC 7160c2 (KPCY – 7160c2) cell line from Kerafast cell bank (Kerafast, Boston, Massachusetts, USA). We cultured TB32048 and KPCY cell lines for 3–4 passages, with a maximum of 80% confluency in...
10% fetal bovine-serum (Invitrogen) in DMEM media and 1% penicillin/streptomycin (ATCC, Manassas, Virginia, USA) in a T175 flask under standard conditions (37°C, 5% CO2, 95% humidity). We regularly tested these cell lines in-house for mycoplasma. To develop mouse models of melanoma, osteosarcoma, and glioblastoma, we used B16F10 (CRL6475TM), K7M2 (CRL-2836) and GL-261 (CRL-1887TM) cell lines purchased from ATCC (ATCC). We cultured these cell lines as described above for TB32048 and KPCY lines.

We purchased fluorochrome-conjugated antibodies for mouse MHC II (M5/114.15.2) and MHC I (28-14-8) were purchased from Thermo Fisher Scientific (Thermo Fisher Scientific, Waltham, Massachusetts, USA). We bought all other antibodies from BD Biosciences (BD Biosciences, San Jose, California, USA).
The rates are: CD3 (145–2C11), CD19 (1D3), CD20 (GOT214A), CD56 (809220), CD80 (1G10/B7), CD62L (MEL-14), CD86 (PO3), CD4 (RM4-5), CD8 (5H10-1), CD25 (7D4), CD44 (IM7), CD11c (HL3), CD40 (3/23), CD45 (13/2.3), CD11b (M1/70, 1C4, WT.1, LS132.1D9, and VIM12), CD11c (HL3), CD54 (3E2), CD83 (Michel-19), CCR2 (475301), Ly6C (AL-21), and Ly6G (1A8).

MRI
The study used MRI to obtain high resolution $T_2$-weighted images of mice using a 7T Bruker Biospec MRI scanner. The mice were first anesthetized with isoflurane (2–3% in 100% oxygen gas). And then placed within the MRI scanner, where they were maintained at 35±1°C and continuously supplied with isoflurane to regulate their respiratory rate at 40–60 breaths per minute. Following initial localization scans, high resolution, coronal $T_2$-weighted images were acquired using a RARE MRI acquisition (TR/TE = 4298/35 ms, 37 slices, resolution = 500 × 117 × 117 microns, matrix = 512×256, 2 signal averages). To assess tumor volumes in each animal at each time point, ROI analysis was used, and ROI values were reported as radiant efficiency ((p/sec/cm²/sr)/(µW/cm²)).

BG34 sample preparation
BG34 samples of 50, 200 and 500 kDa were prepared in our laboratory and characterized according to our previous publications. These samples were dissolved in PBS solution at a concentration of 5 mg/mL as a stock solution for both in vitro and in vivo experiments.

To create biotin-conjugated samples, the BG34 samples were dissolved in an alkaline buffer (boric acid potassium chloride sodium hydroxide) at 1 mg/mL overnight with constant stirring. This process introduced aldehyde groups to the non-reducing end of glucan. The ARP (N-(aminooxyacetyl)-N'-D-biotinoyl) hydrazine) TFA salt (Sigma-Aldrich) was added in excess to the solution to react with the aldehyde group. After the reaction, the samples were purified using $M_v$ cut-off columns (cut-off $M_v$=10k) and centrifugation (5000 rpm for 30 min) to remove unreacted small molecules and salts. The purified samples were freeze-dried using a benchtop vacuum concentrator and freeze dryer (Neutec, Farmingdale, New York, USA). The resulting biotin-conjugated BG34 samples of different $M_v$ were used for peptide overlapping microarrays and AAs substitution scans.

For the preparation of AF647-conjugated BG34 samples, the biotin-conjugated BG34s were further reacted with AF647-tagged streptavidin (Thermo Fisher Scientifics). The resulting samples were again purified using $M_v$ cut-off columns (cut-off $M_v$=10k) and centrifugation (5000 rpm for 30 min) to remove any salts and/or unreacted molecules.

Fluorescence imaging
Fluorescence imaging was performed using IVIS Spectrum (Perkin Elmer, Waltham, Massachusetts, USA). Prior to and during imaging, all mice were anesthetized via isoflurane inhalation (2% isoflurane, oxygen flow rate of 2L/min) using the XGI-8 Gas Anesthesia System. Imaging was performed on both the dorsal and flank positions of the mice on specified days post injection. The acquired images were analyzed using the manufacturer’s Living Image V.4.7.3 in vivo software package. Standard region-of-interests (ROIs) were defined around tumor location and torso, and the ROI values were reported as radiant efficiency ((p/sec/cm²/sr)/(µW/cm²)).

Preparation of plasma and cell samples
To prepare plasma samples, mouse blood was drawn into purple top EDTA-coated BD vacutainer tubes (BD, Franklin Lakes, New Jersey, USA), completely filling the tube whenever possible to avoid dilution from the anticoagulant or preservative. The blood was immediately mixed by gently inverting the tube 5–10 times, then separated by centrifugation for 15 min at 2500 rpm. The resulting plasma was stored at −80°C.

To prepare peripheral blood mononuclear cells (PBMCs), mouse blood was drawn into purple top EDTA-coated BD vacutainer tubes. The whole blood was lysed with ACK lysis buffer, and PBMCs were separated by centrifugation for 15 min at 400 rpm. PBMCs were washed twice in PBS and resuspended in media without serum before we used them for functional assays.

Mouse models of solid cancers, dose, and schedule
In the B16F10 model, one million tumor cells were subcutaneously injected to WT or CD11b$^-$/C57BL/6J mice. In the K7M2 model, one million tumor cells were injected into Balb/c mice. Twice a week for 2 weeks, starting at 5 days after tumor cell injection, either PBS or BG34 samples (50 mg/kg) were intravenously injected. For the spontaneous KPC model, tamoxifen (100 mg/kg in corn oil) was intraperitoneally injected for 5 days to induce tumor growth. PBS or BG34 samples (50 mg/kg) were injected weekly for 8 weeks.

For the orthotopic pancreatic tumor models, half a million TB32048 or KPCY cells were dissociated with 0.25% trypsin-EDTA (PBS) (Thermo Fisher Scientific), washed twice in phosphate-buffered saline (PBS), and resuspended in a half-PBS half-Matrigel Basement Membrane Matrix High Concentration (BD Biosciences, New Jersey, New York, USA) matrix at 10,000 cells/µL. Fifty microliters (µL) of cell suspension was injected directly into the pancreas. The peritoneal wall was sutured using 6/0-gage coated vicryl sutures (Harvard Apparatus, Holliston, Massachusetts, USA) and the skin closed using two surgical clips and an Autoclip applier (MedVet, Brook Park, Ohio, USA).

For the mice that were used to collect blood and tumor cells for kinetic analysis, the mice were sacrificed at different time points after intravenous injection of either PBS or BG34 samples to collect the corresponding samples. Age-matched (6–12 weeks) and sex-matched mice were used for all experiments. All mice were maintained under specific pathogen-free conditions in the Animal Research Center of Case Western Reserve University (CWRU). The Institutional Animal Care and Use Committee (IACUC) of CWRU approved all animal protocols (protocol # 2016–0018).
was centrifuged at 300 g for 5 min to discard the plasma layer, and dilute the remaining cells were diluted with red blood cell (RBC) lysis buffer at a ratio recommended by the manufacturer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The RBC lysed blood cell suspension was processed to collect PBMCs by gradient centrifugation according to the standard protocol.

To prepare THP-1 cell-derived inflammatory monocytes, the cells were cultured in complete RPMI-1640 medium until they reach a density of 2–3 x 10^6 cells/mL. Then, add PMA to a final concentration of 20 ng/mL and incubate the cells with PMA for 48 hours at 37°C and 5% CO₂. After 48–72 hours, check the cells under an optical microscope to assess their adherence and spreading. Cells with increased adherence and spreading were used as inflammatory monocytes. For the culture of murine BM-derived inflammatory monocytes, follow the same procedure, but add PMA at a final concentration of 10 ng/mL. To prepare the peripheral blood derived monocytes, PBMCs were sorted by microbeads (Miltenyi Biotec, Cambridge, MA) to collect the CD11b⁺CCR2⁺ monocytes.

To prepare the monocyte derived DCs, the monocytes were cultured at 0.5–1 x 10^6 cells/mL in DC differentiation medium supplemented with 20 ng/mL recombinant mouse GM-CSF and IL-4 (R&D Systems, Minneapolis, Minnesota, USA) for 7 days. Fresh medium with GM-CSF and IL-4 were given on day 3 or 4. Non-adherent and loosely adherent clusters of immature DCs were harvested on day 7 for studies. The immature DCs were resuspended in fresh complete medium (CM) containing 10 ng/mL GM-CSF and IL-4 and stimulating cytokine TNF-α for an additional 2 days to generate mature DCs.

To prepare single cell suspensions of tumors, tumor samples were collected and immediately placed into cold complete medium. The tumor tissue was dissociated for 4–6 hours at 37°C using a cocktail of collagenase III, DNase I, and trypsin. Cell suspensions were washed, resuspended and lysed by RBC lysis buffer. For the preparation of BM cells, the BM was washed three times using complete medium, resuspended and lysed by RBC lysis buffer.

To stimulate cells with iC3b or ICAM-1 coated beads, we diluted the silicon beads (Sigma) to 10⁷ beads/mL and added 0.5 μg of iC3b (Sigma) or ICAM-1 (Abcam) proteins (in binding buffer for 4 hours at 4°C). The beads were then washed to remove any unbound protein and added to the cell culture at beads : cell ratio=2 : 1. After cells were cultured with iC3b beads for 4–6 hours, and then harvested for downstream applications.

**Surface stain and intracellular stains of cells**

To perform surface stain and intracellular stains of cells, single cell suspensions were first stained with surface molecules for 20–30 min at room temperature. Following staining, the cells were washed twice with PBS and then centrifuged to collect cell pellets. These pellets were then fixed in 20 volumes of fixation buffer (BD Bioscience, San Jose, California, USA).

For intracellular stains, the fixed cells were permeabilized using a permeabilization buffer according to the manufacturer’s protocol (R&D Systems). The permeabilized cells were then washed twice with a perm/wash buffer (BD Bioscience, San Jose, California, USA) to collect cell pellets. These pellets were then stained with antibodies for 20–30 min at room temperature. Once staining was complete, the cells were washed with the perm/wash buffer to remove unbound antibodies.

**FACS and sorting**

The stained and fixed cells were subjected to FACS analysis or sorting. For the single and multicolor FACS, BD LSR II (BD Biosciences) was used for data acquisition, and WinList V.10.0 was employed for data analysis. The fixed cells were sorted according to their marker on a BD FACS Aria III cell sorter (BD Biosciences).

For the 15-color multicomponent FACS analysis, we used LSRII FORTESSA flow cytometer. We obtained a customized flow panel from Dr. Rafiek’s laboratory of System Immunology Core laboratory of Case Western Reserve University. After administering a high dose of BG34:200 (150 mg/kg) to the tumor-bearing mice, we collected the blood and tumor samples at different time points and stained the cells with the validated 15-color flow panel. Data analysis was carried out using i-sNE to generate unbiased flow analysis using R studio packages.

**ELISA and ROS assays**

The concentration of CCL2 in plasma, as well as the concentrations of IL-1β, IL-6, and TNF-α in the THP-1 cell culture samples were determined using ELISA kits for mice (Abcam, Boston, Massachusetts, USA) following the manufacturer’s protocols. Plasma samples were diluted 1:5 in the binding buffer. Samples were added to wells that had been precoated with immobilized antibodies. Standards were also added to the wells. Next, any unbound biotinylated antibodies were washed away, and horse-radish peroxidase (HRP)-conjugated streptavidin was added to the wells. After three more washes, a tetramethylbenzidine (TMB) solution was added to develop the color, which was measured at 450 nm using an ELISA microplate reader (Promega, Madison, Wisconsin, USA). For ROS assays, we used a total reactive oxygen species assay kit from Invitrogen. Samples were prepared and assayed following the manufacturer’s instruction.

**Total RNA isolation and quantitative real-time PCR**

The isolation of total RNA and quantitative real-time PCR (RT-PCR) were performed as follows. First, total RNA was isolated from fresh cells using the RNeasy Plus Mini Kit (QIAGEN, Valencia, California, USA) according to the manufacturer’s protocol. During the column washes, an on-column DNase digestion was performed using the RNaSe-Free DNase kit (QIAGEN) to eliminate DNA contamination. Additionally, total RNA was isolated from...
fixed cells using reagents from the RNeasy FFPE kit and the RNeasy Plus Mini Kit (QIAGEN) following the manufacturer's protocol. The quantity of RNA was evaluated using the Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, California, USA). Subsequently, RT-PCR was conducted using a SYBR Green real-time PCR kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and LightCycler 1.5 instrument (Roche Life Sciences, Pleasanton, California, USA). Primers were purchased from Genentech (Genentech, San Francisco, California, USA). For PU.1, the forward and reverse sequences were GGG AGA GCC ATA GCG ACC AT (forward 5' – 3') and TAG GAG ACC TGG TGG CCA AGA (reverse 3' – 5'), respectively. For MafB.1, the forward and reverse sequences were TACTGGATGGGAGCACTACC (forward 5' – 3') and ACTACGGAAAGCGTCAAGCTC (reverse 3' – 5'), respectively. The expression level of each gene was normalized to the expression level of mouse β-actin.

**Cell internalization/phagocytosis assays**

To investigate the internalization rates of binding complexes, we cultured immature DCs with AF647-tagged anti-CD11b antibodies clone 1C4 (Abcam, Waltham, Massachusetts, USA) and AF647-tagged BG34 samples for up to 180 min. Following the culture period, we surface stained the cells with anti-CD11c-PE (R&D Systems), fixed, permeabilized, and intracellular stained them using anti-CD11b-AF488 antibodies (BG Biosciences), followed by FACS analysis. To investigate the phagocytosis rate, we cultured the immature DCs with AF488-tagged E. coli microparticles for up to 180 min, according to the Vybrant Phagocytosis Assay kit protocol (Thermo Fisher). Following the culture period, we quenched the cells with trypan blue, washed them with PBS thrice, surface-stained them with anti-CD11c-PE, and fixed, followed by FACS analysis.

**Cross antigen presentation capability assay**

The mature DCs were exposed to irradiated B16F10-OVA cells (20 Gy) at 1:4 ratio for 45 min. The pulsed DCs were cultured with naive OT I CD8+ T-cells at 5:1 ratio. The OT I mice (C57BL/6-Tg (TCRaTCRb) 1100Mjb/CRL) were purchased from Charles River Laboratory (Charles River, South San Francisco, California, USA). On sacrificing the mice, spleen collection took place. The CD8+ T cells were sorted using CD8 microbeads (Miltenyi Biotec). The T-cells proliferation was assessed through the e-Flor 660-Ki67 proliferation assay (Thermo Fisher), in accordance with the manufacturer’s guidelines.

**STED imaging**

Cells were cultured onto non-coated coverslips (ChemGlass Lifescience) in untreated tissue culture plates. The culture medium was gently and quickly removed by rinsing the cells with PBS, followed by treatment with 2% parafomaldehde in PBS for 15 min. The cells were then incubated with blocking buffer (Bio-Technne) and PE-tagged anti-ICAM-1 antibodies (BD Biosciences) for 2 hours, washed by PBS, and permeabilized with 0.1% Triton in PBS for 10–15 min. CF568 Phalloidin (Biotium) were added for 10–15 min and rinsed off by PBS. The cells were mounted on premium microscope slides (Thermo Fisher) using EverBrite mounting medium for STED imaging.

The STED microscopic imaging was performed using a Leica TCS SP8 gated STED microscope from the Light Microscopy Imaging Core at Case Western Reserve University. The microscope was equipped with a pulsed white-light laser (WLL) for tunable excitations, a 592 nm continuous-wave STED laser, a 775 nm pulsed STED laser, and the objective (HC PL APO CS2 100x/1.40 oil). For STED imaging, the WLL laser intensities were set at 70%, while both 592 nm and 775 nm STED lasers were used 100% to obtain a strong fluorescence depletion signal and improve optimal resolution. Images were acquired on the sequential Leica scan with the corresponding format and zoom factors, line average selected at 4 and frame average selected at 1, along with signal gain around 100%. In addition, all images were acquired on the Leica HyD detectors using time-gated detection from 1.0 to 6.0 ns.

Using the Huygens STED Deconvolution Wizard (Huygens Professional), a moderate degree of deconvolution was manually applied to the acquired STED images to avoid artifacts while to improve deconvolution. The fluorescence density distribution pattern of the PE-tagged ICAM in the treated and untreated cells were collected and quantified to evaluate the clustering status. The actin cytoskeletal patterns in the treated and untreated cells were collected and quantified to evaluate the changes in arrangement.

**Statistics**

The number of cell cultures and animals used in the experiments are documented in both the figures and figure legends. Although no statistical methods were employed to determine sample sizes beforehand, our sample sizes were consistent with those commonly used in the field. For comparisons of multiple groups, we used one-way analysis of variance followed by Holm-Sidak multiple comparison tests were used. Two-tailed Student’s t-test was used for in vitro comparison. Log-rank (Mantel-Cox) test was used to compare survival curves. All statistical analyses were performed using GraphPad Prism V.9.0 (GraphPad). Significance was set at p<0.05.

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