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N-dihydrogalactochitosan as a potent immune activator for dendritic cells

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Abstract

Immunotherapy has become one of the fastest growing areas of cancer research. A promising *in situ* autologous cancer vaccine (inCVAX) uses a novel immune activator, *N*-dihydrogalactochitosan (GC), that possesses the ability to stimulate dendritic cells (DC). inCVAX is a combination treatment procedure involving treatment of the tumor with a thermal near-infrared laser to liberate whole cell tumor antigens, followed by injection of GC (a glucosamine polymer with galactose attached to the amino groups) into the treated tumor thereby inducing a systemic antitumor immune response. Regression of both the treated tumor and distant untreated metastases has been observed in both nonclinical and clinical settings following inCVAX. We studied the stimulatory action of GC on relatively immature DCs (DC2.4 cell line) *in vitro*. GC at 1 mg/mL was a potent stimulator for DC with limited toxicity, giving increased expression of major histocompatibility complex class 2, CD80, and CD11c. Confocal imaging also revealed qualitatively increased uptake of antigen (Texas red-labeled ovalbumin) by DCs after the introduction of GC. To visualize cellular uptake, GC was conjugated with FITC-fluorophore revealing its cellular internalization after 8 hours. In some cases GC was more effective than the toxic TLR4 agonist, lipopolysaccharide.

Keywords

N-dihydrogalactochitosan; glycated chitosan; *in situ* cancer vaccine; inCVAX; dendritic cells; MHC II; flow cytometry; confocal microscope

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INTRODUCTION

For several decades researchers have been searching for ways to trigger the immune system to recognize and destroy cancer. Despite a few successful reports, the search has been relatively unsuccessful.¹ In recent years, however, astonishing advances have revolutionized the field; these have even been termed a “paradigm shift”.² Discoveries have led to the development of effective check-point inhibitors³ including, anti-CTLA4 or Ipilimumab,⁴ anti-PD1 Nivolumab,⁵ and anti-PDL1.⁶ Other advances include patient-specific engineered T-cells for autologous T-cell therapy, such as chimeric antigen receptor (CAR)-T cells⁷ or affinity-enhanced TCR-T cells.⁸ Many of these immunotherapies are combined with tumor-specific approaches⁹ that kill tumor cells in a manner to produce immunogenic cell death¹⁰ such as radiotherapy¹¹ or specific kinds of chemotherapy.¹² Immunogenic cell tumor cell death is characterized by induction of a variety of “damage associated molecular patterns” that are proposed to be recognized by certain cells of the immune system, particularly dendritic cells (DC).¹³

Since the first reports of Coley’s toxins (which were a mixed bacterial vaccine),^{14,15} a wide array of different immune activators have been tested for their ability to stimulate the immune system to combat cancer. The best known anticancer immune activators are probably BCG (used clinically for bladder cancer¹⁶) and CpG oligodeoxynucleotide,¹⁷ but polysaccharides have also been tested.^{18,19} *N*-dihydrogalactochitosan (GC) is a new immune activator that was originally prepared by attaching galactose molecules to the amino groups of chitosan, a glucosamine polymer, in order to improve solubility in water at neutral pH, and to overcome problems of sterile filterability in the manufacturing process.

InCVAX is a novel *in situ* autologous cancer vaccine for treatment of metastatic cancer, which stimulates an endogenous antitumor immune response.²⁰ Animal^{21–31} and early human studies³² demonstrate that inCVAX has strong therapeutic effects on cancers even with distant metastases, and in animals induces long-term protection against rechallenge with the same tumor. By training the immune system to search for and destroy micrometastases, inCVAX may prove to be a clinically powerful approach for managing and possibly eradicating cancer, even in late-stage settings. InCVAX is a two-step procedure involving delivery of low-power thermal laser (for 10 minutes) to any accessible tumor to cause tumor cell death, release of antigens, and to increase the immunogenicity of tumor, followed immediately by injection of GC into the laser-treated tumor region to stimulate antigen presenting cells. The two-step procedure works synergistically to induce systemic antitumor T cell response that is capable of eliminating both primary and metastatic cancers in some patients with advanced, stage III/IV, breast cancer with minimal toxicity.³³

To induce systemic antitumor response, tumor antigens have to be taken up by antigen presenting cells (APCs) and presented to naïve T and B cells. Among different APCs, DC are most potent in eliciting a strong antitumor CTL response. DCs were first observed in 1868 in the skin and called Langerhans cells (LC), yet their major role as antigen presenting cells was not discovered for another quarter of a century. The main functions of DC are to capture, process, and present antigens to T-cells, activating T-cells or inducing peripheral

tolerance. The type of immune response induced by DCs depend on their maturation state, cytokine profile, quality of antigens, and the type of tissues involved. Immature DCs circulate throughout the body where they can capture and uptake either self-derived or exogenous antigens³⁴ by phagocytosis, micropinocytosis, and receptor-mediated endocytosis. Different pattern recognition receptors (PRR) are expressed on DCs including Toll-like receptors, C-type lectin receptors (CLRs), and others, which sense the presence of specific molecular patterns, particularly those originating from pathogens. In the absence of infection or inflammation, the circulating immature DCs normally express lower levels of major histocompatibility complex class II (MHCII), and co-stimulatory molecules CD80 and CD86. Upon ligand binding to PRRs, DCs are stimulated to undergo maturation. During maturation, the antigens are processed and rapidly loaded onto the MHC molecules on the DCs as they migrate to secondary lymphoid organs such as lymph nodes. The DC lose the ability to take up new antigen but the expression of MHCI and MHCII go up, as well as the expression of co-stimulatory molecules such as CD40, CD80, CD86, and so forth. Morphologically, DCs have more elongated cytoplasmic processes. These changes enable DCs to optimally present the antigens as they arrive from the lymph node to naïve T cells/B cells to provoke the suitable immune response.³⁵ Other factors such as adhesion molecules and specific chemokines play regulatory roles in the differentiation and migration of DCs, as well as the migration of naive T cells for maximal exposure and interactions.

CLRs have attracted much research interest since they were found to be the key recognition receptors for a broad spectrum of carbohydrate molecules found on self-antigens as well as exogenous antigens.³⁶ Extensive studies have been made to classify and to recognize the antigen specificity and the recognition behavior of CLRs, but still, what is known is much less than what remains unknown. Recent studies suggested a regulatory and signaling role for different subclasses of CLRs; Dectin-1, DC-SIGN, and the mannose receptors (MRs) beside their adhesion function. GC is a plausible candidate for binding to CLRs, since the galactose molecules that are chemically attached to the polymer backbone in GC can be recognized by CLRs. Additionally, Zaharoff et al.³⁷ suggested that chitosan itself could effectively stimulate humoral and cell-mediated immune responses, and to that extent it can behave as an antigen depot.

However, the exact role of GC on cancer cells and on other immune cells remains unclear. The aim of the current work was to study the stimulating effect of GC on the professional antigen presenting cells, DCs *in vitro* and to visualize the binding (if possible) as well as comparing such activation (if any) with lipopolysaccharide (LPS) as a positive control that activates DCs via binding to Toll-like receptors (TLR4).

RESULTS

Determination of optimum concentration of GC

Our initial studies were directed toward determining the appropriate concentration of GC and the time course of incubation that could stimulate the DC without causing any undesirable toxicity. We tested a broad range of concentrations of GC, comprising 0.01, 0.1, and 1 mg/mL, and compared these GC concentrations with LPS at 50 µg/mL. Figure 1(A–J) shows the cellular morphology of the DC2.4 cells under the tested conditions captured by

bright field microscopy. Untreated cells [Fig. 1(A,F)] mostly had a round shape with a fairly non-adherent behavior, but then became slightly more adherent with longer culture times. Figure 1(B,G) shows that GC at 0.01 had little if any effect on the morphology. Figure 1(C,H) shows that GC at 0.1 mg/mL began to have the effect of producing cells with a more elongated morphology and a more adherent phenotype at 4 hours incubation, and this effect was more pronounced at 24 hours incubation. Figure 1(D,I) shows that this trend continued when the concentration of GC was increased to 1 mg/mL with a noticeable change seen at 4 hours and more at 24 hours. Figure 1(E,J) shows that using LPS at 50 µg/mL the change in morphology was even more pronounced than seen with the highest concentration of GC at both time points. Interestingly it can be seen that there are fewer cells present with LPS (especially at 24 hours) suggesting that LPS was exerting some toxicity at this concentration.

We next tested the viability of the DC2.4 cells under the conditions tested above as it appeared that LPS in particular showed some toxicity to the DC. Figure 1(K) shows the results of the Presto Blue viability assay carried out at 24 hours of incubation. Although there was a loss of viability with GC used at 1 mg/mL, the viability was still significantly higher than that seen with LPS at 50 µg/mL ($p < 0.001$). GC at 0.1 and 0.01 mg/mL did not show any significant loss of viability. Taking the data in Figure 1 together with those in Figure 2, where the highest expression of major histocompatibility complex class 2 (MHC II) was observed at a GC concentration of 1 mg/mL, the latter was chosen in the following sets of experiments.

Comparison of GC with LPS by confocal microscopy

We used confocal microscopy imaging to compare the effects of GC at 1 mg/mL with LPS at 50 µg/mL on DC2.4. We tested the cellular uptake of a model antigen (FITC-labeled ovalbumin, FITC-OVA), expression of MHCII, and expression of CD11c and the results can be seen in Figure 3. There was a high uptake of FITC-OVA by DC2.4 in their unstimulated state [as expected, Fig. 3(A)] and this appeared to be reduced more by GC [Fig. 3(B)] than it was by LPS [Fig. 3(C)]. MHCII expression was low in unstimulated DC2.4 [Fig. 3(D)] and appeared to be increased approximately equally by GC [Fig. 3(E)] and LPS [Fig. 3(F)]. CD11c expression was moderate in unstimulated DC2.4 [Fig. 3(G)] and was increased in both GC [Fig. 3(H)] and LPS [Fig. 3(I)].

Comparison of GC with LPS by FACS

In order to verify the effect of GC and provide quantification that could not easily be obtained from the confocal imaging results, we used FACS analysis to measure the expression of three DC specific markers, CD11c, CD80, and MHCII at two separate time points, 4 and 24 hours, as shown in Figure 3. DC2.4 cells that were treated with GC expressed higher CD11c levels compared to control cells and cells that received LPS ($p < 0.001$) at both time points. DCs that were treated with GC expressed much higher CD80 levels compared to control cells ($p < 0.001$) and higher levels compared to cell treated with LPS ($p < 0.001$) at both time points. GC induced much higher level of MHC class II expression on DCs compared to control ($p < 0.001$) and higher levels compared to LPS ($p < 0.001$) at both time points. Interestingly GC increased MHCII expression to a higher degree

at 24 hours compared to that found at 4 hours ($p < 0.05$), but this was the only significant difference between time-points.

Secretion of IL12

Interleukin 12 is a cytokine secreted by activated DC, which is crucial for initiating and propagating Th1 response. Figure 5(A) shows the calibration curve for the ELISA assay, and Figure 5(B) shows that both GC and LPS induced a large increase in IL12 expression ($p < 0.001$) compared to the level from control cells that was undetectable.

Uptake of FITC-labeled GC

We obtained FITC-labeled GC from Immunophotonics in order to able to detect the uptake and binding of GC to the DC2.4 cells, and we decided to co-incubate the green-fluorescent GC with Texas Red-labeled OVA (red) in order to carry out a time course of antigen uptake, and to see if the GC stimulation made any difference to the uptake or subcellular localization of the OVA antigen. Figure 6(A) shows that good uptake of OVA by nonstimulated DC2.4 after 60 minutes. After 4 hours of the co-incubation of GC and OVA the green FITC-GC was hardly binding to the surface of the cells [Fig. 6(B)]. At 8 hours [Fig. 6(C)] it could be seen that the FITC-GC had agglomerated together into larger particles, and appeared to be binding more to the cells than at 4 hours. After 24 hours [Fig. 6(D)] a significant amount of the FITC-GC had been internalized into the cells, and was co-localized with the OVA (yellow overlap). Interestingly at 24 hours the uptake of OVA was increased and also the intracellular processing was increased (no punctate red spots).

DISCUSSION

inCVAX is a promising therapeutic approach for the treatment of primary and metastatic cancer.³³ The procedure involves the local application of photo-thermal energy from a NIR laser to a visible tumor deposit, which is immediately followed by a local injection of GC into the tumor region as an immune activator. Our data shows that intratumoral injection of GC could stimulate immune antigen presenting cells (APC) such as DC that would lead to increased uptake of tumor antigens that had been liberated by the thermal laser treatment to the tumor. This pathway would be expected to lead to the maturation of DC that eventually results in up-regulated presentation of antigens and activation of naive T cells, leading to induction of an adaptive tumor-specific immune response. Provoking an efficient, strong host immune response against the tumor could be the determinant of the patients' survival in the course of treatment for advanced cancer. This type of immunotherapy could have synergistic effect when combined with other cancer therapies like photodynamic therapy³⁸ or other conventional treatments like radiotherapy or chemotherapy.

Immunotherapy is now being considered as a renewed approach for fighting cancer, since the relatively recent advent of the so-called "check-point inhibitors".³ These inhibitors are monoclonal antibodies that release the blockade against host T-cells to allow them to attack the tumor (and to some degree attack normal host tissue). Since tumor antigens are considered to be weak and "self-like",³⁹ strong immune responses against tumor-associated antigens are the exception rather than the rule. Therefore, many approaches are used to

augment the antitumor immunity (such as that induced by checkpoint inhibitors) such as, various vaccine strategies, cytokines, and chemokines. Immune activators can also enhance systemic antitumor immunity. Indeed this was the original hypothesis underlying “Coley’s toxins” already in 1891.¹⁴ An immune activator, also referred to as immune stimulants or immunoadjuvants, can be defined as a substance that stimulates a response of the host to a vaccine or other source of antigens, while it has no antigenic effect on its own. Ideal immune activators would be the ones who can induce an immune response with a high affinity to a specific antigen but without being toxic. Moreover they should be biodegradable and with reproducible production methods. There has been a long-term need to identify more effective and less toxic immune activators for application in cancer immunotherapy.

Several studies have been conducted to explore chitosan as an immune activator. Zaharoff et al. tested chitosan in mice vaccination with β -galactosidase as an antigen.³⁷ Their results revealed many-fold increase in the antigen-specific antibody titers and antigen-specific splenic CD4+ proliferation. It has been suggested as well that chitosan can act as an antigen depot and can induce both innate and adaptive immune response as shown by the observation of increased antibody titers with robust delayed-type hypersensitivity (DTH) responses. Finally they concluded that chitosan was more effective than aluminum hydroxide and had the same potential as incomplete Freund’s adjuvant (IFA). Our data have shown that GC, an improved derivative of chitosan developed for clinical application, has potent immunostimulatory effects by stimulation the maturation of DCs, increasing its upregulation of MHCII, CD80, CD86, and IL-12 production without inducing significant cellular toxicity.

DC behavior varies according to their maturation and differentiation status, and these parameters consequently affect their role as regulators of the immune system. There are nowadays many proposed signaling pathways for activation of DC that could be synergistic or additive with each other, or conversely could have negative or antagonistic effects. Several studies have been conducted on the activation of DC2.4 cells. One study carried out by Rhule et al. (2009) showed that the natural extract notoginseng was an immune-modulator that could selectively affect the production of pro-inflammatory mediators such as TNF- α and IL-6 by DC2.4 cells after their activation by LPS as a TLR ligand.⁴⁰ Sheng et al.⁴¹ demonstrated for the first time the effect of IFN- γ on DCs *in vitro*, where the IFN- γ -treated cells were then incubated with TLR2, TLR4, or TLR9 agonists that resulted in higher DC activation when compared to TLR ligation only. They also increased expression of CD40 after LPS treatment and increased CD86 after CpG activation. Our current results are in good agreement with such studies since an LPS stimulus resulted in increased expression of MHCII, IL-12, and CD80 (Fig. 4), but CD11c was reduced by LPS action on DC2.4. The CD11c reduction can be attributed to the hypothesis suggested by Singh-Jasuja et al.⁴² where such down regulation of CD11c on mouse DCs might be attributed to strong stimuli such as TLR ligands like LPS. LPS is known to activate DC through the TLR4 pattern-associated molecular pattern (PAMP). One common proposed signaling model is that TLR4 is activated upon ligand binding and starts a cascade of signaling events via the adaptors called MyD88 (myeloid differentiation primary response gene 88) and TICAM-1 (Toll-like receptor adaptor molecule 1) leading eventually to the activation of NF- κ B (nuclear factor kappa B), TRIF (TIR = Toll/interleukin-1 receptor-domain-containing adapter-inducing

interferon- β), and possibly other transcription factors. Shotorbani et al.⁴² proposed that by optimizing DC activation and antigen presentation, activation of effector T cells could be accomplished. They studied the effect of Hlx gene as a Th1-specific transcription factor on DC2.4 maturation. They concluded that over-expression of Hlx gene in DC2.4 cells led to their increased maturation as shown by up-regulation of CD40, CD80, CD86, MHC-I, and MHC-II as well as IL-12. This observation corroborates our finding that GC has enhanced DC2.4 maturation as our results also showed upregulation of CD11c, CD 40, MHC II (Fig. 4), and as well as IL-12 secretion (Fig. 5). To better define the molecular pathway of GC, further research will look at the receptors and the downstream signaling events. It would be of interest to investigate whether GC's induced downstream effects synergizes with other TLR ligands and cytokines. Such experiments could possibly yield to the design of a cocktail immune activators using GC as the backbone for optimal DC stimulation.

Calcium-dependent lectins (C-lectin) receptors are another family of important receptors, besides the TLR family, on DCs that are involved in antigen uptake and DC maturation.⁴³ These transmembrane receptors are believed to recognize carbohydrates and polysaccharides that are found in such organisms as pathogenic fungi and mushrooms. Because of the presence of galactose in our GC, CLR member is the prime candidate receptor for GC. C-lectins employ strongly conserved carbohydrate-recognition domains (CRDs). The C-lectins expressed on DCs are subdivided into two main groups, where group 1 has many CRDs and includes those whose N-terminus is extracellular, while group 2 has only a single CRD that is located at the carboxyl terminus, and the N terminus is inside the cytoplasm. Two important examples of type 1 C-Lectin family on DCs are macrophage mannose receptor (MMR) and DEC-205 and both of them share the presence of a tyrosine-based motif for antigen intake into vesicles. Recent studies have shown many DC receptors that belong to the type 2 C-lectin family like DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN), DC immunoreceptor DCIR, the DC associated C-type lectins 1 and 2 (dectin-1, dectin-2), C-type lectin receptor 1 (CLEC-1), and lectin-like immunoreceptor (LLIR).⁴⁴ The expression of CLRs varies with the tissue localization and the activation status of DCs. For instance, DEC-205 is believed to be involved in DC activation because of its increased expression on activated DC, while the expression of MMR and DC-SIGN are decreased upon the activation of DC that are derived from monocytes. Such variation of expression could indicate different antigen uptake as well as migration behavior in different DCs.⁴⁵

CLRs have been considered more likely to be involved in antigen binding and uptake rather, than in activating and inducing maturation of DC, as is more typical of Toll-like receptors. Cell-surface C-type lectins may function as endocytic receptors, as they can be internalized directly like MMR, or else are internalized after binding to ligands, as is the case for DEC205 and DC-SIGN. C-lectins undergo recycling by returning to the cell surface to repeat the process. C-type lectins have also been found to be involved in DC trafficking, and in aiding DC to migrate to germinal centers (lymph nodes) during the immune response. Moreover C type lectins are involved in the cross-talk between DCs and T cells.⁴⁶ Recent studies have shown binding between DC-SIGN and ICAM-3 (an adhesion molecule that is significantly expressed on resting T cells). It has been proposed that dectin-1 plays a role in DC binding to T cells that leads eventually to their activation.⁴⁷ Group 1 C-type lectins are associated with a signal transduction pathway that results in the production of tumor

necrosis factor α (TNF α) and IL-12. Since C-type lectins are a relatively recent discovery, their role in tumor immunotherapy has not been studied to the same extent as other DC receptors.⁴⁸ Many studies, however, have reported the immune-modulation effects that can be exerted by the carbohydrate-derived materials derived from mushrooms, schizophyllan, and β -glucans, where both innate and adaptive immune response can be enhanced.⁴⁹ It is believed that dectin-1 receptors on DC together with ICAM-3-grabbing nonintegrin (DC-SIGN) homolog, SIGN-related 1 (SIGNR1) expressed on macrophages work together in the nonopsonic recognition process of β -glucans and to lesser extent schizophyllan. These substances (schizophyllan and β -glucans) have been suggested to have anticancer activity through immune-modulation acting via cytokine production such as IL-6, IL-10, IL-12, and TNF- α .⁵⁰ Cobb et al. have shown the activation of CD4 (+) T cells via the endocytic MHC II pathway rather than via MHC I after the uptake of β -glucans by DC that eventually results in a Th-1 response.⁵¹ Future work is clearly required to identify the precise receptors that are involved in binding of GC to DC as it is of crucial importance to deduce the consequently signaling pathways. A better understanding of the cross-talk between CLRs and TLRs on DC, and how the former can induce gene-expression of activator proteins is needed. Eventually it could help in maximizing the usage of GC in various medical applications one of which could be vaccination.

MATERIALS AND METHODS

One of our collaborators provided the lab with mouse DC line DC2.4 as a kind gift. RPMI 1640, Trypsin, Presto blue, Hoechst 33342, Lipopolysaccharides (LPS), Fluorescein isothiocyanate (FITC), and Texas red conjugated Ovalbumin (OVA) were purchased from Gibco, ThermoFisher Scientific (Cambridge, MA). GC was provided by Immunophotonics. PE-Cy7 conjugated MHC Class II (I-A/I-E) antibody (M5/114.15.2), FITC conjugated CD80/B7-1 antibody (16-10A1) and its FITC conjugate isotype control antibody Hamster IgG, PE-Cy7 conjugated Integrin α X/CD11c antibody, CD16 + CD32 antibody for blocking unspecific binding were all bought from ThermoFisher Scientific. PE-Cy7 conjugated Hamster IgG antibody isotype control for CD11c was brought from Biolegend (San Diego, CA), and PE-Cy7 conjugated Rat IgG2b K isotype control for MHC Class II was from eBioscience (San Diego, CA).

Cell culture

DC2.4 cells were cultured in RPMI 1640 medium which was supplemented with 10% fetal bovine serum, 55 μ M beta-mercaptoethanol, 100 U/mL of penicillin/streptomycin antibiotic, 2 mM L-glutamine, 1% HEPES, and 100 μ M nonessential amino acid. DC2.4 cells were cultured with the abovementioned supplemented media and three different concentrations of GC that was originally provided by Immunophotonics (Columbia, MO). The used GC concentrations were 0.01, 0.1, and 1 mg/mL for 4 and 24 hours to investigate by flow cytometry and confocal imaging if GC had any stimulating effect on these cells. LPS at 50 μ g/mL was used as a positive control since it is known for its stimulating effect on DC2.4 cell line.

Viability test

The toxicity of the different used concentrations of GC and the 50 µg/mL of LPS was evaluated by Presto Blue test. The test relies on the well-known fluorescent redox indicator called resazurin that has a blue color in its oxidized form. The test is very specific since only viable cells can reduce this indicator to the red fluorescent resorufin, whereas non-viable cells can do nothing. The fluorescent emission was measured by a microplate reader at 590 nm with an excitation wavelength of 570 nm.

ELISA test

Enzyme linked immunosorbent assay (ELISA) was used for the detection and quantification of secreted IL-12 as a marker for DC2.4 maturation and differentiation. Mouse IL-12 p70 DuoSet ELISA Kit and the ELISA Clear Polystyrene 96 well plates were bought from R & D systems (McKinley Place NE, MN) were employed. The optical density of each well was determined using a microplate reader that was set at 450 nm and a wavelength correction was done by subtracting the reading of 540 nm from that of 450 nm. Standard known IL-12 concentrations were used to plot a standard curve for identifying the secreted IL-12 concentrations from DC2.4 culture supernatants in the presence of GC or LPS after 24 hours incubation.

Flow cytometry

Cells were harvested after culturing in the presence of GC 1 mg/mL or LPS 50 µg/mL for 4 and 24 hours. FACS Buffer was used for cell resuspension after they had been incubated for 20–30 minutes with CD16 + CD32 Antibody to block Fc receptors and hence any unspecific binding to DC 2.4 cells. Cells were then washed, centrifuged, and resuspended and incubated with FACS Buffer containing the working concentration of the fluorescent conjugated monoclonal antibodies for CD11c, MHC class II, and CD86 on ice and avoiding any direct light interaction for 30 minutes. After washing the cells three times with phosphate-buffered saline (PBS), flow cytometry (FACSaria, BD Biosciences, Sparks, MD) was used to quantify the labeled cells and the analysis was done using FACSDiva and Flowjo softwares. Isotype monoclonal antibody for each monoclonal antibody was used as a background control in addition to unstained cells.

Confocal microscopy

DC2.4 cells were cultured in the presence and absence of GC with the three mentioned concentrations or 50 µg/mL LPS in glass bottom plates at a density of 1×10^5 cells/cm². FV1000-MPE Olympus confocal microscope was used to visualize the stimulating effect of GC or LPS on DC2.4 cells using only the bright field imaging. The differentiated cells have a different morphology from the floating rounded shape, which is characteristic of immature DC. The confocal microscope was also used to investigate the effect of GC or LPS in changing the rate of antigen uptake after 4 and 24 hours by DC2.4 cells via incubating cells for 1 hour with 1 mg/mL FITC conjugated OVA and 30 minutes with 4 µg/mL Hoechst blue that stains DNA in cell nuclei. GC itself was conjugated with FITC for localizing its uptake at a concentration of 1 mg/mL by DC2.4 cells after 4, 8, and 24 hours and by using 1 mg/mL Texas red conjugated OVA and 4 µg/mL Hoechst blue dyes. Finally confocal microscopy

and immuno-staining were used to visualize the expression of MHC class II and CD11c by DC2.4 cells with and without the presence of GC 1 mg/mL or LPS of 50 µg/mL for 4 and 24 hours.

Statistics

Each set of experiments was repeated four times ($n = 4$), while each assay was done in duplicate; Analysis was performed to obtain all the mean, standard deviation, and standard error values as well as designating p values which were significant if < 0.05 using one-way ANOVA (Originpro 8).

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FIGURE 1.

Determining the optimum concentration of GC by microscopy. Morphological changes in DC2.4 cells at 4 hours (panels A–E) and 24 hours (panels F–J) after treatment with control (panels A & F); GC at concentrations of 0.01 mg/mL (panels B & G); 0.1 mg/mL (panels C & H); 1 mg/mL (panels D & I); 50 µg/mL LPS (panels E & J). (K) Histogram showing Presto Blue viability test for in DC2.4 cells for 24 hours with agents as described above. Experiments were repeated seven times.

FIGURE 2.

Determining the optimum concentration of GC by flow cytometry. Relatively immature DC2.4 cells were cultured in the absence and the presence of 0.01, 0.1, and 1 mg/mL GC and then were analyzed by flow cytometry gating out dead cells. (Panels A and B) cells were unstained and stained with PE-Cy7 conjugated-rat IgG2b K isotype control as background control, (panels C–E) cells were incubated with 0.01, 0.1, and 1 mg/mL GC and then stained with PE-Cy7 conjugated MHC Class II (I-A/I-E) antibody (M5/114.15.2) and (F) Histogram showing MHC class II expression level (means + SD) for treated DC2.4 as described above. Experiments were repeated three times.

FIGURE 3.

Confocal imaging for expression of activation markers showing DC2.4 cells after 24 hours of incubation. FITC-conjugated ovalbumin (green) uptake (panels A–C); MHC class II expression by PE-CY7 conjugated MHC Class II (I-A/I-E) antibody (red) (M5/114.15.2) (panels D–F); CD11c expression by PE-CY7 conjugated integrin alpha X/CD11c antibody (red) (panels G–I); in control DC2.4 cells (panels A, D, G); DC2.4 incubated with 1 mg/mL GC for 24 h (Panels B, E, H); DC2.4 incubated with 50 µg LPS for 24 h. Blue staining is Hoechst nuclear stain. Scale bar = 10 µm.

FIGURE 4.

Expression of CD11c, CD80 and MHC II by flow cytometry. Histogram shows DC2.4 cells expression levels of CD11c, CD80, and MHC II after incubation with 1 mg/mL GC and 50 μ g/mL LPS for 4 and 24 hours compared to control group.

FIGURE 5.

Secreted IL12 measured by ELISA. (A) calibration curve obtained from standard concentrations of mouse-IL-12 p70. (B) Concentrations of IL-12 secreted from untreated (control) and DCs incubated with 1 mg/mL GC and 50 μ g/mL LPS for 24 h. Data are mean of six experiments.

FIGURE 6.

Time course by confocal imaging of DCs treated with OVA and GC. DC2.4 were treated with 1 mg/mL Texas red-labeled OVA and FITC-labeled GC. (A) Control (no FITC-GC); (B) 4 hours; (C) 8 hours; and (D) 24 hours. Experiment was repeated three times.