Regulation of phagosome processing & antigen presentation by macrophages and dendritic cells based on target stiffness

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Abstract

Cancer cells are deviant cells with an altered genetic make-up allowing them to bypass standard cell life. These cells possess unique characteristics which contribute to their ability to evade the immune cell response. It has recently been shown that physical properties such as stiffness could impact how these cancer cells are able elude immune surveillance of antigen-presenting cells (APCs), particularly macrophages (J774) and dendritic cells (DC2.4). This study focuses on the influence of target stiffness in the context of proteolytic degradation and antigen presentation. Using cell-mimicking deformable poly-AAm-co-AAc microparticles (DAAM-particles) of varying stiffness (5, 1 & 0.3 KPa) conjugated with appropriate proteins to evaluate the impact on APCs to internalize, process and present antigens. A new innovative method to distinguish internalized particles against the external ones was introduced by utilizing secondary antibody staining. Additionally, the concentration and functionalization method were optimized for assessing proteolytic degradation. This study demonstrated J774 macrophages exhibited significant interaction efficiency when compared with DC2.4 dendritic cells. We show that both J774 and DC2.4 are capable of degrading DQ-BSA. Our results suggest that degradation may not depend on stiffness. Furthermore, we did not find evidence of cross-presentation of ovalbumin (OVA) from phagocytosed DAAM-particles peptide on the major histocompatibility complex -I (MHC-I). Most interestingly, our results comparing a dendritic and a macrophage cell line, imply that dendritic cells degrade targets faster than macrophages. This is in contrast to prior evidence, and may be related to their proteolytic system. Although the findings were not conclusive, understanding the role of stiffness in affecting APC activity could potentially lead to reasons as to how cancer evasion occurs, with broad implications for prospective cancer immunotherapy.

1. Introduction

Cancerous cells are mutated cell types that have been difficult to fully understand and treat effectively. The development of this disease begins with the genetic alteration of an individual cell leading to unregulated proliferation. These deviant cells ignore canonical cellular machinery resulting in different hallmarks such as resisting cell death and enabling replicative immortality. One hallmark of cancer of particular interest is the ability to evade immune recognition and destruction. Ideally, cancer immune responses involve the inhibition of tumor progression in multiple stages. Immunosurveillance is the initial stage in which innate and adaptive immune cells persistently skim through for abnormalities [1,2]. However, there is a probability of cancer cells dodging the selection pressure by the immune system. This evasion occurs due to multiple well-known possibilities, including lack of tumor antigen perception due to neoplastic modification, resisting immune-induced cell death, or developing immune tolerance through the release of immunosuppressive factors [3,4]

Research has also shown that cancer cells change their stiffness [5,6]. Stiffness is measured as the resistance of a material to deformation under the influence of mechanical forces [7]. In other words, it demonstrates how capable cells can withstand the forces exerted by for example the extracellular matrix (ECM) [8]. The lowered stiffness of cancer cells can have large impact on cancer cell functions, it can hinder the infiltration of the immune cells in the tumor microenvironment, alter signaling pathways by suppressing antigen expression required for immune activation [5,6,9]. A domain of research with respect to immune evasion focused on studying the activity T regulatory cells. Cancer cells produce cytokines such as transforming growth factor (TGF)-β which is responsible for the conversion of CD4+ T cells into suppressive T regulatory cells. These type of T cells express a transcription factor FoxP3 which are known to highly immune suppressive by inhibiting anti-tumour T effector response. In other words, cancer immune evasion occurs through crippling T cells activity by the production of tumor necrosis factor (TNF)-α, IL-1, IL-6 and type I IFN. Cancer cells are also capable of suppressing dendritic cell maturation through the release of vascular endothelial growth factor (VEGF) [1,9-11]. There has been numerous research performed on the many molecular pathways cancer cells use to evade immunosurveillance. However, physical properties of cancer cells are a domain with a potential to give rise to new perspective in comprehending this hallmark of immune evasion by the cancer cells

Macrophages and dendritic cells, called antigen-presenting cells (APCs) are innate immune cells responsible for the initial immunosurveillance. These phagocytic cells express a myriad of pattern recognition receptors (PRR) for the recognition of pathogens or damage-associated molecular patterns (PAMPs / DAMPs) [12,13]. Binding of ligands to such receptors can induce phagocytosis, a pivotal mechanism used by APCs to facilitate internalization of antigens. Internalization is the first step followed by phagosomal maturation during which interactions with lysosomes and other organelles occur [14]. The lumen of the phagosome is thereby acidified, making it hostile environment for the antigens carried within, which are eventually degraded by proteolytic enzymes [15,16]. The processing of extraneous proteins leads to the transformation into immunoreactive peptides and is presented via the major histocompatibility complex (MHC) to T cells and other adaptive immune cells [17].

MHC-II molecules are solely presented on APCs specific to exogenous antigens while MHC-I molecules present endogenous antigens residing in the cytosol [18]. These antigens are presented to T cells causing the activation of different T cell lineages such as helper T cells (Th) and cytotoxic T cells (Tc). Specifically, antigens presented on MHC-I trigger Tc cells expressing the CD8 marker, and antigens presented on MHC-II trigger Th cells expressing the CD4 marker [17]. Lineages of T cells have unique functions, Th regulates immune response by releasing cytokines and modulates cell surface expression, while Tcs release cytotoxic compounds to induce target cell apoptosis [15,16]. Additionally, cross-presentation may transpire, when the peptide relocates to the cytosol and is presented via MHC-I instead of customary presented via MHC-II [19]. However, the fundamental mechanism of the peptide translocating to the cytosol is still obscure [20-22].

The core objective of this study is to provide some insight on how target stiffness influences immune cell response. Research has already shown that stiffness has an effect on internalization [23,24]. This study dives deeper into the downstream immunological processes which include proteolytic degradation and antigen presentation. To this end, we will employ biophysical strategies such as deformable poly-AAm-co-AAc microparticles (DAAM-particles) with various rigidities [24]. Comprehending how stiffness impacts immune cell activity might lead to a better understanding of how cancer cells evade immunosurveillance, and potentially identify new molecules and processes that can be targeted in cancer immunotherapies.

2. Materials and Methods

2.1. Cell culture

Murine J774.1 macrophage were obtained from the Cell Biology and Immunology department (Wageningen University and Research). The cells were expanded at 37°C in DMEM media (Gibco #11965092) containing 10% FBS (Gibco, #10082147), Pen-Strep (obtained from Cell Biology and Immunology department). The cells were physically scraped to get subcultures required for phagocytosis assay as well as for further regular passages.

DC2.4 dendritic cells, murine cell lines were obtained from the Cell Biology and Immunology department (Wageningen University and Research). The cells were cultured and maintained 37°C in Expansion Medium containing RPMI-1640 (Gibco, #61870036), 10% FBS, non-essential amino acids (Gibco, # 11140050), HEPES Buffer solution, Glutamax and Antibiotic-Antimycotic (anti-anti) (obtained from Cell Biology and Immunology department). The cells were initially aspirated with 1X PBS followed by the incubation with Trypsin (Gibco, #25200072) at 37°C for 5 mins. The detached subculture was resuspended in expansion media and the entire volume was transfered into a falcon tube for centrifugation at 300g for 5 mins. The supernatant was discarded and expansion media was added as required for phagocytosis assay as well as for regular passages.

2.2. Functionalization of DAAM particles

2.2.1. Functionalization of DAAM particles with DQ-BSA and BSA

Different stiffness of DAAM particle stocks (5, 1 & 0.3 kPa) were diluted to get 5% (v/v) solids in activation buffer (100mM MES, 200 mM NaCl, pH 6). DAAM-particles were then twice washed with activation buffer and then mixed at room temperature for 15 mins with 0.1% tween 20, ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC, 40 mg/ml), and N-hydroxysuccinimide (NHS, 20 mg/ml). The DAAM particles were washed (Table 1) thrice with 0.1x PBS (pH 6) + 0.2% tween 20 and resuspended in half the volume along with 2x PBS pH 8.5.

	Stiffness (KPa)	*Size (micron)*	*Centrifugation speed (g)*	*Centrifugation time (mins)*
Stiff	5	8.6	10000	1
Intermediate	1	11.3	10000	1
Soft	0.3	13	16000	2

Table 1. Comprehensive table on which DAAM-particles were used along with their stiffness, size, centrifugation speed and time which were followed during washing steps

Various concentrations of DQ-BSA (Invitrogen, #D12050) and bovine serum albumin (BSA, 10 mg/ml) were added together or consecutively (Figure 1, Table 2) and mixed at room temperature for respective time periods (Table 2). Subsequently, Tetramethylrhodamine Cadaverine (TMR-Cad, 0.1 mM) was added and mixed for 30 min at room temperature.

DQ-BSA concentration (mg/ml)	*Functionalization setup*
0.2	Separately
0.2	Together

Table 2. Categorization of the different groups of DAAM-particles functionalized with various concentration of DQ-BSA

DQ-BSA concentration (mg/ml)	*Functionalization setup*
0.1	Together
0.2	
0.5	

Table 3. Categorization of the different groups of DAAM-particles functionalized with various concentration of DQ-BSA

To this mixture, a blocking buffer was added (100 mM Tris pH 9 and 100 mM ethanolamine) and again mixed for 30 min at room temperature. Next the DAAM particles were washed thrice with 1x PBS pH 7.4 + 0.1% tween 20. Finally, the functionalized DAAM particles were mixed for 1 hr at room temperature with anti-BSA rabbit IgG (0.1 mg/mL) dissolved in 1x PBS pH 7.4. After incubation, the DAAM particles were washed twice with 1x PBS pH 7.4 + 0.1% tween 20 and resuspended in 1x PBS pH 7.4 then stored at 4C.

Figure 1. Illustration of how the DQ-BSA functionalization was performed

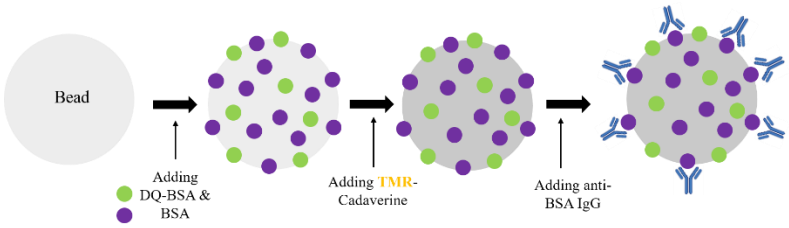

Figure 2. General overview of the functionalization procedure of DQ-BSA, BSA and the dye used for all degradation studies

2.2.2. Functionalization of DAAM particles with Ovalbumin

The protocol from Materials & Methods section 2.2.1 was followed with slight modifications. The DAAM particle stocks (5KPa) appropriately diluted to get 5% (v/v) solids and was subjected to activation with EDC followed by NHS. Ovalbumin (OVA, 4 mg/ml) (Invivogen, #vac-stova) and bovine serum albumin (BSA, 10 mg/ml) were added immediately (Figure 3) and mixed at room temperature for 1 hr. Subsequently, Tetramethylrhodamine Cadaverine (TMR, 0.1 mM) and Fluorescein isothiocyanate (FITC, 0.1 mM) were added and mixed for 30 min at room temperature. The DAAM-particles were washed and followed by blocking. The DAAM-particles were then coated with anti-BSA IgG and finally stored at 4°C.

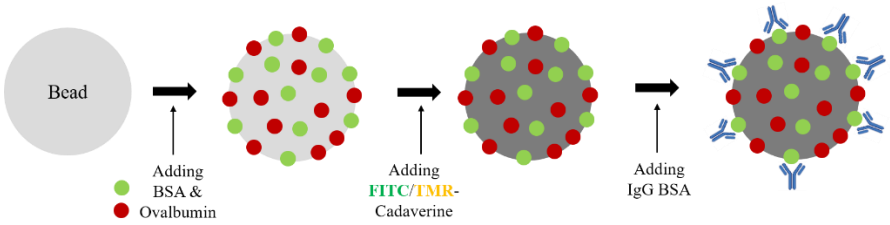

Figure 3. General overview of the functionalization procedure of Ovalbumin, BSA and the dyes used for all degradation studies

2.3. Phagocytic assays

J774s or DC2.4s were cultured from T75 flask and counted using hemocytometer. Cells were then seeded in a 24 well plate at a density of 7.5*10^4 cells in 0.5 ml media per well. The plate was incubated overnight at 37°C. The next day, the DAAM particle concentration was counted using hemocytometer and diluted with media for a final count of 5*10^5 particles per well. The media in each well was replaced with 200 µL media composing the functionalized DAAM particles (DQ-BSA/BSA and OVA/BSA) and the plate was spun at 30g for 1 min. The plate was then incubated at 37°C for different points based on experiment (Table). Additionally, for positive controls in antigen-presentation assays, the cells were directly incubated with

SIINFEKL peptide (100µM). At each time point, the cells were scraped with a pipet tip and transferred into Eppendorf tubes.

For degradation studies, the harvested cells were incubated with Alexa Fluor 647 donkey anti-rabbit IgG (0.1 mg/ml) (Invitrogen, #A31573) for 30 mins at 4°C.

For cross presentation studies, one group of the harvested cells were incubated with 2% Goat serum (Agilent, #X0907) for 15 mins at 4°C. The other group and after 2% goat serum incubation were followed by incubating with OVA257-264 (SIINFEKL) peptide bound to H-2Kb Monoclonal Antibody, APC, eBioscience™ (1.25 µg/ml) (Invitrogen, #17-5743-82) for 30 mins at 4°C (Figure 4).

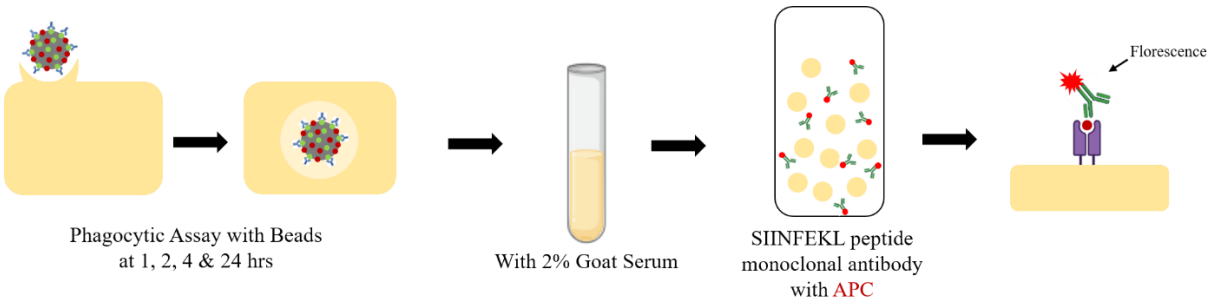

Figure 4. Schematic overview of how the cross presentation study was executed

2.4. Immunostaining and Flow Cytometry

Post-phagocytic assays, the samples were incubation with either the secondary antibody for distinguishing internal and external DAAM-particles and/or the monoclonal antibody for cross-presentation was transferred to FACS tubes with ice cold 1x PBS pH 7.4. These samples were analyzed using Cytoflex LX flow cytometer (Beckman Coulter) with a fixed acquisition volume of 50 µL. The gains of individual lasers were optimized for visualization and analysis. The data was analyzed using FlowJo (v.10.9.0) and raw data was exported to excel. The gating strategy is attached in the Appendix section.

3. Results

3.1. Phagocytic potential of dendritic cells

Based on previous research, it was established that J774 is capable of internalizing IgG-opsonized DAAM-particles [25. The objective was to understand if dendritic cells, which are regarded as the most efficient antigen-presenting cells, would also efficiently internalize DAAM-particles. To this end, we explored the phagocytic potential of DC2.4 using BSA, TMR-, and FITC-labeled DAAM-particles opsonized with anti-BSA IgG. Phagocytic assays were performed alongside J774 macrophages. We identified phagocytic events based on the quenching of the DAAM-particle FITC signal, due to acidic pH in the phagosome (Appendix 6.1). This indicated that DC2.4 was able to phagocytose DAAM-particles. The interaction efficiency was calculated based on the fraction of J774 and DC2.4 that are associated with the DAAM-particles (i.e. fraction of cells with adhered and internalized DAAM-particles) (Appendix 6.2). When comparing the interaction efficiency, there was a significant difference in between J774 and DC2.4 (Appendix 6.3). It can be observed that the interaction efficiency of J774 is 45% at 15 mins and increases to 60% at 60 mins. Meanwhile the DC2.4 have a lower interaction efficiency of 20% at 15 mins and barely hits 30% at 60 mins (Figure 5a). Additionally, internalization efficiency was calculated with respective to the fraction of J774 and DC2.4 with at least one internalized DAAM-particle. The difference in internalization efficiency between J774 and DC2.4 was found to be insignificant (Appendix 6.3). However, there is a gradual increase in both J774 and DC2.4 peaking at 40% and 60% at 60 mins respectively (Figure 5b). It is worth noting that the interaction efficiency is significantly greater for J774 which would translate into higher phagocytic activity. Nevertheless, it shows us that these dendritic cells, are capable of DAAM-particle phagocytosis. Dendritic cells are most relevant for our further studies planned around protein degradation and antigen presentation.

(a) **(b)**

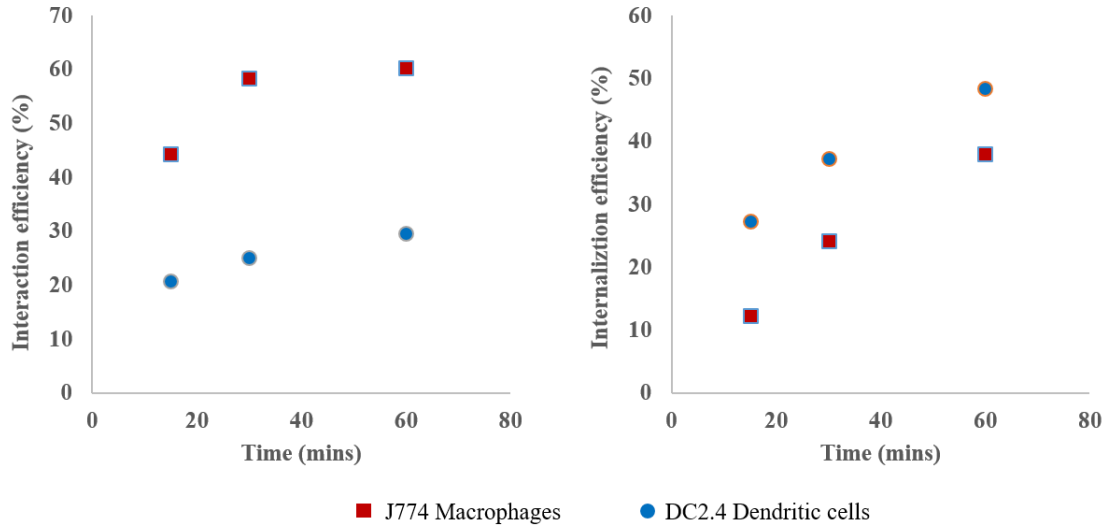

Figure 5. Comparative analysis of the (a) interaction efficiency and the (b) internalization efficiency of both DC2.4 and J774 after performing phagocytic assay using DAAM-particles coated with BSA, FITC & TMR dyes opsonised with anti-BSA IgG.

3.2. Differentiating internal and external DAAM-particles using antibodies

Self-quenching fluorogenic proteins like DQ™ Green BSA are commercially available and are ideal for studying the degradation potential of antigen-presenting cells like macrophages and dendritic cells. DQ™ Green BSA, however, has overlapping wavelength with FITC, which quenches in acidic phagosomes and is frequently used for discriminating between adherent and internalized particles [26]. Therefore, we sought alternatives for discriminating between adherent and internalized particles. Firstly, Bodipy 630/650 amine (BDP) was used as a substitute for the pH-sensitive FITC as previous research used this dye for lysosomal studies [27]. So, DAAM-particles were functionalized with BSA, FITC, BDP, and TMR. After performing the phagocytic assay at different time points (30, 60 & 90 mins), it was clear that the BDP dye was not pH sensitive like FITC (Appendix 6.4). This is likely because BDP630/650 pH-sensitivity depends on the functional group. Based on correspondence with the manufacturer, it is likely that the obtained carboxylic acid BDP is pH stable whereas the NHS ester BDP is pH sensitive. Hence, labelling DAAM-particles with carboxylic acid BDP is not suitable for differentiating "inside-outside".

Next, an approach in which DAAM-particles were labelled with only BSA, anti-BSA antibody and TMR (i.e. no pH dependent dye) was performed. Following the phagocytic assay, the harvested cells were incubated with Alexa Flour 647 secondary antibody at 4C. The rationale being that internalized DAAM-particles, unlike adherent DAAM-particles, are not available

for the secondary antibody to bind to and would hence show a lower fluorescence. Indeed, clear distinction between internalized DAAM-particles and the ones adhered on the cell surface was visible based on this antibody signal (Figure 6a). This distinction was also validated because by observing the fluorescence of FITC (Figure 6b). Hence, secondary antibody staining is a viable alternative to functionalizing DAAM-particles with FITC dye as an "inside-outside" differentiator.

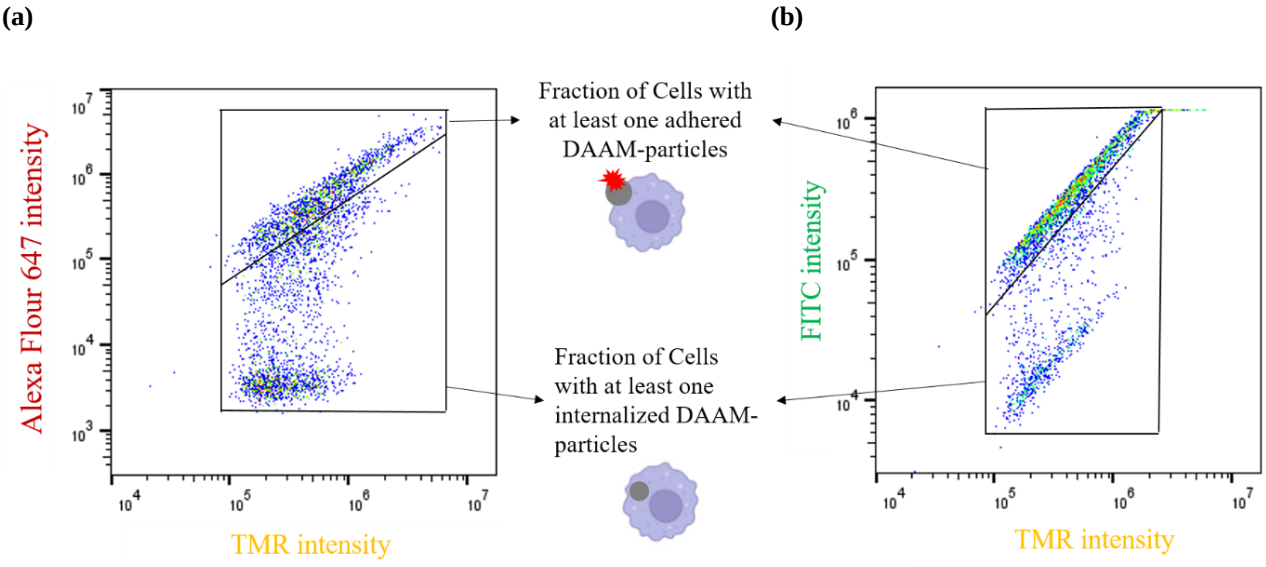

Figure 6. The analysis extracted from flow cytometer after phagocytic assay. (a) Alexa Flour 647 secondary antibody staining was used to differentiate the fraction of cells with internal and external DAAM-particles, which is validated with respect to the quenching of (b) FITC fluorescent signal.

3.3. Degradation of DQ-BSA by Antigen-presenting cells

3.3.1. Optimizing DQ-BSA functionalization

DQ-BSA possess as an ideal option for studying degradation potential of antigen-presenting cells like macrophages and dendritic cells. The rationale behind using DQ-BSA was that during degradation within the phagosome, the proteolytic enzyme breaks down DQ-BSA. As this breakdown occurs the self-quenched fluorophores conjugated onto the protein disassociates to emit green fluorescence [28,29]. There was a challenge that we faced while functionalizing the DAAM-particles with DQ-BSA alongside BSA. Both proteins are conjugated via the same amine functional group and this likely results in competitive binding between DQ-BSA and BSA, where the latter needs to be used at a high concentration (10 mg/ml) to efficiently trigger phagocytosis. To optimize functionalization, one set of DAAM-particles were incubated with DQ-BSA followed BSA for 30 mins for each protein and the other set of DAAM-particles were

incubated with DQ-BSA and BSA together for 30 mins. In order to confirm that binding of both proteins has taken place, these particles were directly run in flow cytometer with respect to green signal. Interestingly, the mean fluorescence intensity of DQ-BSA that was coated simultaneously or before BSA were similar. This suggested that functionalizing DQ-BSA and BSA together produced equivalent results to being functionalized separately and was considered to simplify the experimental setup (Appendix).

To narrow down on the concentration of DQ-BSA, different concentrations were chosen (0.1, 0.2 & 0.5 mg/ml) based on previous studies [28]. Phagocytic assay was performed using DQ-BSA/BSA coated DAAM-particles with the J774. Following the gating on the internalized with the secondary antibody staining (Figure 6a), there was evident distinction between degraded protein and the intact protein according to the increase in green fluorescence signal emitted by the disassociated fluorophores of DQ-BSA (Figure 6b). Based on the fluorescence emitted, 0.1 mg/ml and 0.2 mg/ml of DQ-BSA displayed a 3-fold and 4-fold increase. Meanwhile, 0.5 mg/ml of DQ-BSA had an 8-fold increase in green fluorescence, which was then chosen to be the most optimal concentration for further degradation studies.

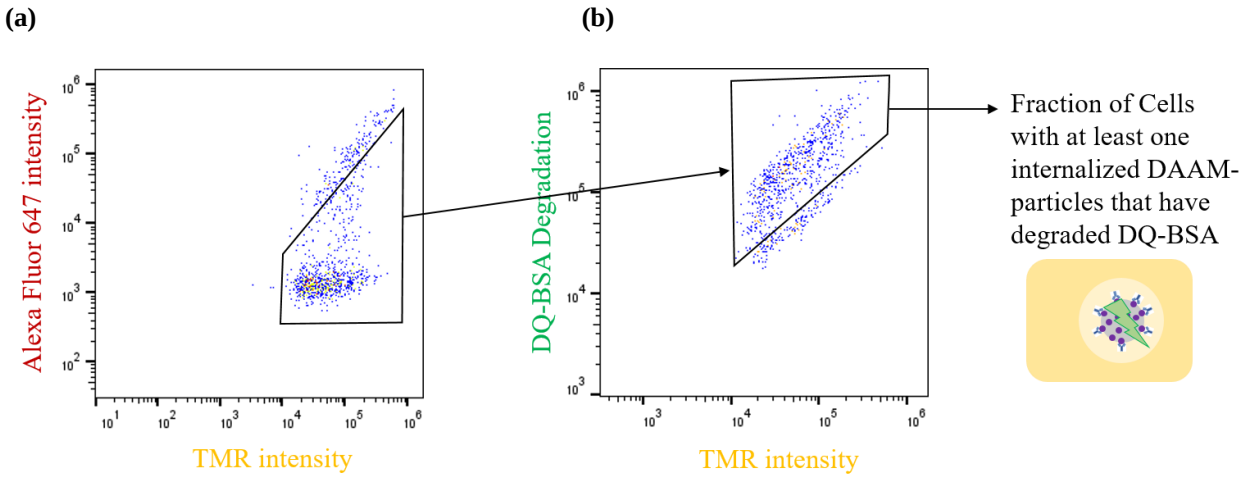

Figure 6. The analysis extracted from flow cytometer after phagocytic assay. The fraction of cells with at least one internalized DAAM-particle using (a) Alexa Fluor 647 antibody staining. Then the increase in green fluorescence signal highlights fraction of cells that have degraded DQ-BSA protein.

After determining the optimal method of functionalizing DQ-BSA onto DAAM-particles, three different stiffness of DAAM-particles were functionalized with TMR, 0.5 mg/ml DQ-BSA and BSA (10mg/ml) together. These particles were then incubated with the J774 macrophages. Following the antibody staining, the cells that have at least one internalized DAAM-particle were gated and observed for the green signal as previously mentioned. It can be observed that

there was a similar magnitude of increase in the percentage of DQ-BSA degradation across the three stiffness, starting at 30% of cells with degraded DQ-BSA at 30 mins and plateau between 2-4 hrs with 70% of cells with degraded DQ-BSA. Furthermore, it was shown the both the internalization (Appendix 6.5) and the degradation of DQ-BSA was not dependent on DAAM-particle stiffness (Figure 7).

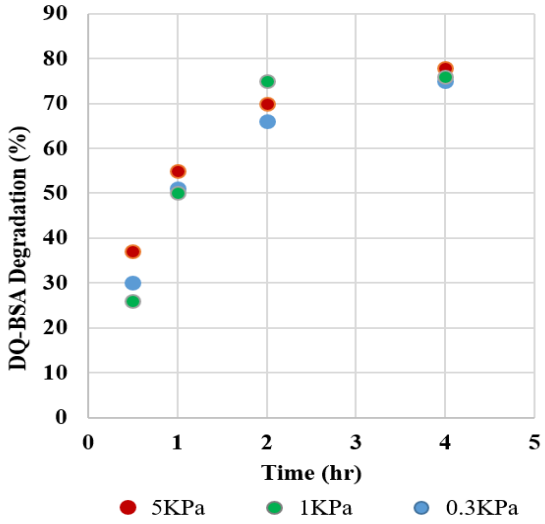

Figure 7. Graphical analysis of the DQ-BSA degradation with respect to different stiffness of DAAM-particles. The fraction of cells that have at least one internalized DAAM-particle were gating for the increase in green fluorescence signal emitting from the disassociated DQ-BSA fluorophores.

In order to compare to stiffness-dependent degradation potential of both antigen-presenting cells, phagocytic assays were performed with DC2.4 cells using the same DQ-BSA coated DAAM-particles of varying stiffness. Following the secondary antibody staining, based on the green signal, it indicated that the DC2.4 were capable of protein degradation. There was a clear distinction degraded protein and the whole protein. However, the degradation seemed to occur very quickly, since already 60-70% of cells with internalized particles had degraded DQ-BSA after 30 minutes (Figure 8a). After 1 hour, a plateau was already reached (Figure 8a). This is in stark contrast to the J774 macrophages, which had only 30% of cells with degraded DQ-BSA after 30 minutes and where reaching a plateau took at least 2 hours. To investigate these fast dynamics, we repeated the phagocytic assay with earlier time points of 10, 20, 30 & 60 mins (Figure 8b). Additionally, it was observed that the internalization efficiency was found to be stiffness dependent (Appendix 6.7). The DAAM-particle of 0.3KPa stiffness was found to be significantly lower as compared to 5KPa and 1KPa (Figure 8a). Additionally, The DAAM-particle of 1KPa stiffness was found to be significantly hgher as compared to 5KPa and 0.3KPa

(Figure 8b). However, it does not provide a definite indication that the DQ-BSA degradation is stiffness dependent.

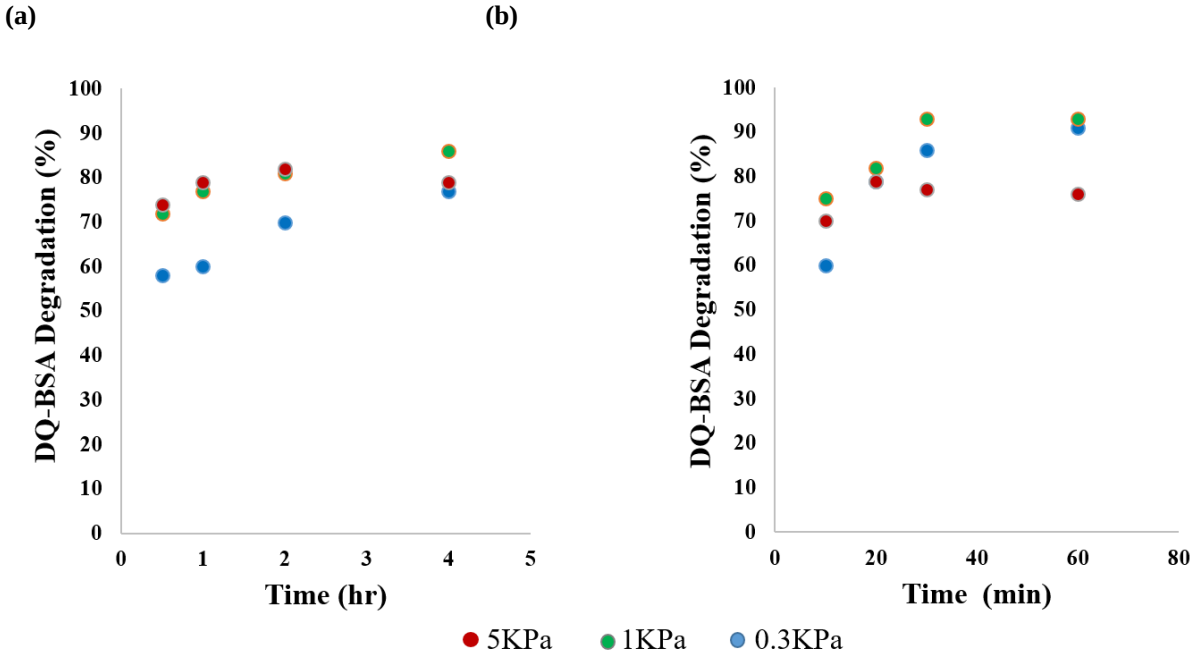

Figure 8. Graphical analysis of the DQ-BSA degradation study performed with DC2.4 with varying DAAM-particle stiffness, (a) representing the percentage of DQ-BSA degradation at time points 30 mins, 1,2 & 4 hrs then (b) plot representing the percentage of DQ-BSA degradation at time points 10, 20, 30 & 60 mins.

3.4. OVA cross-presentation

The next stage of the downstream immune processing we investigated, was e antigen presentation. To investigate the antigen presentation potential, ovalbumin was used and the presentation was tested by incubating the harvested cells with antibody specific to ovalbumin. For any antigen to be presented, it must go through either the MHC-I or MHC-II pathway. As the MHCs are highly polygenic and polymorphic leading to a number of possible combinations. So having an antibody compatible with the cell line's MHC haplotype is vital for this study. There were previous studies that have tested out the compatibility of OVA257-264 (SIINFEKL) peptide monoclonal antibody conjugated with APC that specifically binds to H-2Kb haplotype of MHC-1, which is present in the DC2.4 cells [30,31].

Phagocytic assays were performed at 1, 2, 4 and 24 hrs with DC2.4 using DAAM-particles coated with ovalbumin, BSA, TMR and FITC. There were two groups of samples; a group that was incubated for with 2% goat serum post-harvest which was commonly used in the prevention of non-specific binding followed by incubating with the ovalbumin monoclonal antibody and the other group was incubated directly with the SIINFEKL monoclonal antibody

post-harvest. The positive control was the SIINFEKL peptide that was directly incubated with the DC2.4 at the same points which were also divided into groups with goat serum and direct monoclonal antibody incubation post-harvest. For the negative control, the monoclonal antibody was directly incubated with the DC2.4 cells. Based on the quenching of FITC signal, it was apparent that the ovalbumin coated DAAM-particles were being internalized (Appendix 6.9). According to the positive control, we were able to observe that the experimental setup worked as the mean fluorescence intensity of red APC signal. This mean fluorescence intensity can be seen to increase rather slowly which can be observed from 4-24 hrs. Furthermore, the baseline (negative control) gives us a minimum threshold at which the SIINFEKL monoclonal antibody conjugated with APC emits fluorescence. However, the red signal from the ovalbumin coated DAAM-particles seems to be below to the baseline. This would imply that the DC2.4s were seemingly not able to present ovalbumin in any capacity (Figure 9).

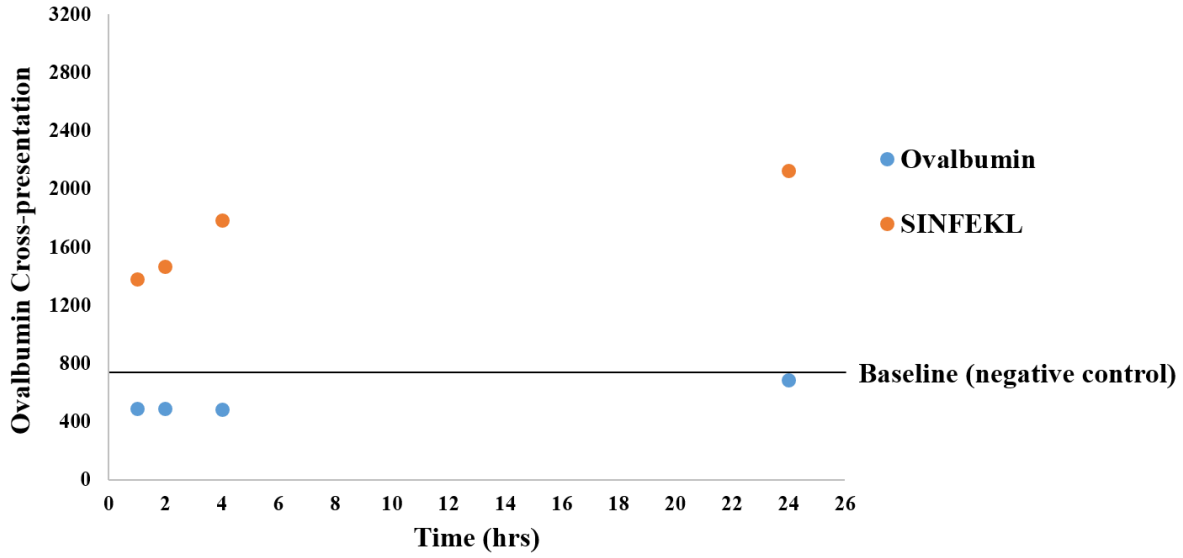

Figure 9. Percentage of cells that have cross presented ovalbumin and/or SIINFEKL peptide which have been categorized into group incubated with goat serum followed by monoclonal antibody at various time points (1, 2, 4 and 24 hrs).

4. Discussion

The process of immune evasion by cancer cells is a major hurdle in developing immunotherapy treatment options. Many immunotherapies focus on targeting molecular mechanisms to eradicate cancer but this disease still proves to be a challenge to formulate a suitable treatment option. To produce novel therapies, we considered biophysical properties of cancer cells namely cellular stiffness as a domain worth exploring. Research has found that cancer cells intrinsically modify their stiffness [5]. We have hypothesized that this alteration in stiffness provides the cancer cells the competency to escape the immune surveillance of immune cells.

The present study aimed to investigate the impact of stiffness on the phagocytic and antigen presentation abilities of antigen-presenting cells (APCs), specifically macrophages (J774 cells) and dendritic cells (DC2.4). According to the results of this study, both the antigen-presenting cells were able to phagocytose the DAAM particles. Interestingly, it also demonstrated unique characteristics between the J774 and DC2.4. The interaction efficiency of macrophages was higher at all-time points, suggesting that macrophages are more prone to adhering to and interacting with the DAAM particles. Physiologically, macrophages are present in seemingly all tissues and play a role in homeostasis, immune response. Due to their omnipotent presence through the tissues, these cells possess a wide range of receptors to recognize and interact with a variety of host cells and microorganisms [32,33]. However, the difference in internalization efficiency was found to be significant between DC2.4 and J774. This indicates that both cells internalize DAAM-particles but the J774 due to their high interaction efficiency would lead to a higher phagocytic potential. Both cell types are categorized as antigen-presenting cells and play crucial roles in immune response but several studies were performed eliciting the notable difference in their mechanisms and uptake efficiencies [32,33].

Macrophages are canonically known for their phagocytic potential as described in numerous researches. This cell type has proven to be highly efficient at internalizing an array of antigens, including the DAAM particles, through the process of phagocytosis and endocytosis. Some studies imply macrophages are capable of exhibiting a higher antigen uptake rate as compared to dendritic cells, particularly concerning rapid pathogen clearance. For instance, research has shown that the macrophages demonstrated higher uptake of antigens such as o β-lactoglobulin and thyroglobulin suggesting a more efficient phagocytic role [34-36]. Conversely, dendritic cells are also capable of phagocytosis primarily by capturing antigens but come at the cost of slower uptake [37,38]. Research has shown that this cell type is adept at capturing antigens but

is more capable in antigen presentation leading the induction of adaptive immune response [34,35]. In context with this study, the findings were consistent with previous studies implying that dendritic cells while being slower to engage with antigens, can process them more efficiently once internalized. This would suggest J774 macrophages to be more into engaging with pathogens and other host cells, while the DC2.4 dendritic cells being slow to interact. However, the difference in the internalization efficiency being insignificant

Recently, it was established that the internalization efficiency of J774 macrophages, similar cell lines (e.g. RAW 264.7 macrophages) and primary macrophages was stiffness-dependent [39]; and results from this study suggested that the DC2.4 posed similar characteristics concerning stiffness-dependent internalization. Regardless, we speculated based on the stiffness dependent uptake of DAAM-particles. We expected that the fraction of cells that degrade DQ-BSA would also be dependent on DAAM-particles of different stiffness. As previously stated the fraction of cells that internalize the stiffer DAAM-particles would display a higher percentage of DQ-BSA degradation. And fraction of cells that internalize the softer DAAM-particles would display a lower percentage of DQ-BSA degradation. The DAAM particle labeled with self-quenching DQ-BSA aided in quantifying the percentage of degradation of both J774 and DC2.4. In contrast to our hypothesis, the results from this study suggest that DQ-BSA degradation is not dependent on stiffness. After scrutinizing the results, it was noted that there was a significantly lower number of particles than targeted which could be a reason for the irregular internalization efficiency of the J774 across different stiffness groups. Similarly, the low amount of particles was also observed with the DC2.4 but the internalization efficiency was stiffness dependent. When working back through how the experiment was performed, the loss could be traced to manual error. Combined with our observation that internalization by J774s doesn't depend on rigidity, which is inconsistent with multiple experiments, this leaves this result to be non-conclusive at present and further repetition could improve the reliability of this result.

DCs are capable of taking up exogenous antigens and cross-present for the activation of CD8+ T cells. The use of ovalbumin is extensively studied cross-presentation and is considered a well-characterized model antigen [12,13]. We expected that the DAAM-particle coated with ovalbumin would be internalized by DC2.4, followed by the degradation and the degraded ovalbumin peptide would be presented via MHC-I to elicit a cytotoxic T-cell response. The results from the cross-presentation experiment suggested that the DC2.4 was not able to cross-present the ovalbumin-coated DAAM-particle. Antigens that have been taken up exogenously

follow the MHC-II pathway but there have been other cases in which these external antigens translocate to the cytosol. However, complete comprehension of this mechanism is still lacking. Based on the results and this prior knowledge, it is likely that the degraded ovalbumin from the DAAM particle did not enter the cytosol [41,42]. This meant that the chances of ovalbumin being cross-presented via the MHC-I pathway were scarce. In contrast, the positive control of directly incubated SIINFEKL (OVA257-264) peptide with DC2.4 demonstrated that cross-presentation by the cell line was possible; this would imply the SIINFEKL was able to translocate to the cytosol and enter MHC-I pathway. The efficiency of cross-presentation could be influenced by the form of ovalbumin used with the dendritic cells. Some studies have demonstrated ovalbumin derived from other sources namely E. coli or chicken eggs would be cross-presented with similar efficiencies, proposing post-translational modifications might not significantly impact cross-presentation [43-45]. We can also speculate the degradation of ovalbumin protein might not have lead the specific OVA257-264 peptide specific for the SIINFIKL monoclonal antibody to bind. Furthermore, the use of DAAM-particle for delivering ovalbumin to the dendritic cells has a lot of scope to improve through optimization of the experimental model or adopt another readout. The well-known method of studying cross-presentation is co-culturing the dendritic cells with T-cell hybridomas and checking for activation. Others consist of investigating the expression of CD69 which is a marker for lymphocyte activation and labeling T-cells with CFSE which can be used to monitor the proliferation [46,47].

According to previous research, protein degradation in dendritic cells occurs at a slower rate compared to macrophages. Phagosomal degradation occurs due to the fusion of lysosomes which consist of protease enzymes responsible for the breakdown of the proteins. Macrophages possess higher levels of lysosomal protease, leading to the rapid degradation of the internalized antigen. Meanwhile, the dendritic cells have a low protease activity which aids in the survival of the long chains of peptides within the phagosome for longer durations. This may be necessary because the cells migrate along the lymph nodes during which the antigens are processed and then presented to naive T-cells [47]. This migration is essential for the activation of the adaptive immune response [38,39]. The antigen is able to be preserved for a long duration due to a lesser antagonistic phagosome. It was also hypothesized that this could contribute to cross-presenting on MHC-I because of the higher probability that the antigen to enter the cytosol. Surprisingly, results from this study observed that degradation of DQ-BSA happened at a faster rate in DC2.4 than in J774. When focusing on the time taken to degrade DQ-BSA,

it was observed that the DC2.4 was able to degrade as quickly as 20 minutes. These results go against the preconceived research done in the past. The dendritic cells are specialized antigen presenting cells that have a sophisticated proteolytic system. These cells utilize both the proteasome as well as autophagy pathways to degrade proteins. This hypothetically should allow the proteins to be degraded rapidly. Research has highlighted that dendritic cells constantly receive signals from autophagosomes which enhances loading of antigens via the MHC pathway. This continuous signals would suggest that the dendritic cells can potentially degrade proteins a lot faster and present them, thus facilitating their function in inducing adaptive immune response.

To summarize, this study explored the impact of stiffness on immune cell activity with a particular focus on protein degradation and antigen presentation potential of APCs. By utilizing DAAM particles functionalized with proteins such as DQ-BSA and ovalbumin, the influence of varying stiffness was investigated on the degradation of phagocytosed particles. The findings were not conclusive to say that both J774 and DC2.4 are not capable of degrading proteins based on the DAAM-particle stiffness. Furthermore, to distinguish internal and external DAAM particles, secondary antibody staining was proven to be a viable option for traditional pH sensitive dye. Next, the functionalization of DQ-BSA together has been demonstrated to be as effective as functionalizing separately; also found the optimal concentration of 0.5 mg/ml was for degradation experiments. Additionally, this study tried to demonstrate the potential of DC2.4 to cross-present DAAM-particle coated with ovalbumin but was not successful. Comprehending how substrate stiffness influences the degradation and presentation by the APCs could shed some insight into cancer evasion mechanisms and potentially guide the development of novel immunotherapeutic strategies.

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6. Appendix

6.1. Gating strategies for understanding the phagocytic potential of DC2.4

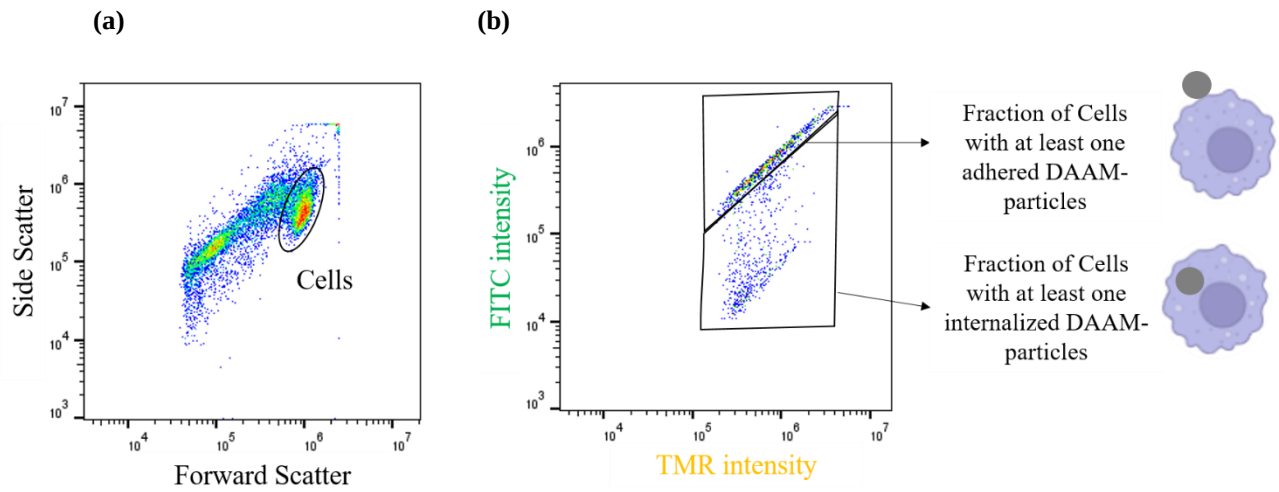

Figure 10. The analysis extracted from flow cytometer after phagocytic assay. The DAAM-particles coated with BSA, TMR and FITC were subjected to phagocytic assay by DC2.4. (a) represents the forward vs side scatter using which the cells are gated. Following that, based on FITC quenching, adherent and internal DAAM-particles were visualized.

6.2. Calculation used to determine Interaction and Internalization efficiency

$$\text{Interaction efficiency} = \frac{(\text{Fraction of cells with at least one adhered DAAM-particle} + \text{Fraction of cells with at least one adhered DAAM-particle})}{\text{Total cell population}} \text{ X } 100$$

$$\text{Internalization efficiency} = \frac{\text{Fraction of cells with at least one adhered DAAM-particle}}{(\text{Fraction of cells with at least one adhered DAAM-particle} + \text{Fraction of cells with at least one adhered DAAM-particle})} \text{ X } 100$$

6.3. Statistical Significance of Interaction and Internalization efficiency

(a)

Interaction efficiency			
Time (min)	15	30	60
DC2.4	20.86	25.05	29.69
J774	44.35	58.35	60.41
Ratio (DC2.4/J774)	0.47	0.43	0.49
p value	0.0011		
	Significant		

(b)

	Internalization efficiency		
Time (min)	15	30	60
DC2.4	27.3	37.3	48.4
J774	12.3	24.2	38.1
Ratio (DC2.4/J774)	0.45	0.65	0.79
p value	0.0643		
	Insignificant		

Table 4. Tabular presentation of the statistical analysis of the (a) interaction and (b) internalization efficiency of both J774 as well as DC2.4

6.4. Gating strategies for studying BDP630/660 coated DAAM-particle to distinguish adherent and internal particles

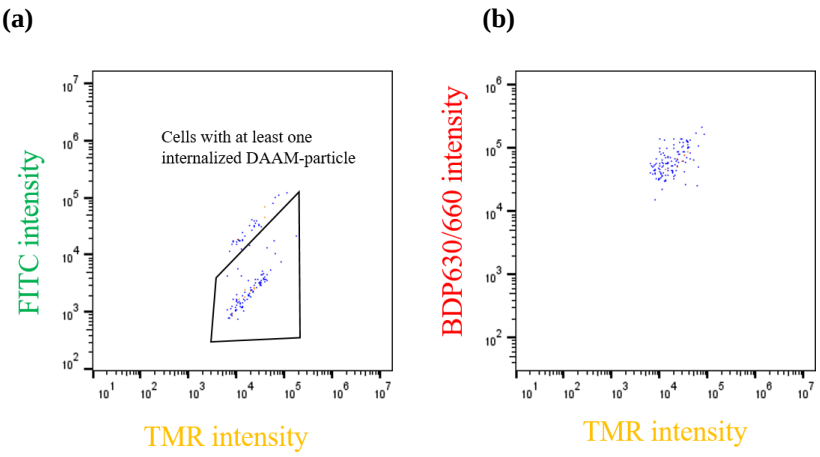

Figure 11. The analysis extracted from flow cytometer after phagocytic assay. DAAM-particles coated with BSA, TMR, FITC and BDP were used to perform phagocytic assay with J774. The quenching of the (a) FITC signal indicated the adherent and internal DAAM-particle. However, (b) BDP was unable to be used to differentiate adherent and internal DAAM-particle.

6.5. Internalization efficiency of J774 during DQ-BSA degradation study

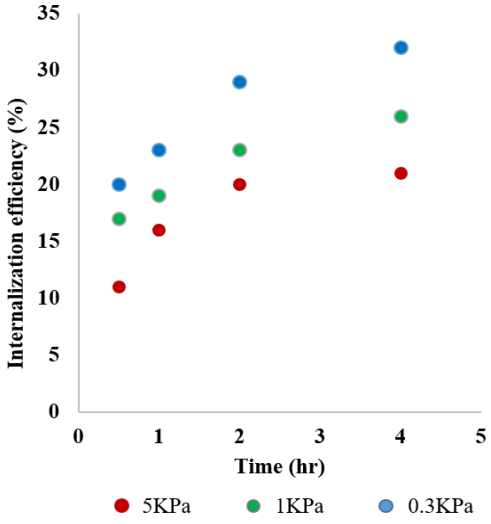

Figure 12. Graphical representation of the internalization efficiency of the J774 based on the gating with the secondary antibody signal while studying the degradation potential using DQ-BSA.

6.6. Statistical Significance of DQ-BSA degradation percentage of the J774

	Stiffness		
Time (hrs)	0.3KPa	1KPa	5KPa
0.5	30	37	26
1	51	55	50
2	66	70	75
4	75	78	76
Ratio (0.3KPa/1KPa)	0.81	0.70	0.87
5KPa/0.3KPa	0.93	0.91	0.98
(1KPa/5KPa)	0.94	0.93	0.88
	0.96	0.97	0.99
p value	0.08	0.14	0.12
Insignificant			

Table 5. Tabular presentation of the statistical analysis of the DQ-BSA degradation percentage by J774 based on the gating for the green signal.

6.7. Internalization efficiency of DC2.4 during DQ-BSA degradation study

(a) **(b)**

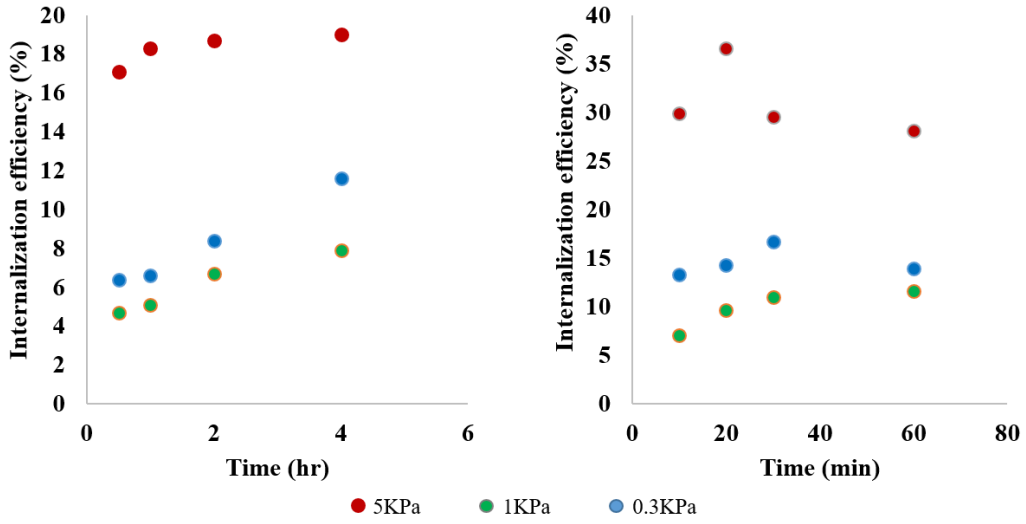

Figure 13. Graphical representation of the internalization efficiency of DC2.4 (a) at 30 mins, 1,2 & 4 hrs and also (b) at 10, 20, 30 & 60 mins based on the gating with the secondary antibody signal while studying the degradation potential using DQ-BSA.

6.8. Statistical Significance of Interaction and Internalization efficiency

(a) **(b)**

	Stiffness		
Time (hrs)	0.3KPa	1KPa	5KPa
0.5	58	72	74
1	60	77	79
2	70	81	82
4	77	86	79
Ratio (0.3KPa/1KPa)	0.81	0.97	0.78
1KPa/5KPa	0.78	0.97	0.76
(0.3KPa/5KPa)	0.86	0.99	0.85
	0.90	0.92	0.97
p value	0.009	0.087	0.043
	Significant	Insignificant	Significant

	Stiffness		
Time (mins)	0.3kpa	1kpa	5kpa
10	60	75	70
20	79	82	79
30	86	93	77
60	91	93	76
Ratio (0.3KPa/1KPa)	0.80	0.93	0.86
1KPa/5KPa	0.96	0.96	1.00
(0.3KPa/5KPa)	0.92	0.83	0.90
	0.98	0.82	0.84
p value	0.13	0.04	0.06
	Insignificant	Significant	Insignificant

Table 6. Tabular presentation of the statistical analysis of the DQ-BSA degradation percentage by DC2.4 (a) at 30 mins, 1,2 & 4 hrs and also (b) at 10, 20, 30 & 60 mins based on the gating for the green signal.

6.9. Gating strategy for ovalbumin cross-presentation assay by DC2.4

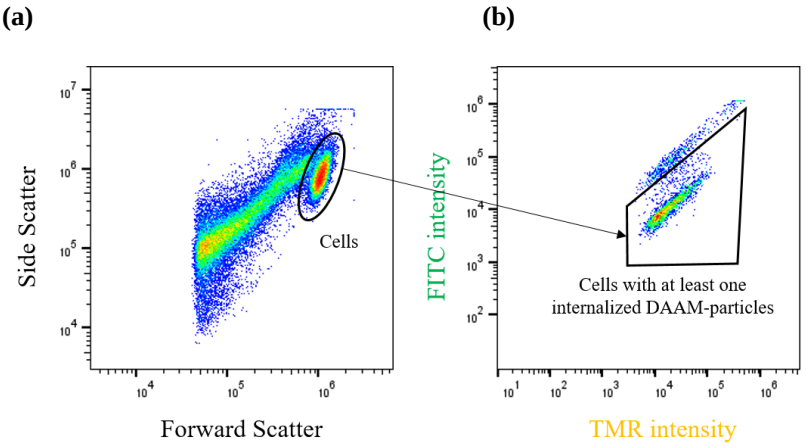

Figure 14. The analysis extracted from flow cytometer after phagocytic assay. The cells were gated based on the (a) forward and side scattering. Followed by the gating of the fraction of cells with internalized DAAM-particles according the (b) FITC signal being quenched.