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Gene expression profile of tumor cell-fused or Noni (*Morinda citrifolia*)-treated dendritic cells

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GENE EXPRESSION PROFILE OF TUMOR CELL-FUSED OR NONI (*MORINDA CITRIFOLIA*)-TREATED DENDRITIC CELLS

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by
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ABSTRACT

Dendritic cell-mediated cancer immunotherapy employs several ways to engage tumor antigens. We have demonstrated both in pre-clinical animal studies and early clinical trials that dendritomas, highly purified hybrids between dendritic cells and tumor cells, are superior activators of anti-tumor immunity. In the present study, we examined the expression profile of several inflammatory chemokine and chemokine receptors of dendritomas by RNA microarray and real-time RT-PCR. The results indicate that dendritomas made from immature DCs and tumor cells express higher levels of CCL3, CCL5, and CCL22 and lower levels of CCR2 and CCR5, which mimics LPS matured DCs, while dendritomas made from mature DCs and tumor cells show a reversed expression profile of these genes: decreased levels of CCLs and increased levels of CCRs. Our data support the notion that dendritomas made from immature DCs and tumor cells may be more effective in migration from the injection site to draining lymph nodes and therefore make them more effective in stimulating anti-tumor immunity.

Morinda citrifolia (Noni) has been used as a folk remedy to treat a myriad of ailments, and is gaining in popularity as a modern dietary supplement to enhance the immune system. Recent studies have shown that Noni juice has anticancer activity. Studies from our lab demonstrated that fermented Noni juice not only prevents mouse sarcoma tumor development but also eradicates existing tumors. Fermented Noni can also directly engage dendritic cells with B cells. Since Noni contains a wide array of microorganisms, and upon fermentation, all but one are killed, it is presumed to contain a plethora of degraded microbial products that would serve as microbial stress signals. We

hypothesized that Noni may activate dendritic cells by engaging their toll-like receptors (TLRs), and investigated genes associated with TLR signaling via real-time RT-PCR. It was determined that Noni stimulates early low levels of inflammatory cytokines, followed by a latent upregulation of anti-inflammatory mediators. Intriguingly, Noni also appeared to trans-differentiate dendritic cells toward macrophage-like cells.

DEDICATION

I would like to dedicate this dissertation to my precious daughter,

Madelyn Marie O’Connor.

ACKNOWLEDGEMENTS

I would like to thank the many people that helped make this possible, but would first like to acknowledge that without God I wouldn't be where I am today. Through Him this was all made possible. I am grateful to my father, William E. Branham, for his desire to grow children strong in character, not accepting less than our best. Your support (and sometimes coercion) has helped me to get where I am today and I deeply thank you. To my mother, Donna L. Branham, whose constant encouragement has helped soften the more difficult times, I appreciate you more than you will know. You have often reminded me that God has great plans for me and have kept me believing in Him and in myself. To my daughter Madelyn, who is my source of joy, and is the best stress-reliever at the end of a tedious work day, I love you and cherish our moments together! And I want you to know that although this accomplishment means so much, it doesn't hold a candle to you. You are and always will be my greatest love! And most of all to my husband, Jerry, without your support I would not have been able to finish – thank you for taking on a role most fathers wouldn't dream of so that I could pursue my passions.

I would also like to reflect on the many others that made this journey enjoyable and bearable: Jamie Korman, Melinda Marquess, Hari Kotturi, Leigh Theofanous, Hilary Bouton-Verville, Keri Nowend, Jenny Nilsson, Angela Houwing, Renuka Persad, Jyothi Rangenini, Jaleh Jalili, Rupal Shah, Neeraj Gohad, and the many others at the Oncology Research Institute and in the microbiology department of Clemson University.

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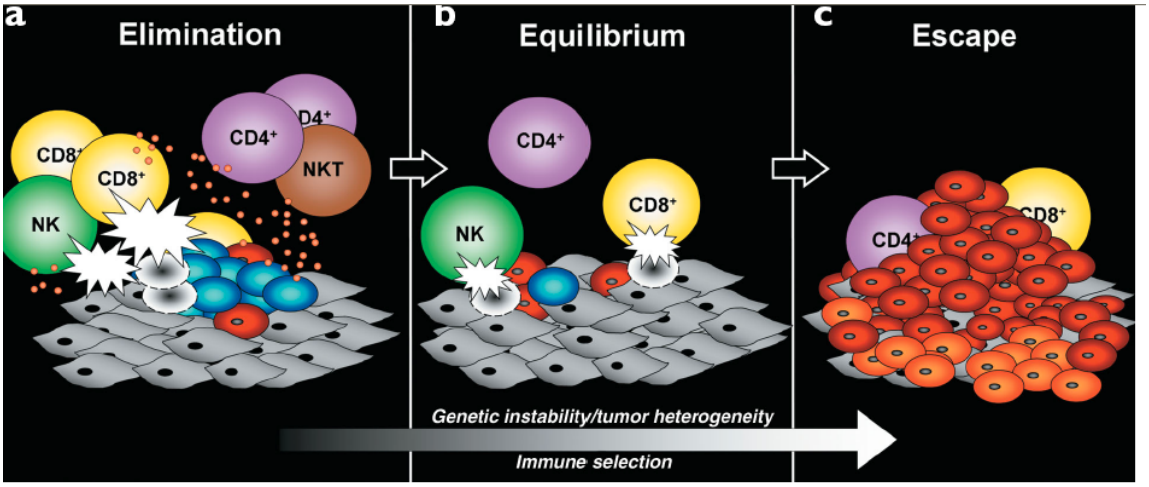
1. LITERATURE REVIEW

1.1. The Immune System and Cancer

In 1909 Paul Ehrlich first hypothesized our immune systems are capable of destroying initial emerging tumor cells (Ehrlich, 1909); however, research in this area had to await the developing field of immunology. Nearly half a century later, Burnett and Thomas separately rebirthed the concept, eventually entitling it 'immunosurveillance' (Burnet, 1957) (Thomas, 1959). Experiments furiously followed aimed at supporting this ideology; however in the late 1970s immunosurveillance was abandoned due to limited understanding of nude mice at the time, and their inability to acquire higher amounts of spontaneous tumor formation (Dunn *et al*., 2002). It is now acknowledged that nude mice have traceable amounts of αβ T cells as well as NK cells and other innate effectors. Although several attempts were made to revive the immunosurveillance concept, it wasn't until the mid to late 1990s that this area was fully rejuvenated. At that point it was further defined as 'immunoediting' in 2002 to more accurately describe the intricate balance between host defense against tumor formation (elimination) and tumor immune evasion (escape) (Dunn *et al*., 2003).

Immunoediting is described as a three-pronged process including elimination, equilibrium and escape (Dunn, 2003) as shown in Figure 1.1. The first phase implies aberrant cells are constantly being transformed in a healthy host, and the immune system duly recognizes and eliminates these cells, hence the title 'elimination'. The second step requires the collection of several genetic modifications and a Darwinian-like selection of tumor cells that have lost their immunogenicity; however, these cells are kept in check by

innate immune cells and T cells, which amounts to a state of equilibrium. The selection of tumor cells that have lost immunogenicity can have detrimental effects. It is these cells that are capable of escaping immune detection and have the potential to acquire all seven hallmarks of cancer. These hallmarks include: (1) self-sufficient, perpetuated growth signals, (2) alluding anti-growth signals, (3) inflammation, (4) uncontrolled replication, (5) evading apoptosis, (6) angiogenesis, and (7) metastasis (Colatta *et al*., 2009; Montavi, 2009; Hanahan *et al*., 2000). The successful treatment of cancer lies in the understanding of how the body initially recognizes and destroys tumor cells as well as how the immune system is commandeered for their protection and proliferation. To eliminate cancer we must tip the scales back toward tumor elimination and minimize the factors allowing for its immune escape.



(Dunn *et al*., 2003. *Ann Rev Immunol*. 22: 329-360)

Figure 1.1. **The three Es of cancer immunoediting.** Cancer immunoediting encompasses three processes. (a) Elimination corresponds to immunosurveillance. (b) Equilibrium represents the process by which the immune system iteratively selects and/or promotes the generation of tumor cell variants with increasing capacities to survive immune attack. (c) Escape is the process wherein the immunologically sculpted tumor expands in an uncontrolled manner in the immunocompetent host. In (a) and (b), developing tumor cells (blue), tumor cell variants (red) and underlying stroma and nontransformed cells (gray) are shown; in (c), additional tumor variants (orange) that have formed as a result of the equilibrium process are shown. Different lymphocyte populations are as marked. The small orange circles represent cytokines and the white flashes represent cytotoxic activity of lymphocytes against tumor cells.

1.2. Cancer Immunotherapy

Cancer immunotherapy hinges on the idea that we can manipulate the body's immune milieu to favor tumor rejection. This idea has developed into one of the most complex areas of cancer research. With countless immune mediators like cytokines, growth factors and 'danger signals' (pattern-recognition receptor ligands), along with inimitable tumor cell profiles and microenviroments, it is impossible to identify one method of treatment effective for all cancer patients; hence the strong desire to advance the field of personalized cancer therapies (Hayden *et al*., 2009). Several different types of immunotherapy are currently being studied for cancer treatment, including monoclonal antibody therapy, radioimmunotherapy, and cell-based therapies (adoptive transfer of T cells and dendritic cell-based immunotherapy). In 1997, Rituximab was the first monoclonal antibody approved by the FDA and it opened the door for cancer immunotherapies (Biotechnology Law Report, 1998). Several more monoclonal antibodies were approved shortly after, and the first radioactive-labeled antibody was approved in 2002, Zevalin (Schilder, 2002). The first vaccine for cancer prevention, Gardasil, was approved by the FDA in 2006 (Zawisza, 2006). It is an immunization against certain HPVs that are associated with increased cervical cancer risk, and is the first drug specifically targeting inflammation-induced cancers. There are, however, no FDA approved cell-based therapeutic cancer vaccines. Here we will further examine dendritic cell-based therapies.

1.3. Dendritic Cells

DCs were first discovered in 1868 in the epidermis by Paul Langerhans, thus termed ‘Langerhans cells’ (Langerhans, 1868). But it was more than a century before they were identified in other tissues and termed ‘dendritic cells’ by Steinman and Cohn in 1973 (Steinman *et al*., 1973). Their scarcity proved them difficult to isolate and study. Consequently, it wasn’t until the early 1990s when DC purification improved and the first clinical trial for DC vaccines, published in 1995, showed promising results (Mukherji *et al*., 1995). DCs are professional antigen-presenting cells poised to bridge innate and adaptive immunity while directing the balance of immunity and tolerance. Their cross-talk with natural killer (NK) cells (Fernandez *et al*., 1999), NKT cells (Fujii *et al*., 2002) and γδT cells (Conti *et al*., 2005) hastens innate immunity, while synchronistically presenting antigen to lymphocytes calling for acquired protection. There is no other cell quite as capable of building this union. Because of this unique property, many immunologists aim to harness the power of the dendritic cell for therapies from cancer treatment and prevention to reversal of type 1 diabetes (Giannoukakis *et al*., 2006) and AIDS vaccinations (Rinaldo *et al*., 2009). Here we will focus on dendritic cell involvement with cancer. The dendritic cell is instrumental in deciphering whether the immune system will eliminate tumor cells or tolerate their existence eventually leading to immune escape and malignant progression. What determines this balance? What factors influence the pivotal decision by the dendritic cell? How can we harness their properties and utilize them against the very disease they are protecting? The next decade of

dendritic cell-based cancer research will, with anticipation, unravel some of these mysteries.

1.4. Dendritic Cells and Cancer

It has been established that the eradication of cancer cells requires both innate and adaptive immunity (Chaudhuri *et al*., 2009; Diefenbach *et al*., 2002), as well as activation of $CD4^+$ and $CD8^+$ T cells. DCs are one of the primary cells capable of helping or hindering this process. Once tumor cells have effectively escaped immune recognition, the tumor microenvironment favors tolerance, and the surrounding and infiltrating immune cells are often usurped for the tumors advantage. Indeed, dendritic cells from cancer patients are functionally compromised (Pinzon-Charry *et al*., 2005; Shurin *et al*., 2006). Often these DCs are inhibited in differentiation and maturation (Almand *et al*., 2000), which could be due to the over-expression of STAT3 by tumor cells and subsequent upregulation of STAT3 by DCs, thus reducing their expression of co-stimulatory and MHC class II molecules, or by the active recruitment of immature DCs to tumor tissues by way of tumor-produced chemokines (Bell *et al*., 1999). It has been shown that most tumors have higher numbers of DC infiltrates than surrounding healthy tissues (Almand *et al*., 2000), so it would be advantageous to develop a treatment aimed at reprogramming the capability of DCs to recognize tumor cells as diseased cells, then process and present tumor antigen to surrounding innate and adaptive effector cells, under an immune stimulating environment.

The most researched immune cells with tumoricidal properties are NK cells and cytotoxic T lymphocytes (CTLs). NK cells recognize tumor cells via downregulation of

MHC class I molecules (in the presence of other activating signals) (Bubenik *et al*., 2004) and upregulation of stress-induced NKG2D ligands (Raulet *et al*., 2009). Although NK cells are capable of direct tumor killing, DCs are involved in cross-talk with NK cells, which enhances NK cytotoxicity (Terme *et al*., 2008). Although $CD4^+$ T helper cells are now receiving more attention, cytotoxic T lymphocytes (CTLs) are the most researched cell in the adaptive immune branch accountable for anti-tumor responses. DCs present antigen to $CD8^+$ T cells via the endogenous pathway, or in the case of tumor antigens, by way of cross-presentation. Based on *in vitro* experiments, there are seven defined modes of cross-presenting tumor antigens to T-cells (Melief, 2008). Transfer of antigens may occur by 1) phagocytosing antigens from necrotic or apoptotic tumor cells, 2) phagocytosing soluble antigens bound to heat shock proteins or other chaperonins, 3) ingestion of soluble proteins secreted from tumor cells, 4) uptake of exosomes secreted by tumor cells, 5) transfer of protein fragments through gap junctions, 6) direct nibbling of tumor cell plasma-membrane by DCs, and 7) 'cross-dressing', where dead tumor cells transfer MHC-I:peptide complexes directly to DCs. Regardless of the method of cross-presentation, if DCs are not properly stimulated, the resulting presentation of antigen to either $CD4^+$ or $CD8^+$ T cells induces tolerance via deletion or regulatory T cell stimulation (Melief, 2008). It was demonstrated that both DCs and NK cells, after cross-talk, were more effective at inducing Th1 and CTL responses in both human *in vitro* and *in vivo* mouse studies (Kalinski *et al*., 2005). Thus, DCs play a vital role in recruiting innate and adaptive tumor-killing cells.

1.4.1. Dendritic Cell Vaccines

Dendritic cells used for cancer vaccines have been researched since the mid 1990's. The first whole-tumor cell DC vaccine clinical trial used autologous DCs pulsed *ex vivo* with the patient's own tumor antigens and administered subcutaneously (Hsu *et al*., 1996). This first trial treated four patients with B-cell lymphoma and was largely successful. All four patients had measurable antitumor cellular immune responses and three patients had positive clinical responses. As clinical trials began for other types of DC cancer vaccines, there were less encouraging results. Broad spectrum statistics of the outcome of all DC vaccine clinical trials yielded an overall clinical response of about 7%; however, other cancer vaccines not utilizing DCs (tumor cell-only, peptide-only or viral based vaccines) have only about a 3.5% response rate (Rosenberg *et al*., 2004). To optimize the DC cancer vaccine, researchers have examined antigen loading strategies, maturation of DCs, and route of administration.

1.4.2. Antigen Loading Strategies

Dendritic cell antigen loading strategies can be broken into three basic categories: RNA or DNA, peptide or protein, and whole tumor loading. Nucleic acid loading of DCs is capable of utilizing the entire repertoire of tumor antigens, if total RNA is extracted from tumor cells (Kalady *et al*., 2004). This method has the unique advantage of avoiding unwanted autoimmune reactions by subtractive hybridization with healthy cell mRNA (Boczkowski *et al*., 1996). Also, it is a promising therapy for patients without identified tumor-associated antigens (TAAs) or that lack sufficient tumor tissues to qualify for whole-tumor cell vaccine approaches. Several drawbacks to this method

include immunodominance, where viral vector antigens dampen the response to the tumor antigens; CTL targeting of the transfected DC eliminating these cells before eliciting the desired antitumor response; and a limited number of repeated vaccinations due to the anti-viral response (Mitchell *et al*., 2000) (Terando *et al*., 2007).

Since most tumor antigens are actually self-derived, they are typically considered weak antigens; therefore, with the discovery of TAAs (Lewis *et al*., 2003) many researchers began to pulse DCs with synthetic peptide antigens such as Her-2/neu, MAGE-1, CEA and many other TAAs (Disis *et al*., 1999; Hu *et al*., 1996; Morse *et al*., 1999; Nair *et al*., 1999). The upside to TAA-pulsed DC cancer vaccines is that these peptides could be synthetically manufactured, eliminating the need for patient tumor samples, and it reduces the possibility of autoimmune induction. This technique is limited quite heavily, however, with the requirement of tumor immunogenic epitope identification and HLA-typing, and it only activates cellular immunity. Some evidence has been given, however, to suggest that immunity against tumor cells not carrying the particular TAA is possible (Disis *et al*., 1999; Bellone *et al*., 1997). To circumvent the need for defined peptide epitopes and MHC restriction, DCs have also been loaded with soluble recombinant or purified tumor proteins (Nonn *et al*., 2003; Shojaeian *et al*., 2009). These proteins are ingested by DCs via macropinocytosis with simple co-culture of DCs and proteins, and although they are primarily presented via MHC class I, they are not restricted to a single MHC class (Svane *et al*., 2003). Overall, peptide and protein pulsing of DCs limits the pulsing antigen to defined TAAs. Unless a potent immune attack follows, immunosculpting may occur to delete tumor cells with the defined TAAs,

but leave behind malignant cells not expressing these antigens, allowing for tumor escape once again. Because of this, many scientists have pursued whole-tumor antigen strategies.

It seems whole-tumor antigen pulsing of DCs would present many self-antigens that would interfere with potent antitumor immune responses, however clinical responses of whole-tumor cell DC vaccines are comparable to peptide, protein and nucleic acid pulsed DCs (Terando *et al*., 2007; Koido *et al*., 2007). Whole-tumor antigen DC vaccines are capable of processing and presenting a wide array of tumor antigens, both known and unknown, to effector T cells, NK cells and other tumoricidal immune cells. This approach appears to have an advantage over the other methods of DC loading since it has the capacity to activate a much larger population of lymphocytes. Within the field of whole-tumor antigen pulsed DC cancer vaccines, there are two main methods of deriving these antigens. DCs can be pulsed with whole-cell tumor lysates (apoptotic or necrotic cells) or DCs can be fused to tumor cells. There is evidence that DCs are more capable of antigen uptake, processing and presentation from necrotic tumor cells, rather than apoptotic cells (Scheffer *et al*., 2003; Sauter *et al*., 2000). Contrary to this observation, apoptotic tumor cells elicited a much stronger antitumor T cell response than necrotic cells (Scheffer *et al*., 2003). This dichotomy could be due to improper methods of 'necrosing' tumor cells. In the body, necrotic tumor cells are in a hypoxic and stress-inducing environment, which would increase the expression of stress signals that augment DC activation. Most methodologies for preparing necrotic tumor cells *ex vivo* employ the freeze/thaw method. This method has recently been shown to diverge from

natural necroses in that the cells become necrotic in the absence of stress (Hatfield *et al*., 2008). The lack of stress signals from the cells may dampen their immunogenicity. Indeed, it was observed that freeze/thaw lysates were not capable of proper costimulatory molecule and MHC class II induction and suppressed Toll-like receptor maturation of DCs (Hatfield *et al*., 2008). The obvious drawback for this method of antigen loading (as well as total tumor RNA transfection of DCs) is the potential to incite autoimmunity; however, clinical trials to date have not observed detectable autoimmune reactions (Zhou *et al*., 2009; Homma *et al*., 2006). Also, there must be a sufficient amount of tumor tissue available for this therapy, limiting it to patients with solid tumors large enough for surgical removal and subsequent *ex vivo* cell culture.

There have been numerous attempts to define whether tumor cell lysate pulsing or DC-tumor cell fusions are superior. So far there is more supporting evidence for the efficacy of DC-tumor cell fusions rather than lysate-pulsed DC vaccines (Galea-Lauri *et al*., 2004; Shimizu *et al*., 2004; Kao *et al*., 2005); however, the rationale behind this has not yet been determined. It is possible that the act of fusion induces enough of a stress response in DCs that enhances their immunogenicity. Dendritic cell-tumor cell fusions have received much attention for DC-based cancer vaccines since they are capable of presenting the entire array of tumor antigens, both known and unknown, to T cells via MHC class I and II molecules. There are two methods for creating DC-tumor hybridomas: electrofusion and chemical fusion with polyethylene glycol (PEG). Electrofusion represents a better choice for designing DC fusion vaccines since there is more control over the process and it gives more consistent fusion rates from day-to-day

and between users than PEG-fusion. PEG fusion tends to be highly unpredictable, giving rise to varying fusion rates critically dependent on constant temperature, precise administration and different administrators. PEG fusion would greatly benefit from an automated system. Regardless of the method of creating DC-tumor fusions, these cells integrate their cytoplasm, but have separate nuclei, allowing for both cells to be partially functional and produce TAAs from the tumor cell which can be complexed with DC-derived MHC molecules (Koido *et al*., 2004). Several studies have used allogeneic DCs fused with autologous tumor cells with promising results (Zhou *et al*., 2009; Lei *et al*, 2009); however, one of the clear disadvantages of using allogeneic DCs is the limitation of eliciting only $CD8^+$ T cell responses. It is becoming more widely acknowledged that $CD4^+$ T cells are necessary for the ultimate antitumor response (Marzo *et al*., 2000; Tanaka *et al*., 2005). New methods of combining DC-tumor fusions with adjuvants, such as OK-432 (Koido *et al*., 2007) and HSP70 (Karyampudi *et al*., 2008), or inhibiting T regulatory cells (Li *et al*., 2007) suggest even more effective strategies for DC cancer vaccines.

1.4.3. Maturation State of Dendritic Cells for Fusion Vaccines

The maturation state of DCs for cancer vaccines has been an area of intense scrutiny over the last several years. In general, immature dendritic cells are more effective at antigen uptake (Rovere *et al*., 1998) and mature dendritic cells are capable of presenting these antigens to effector cells and eliciting stronger immunologic responses (Guermonprez *et al*., 2002). In fact, the presentation of antigens by immature dendritic cells often skews the immune response toward tolerance (Steinman *et al*., 2003). So,

presumably, immature DCs would be most effective to utilize when pulsing or fusing with antigen; however, the vaccine end product should consist of properly matured dendritic. This clear distinction is not as defined in animal and clinical settings, however. Indeed, immature DCs were demonstrated to induce potent cytolytic activity by splenocytes, when fused with MC38 tumor cells (Takeda *et al*., 2003). And other experiments have shown no attributing difference between the effectiveness of immature or mature DC-tumor fusions (Vasir *et al*., 2008), while others clearly argue the necessity of mature DCs for vaccine therapy (Baggers *et al*., 2000). Although general upregulation of CD80, CD86, CD83, CD40 and MHC class II molecules correspond with a maturing DC phenotype, the necessary profile of these proteins and the extent to which they must be expressed for maximum efficacy has not yet been fully elucidated. Thus, the plasticity of DCs has been a complicating factor in designing optimal DC vaccines.

1.5. Dendritic Cells and Chemokines

Chemokines are small chemotactic cytokines that direct the trafficking of immune cells, regulate angiogenesis/angiostasis, and can be involved in the promotion of metastasis. Here we will focus on their chemotactic effect on immune cells, specifically dendritic cells. During an infection or inflammatory response, inflammatory chemokines are rapidly produced (CCL2, CCL4, CCL5, CXCL8) (Sallusto *et al*., 1999). Circulating immature DCs express inflammatory chemokine receptors (CXCR1, CCR1, CCR2, CCR5) (Allavena *et al*., 2000), which allow recruitment to sites of inflammation to take up antigen and aid the immune response against infection. As shown in Figure 1.2, when DCs reach these sites of inflammation and are exposed to maturation stimuli (CD40L,

PRR ligands, inflammatory cytokines), they undergo maturation, decreasing their antigen capturing abilities and increasing MHC class I and II and costimulatory molecules, with a concomitant decrease in inflammatory chemokine receptor expression (Dieu *et al*., 1998). This downregulation of CCRs occurs at both transcriptional and post-transcriptional levels and is accompanied by an increase in CCR7 expression (Hirao *et al*., 2000). This increase in CCR7 and decrease in inflammatory CCRs allows the mature DCs to migrate away from the inflamed site toward nearby draining lymph tissues. CCR7 binds CCL19 and CCL21, which are constitutively produced on endothelial and stromal cells in B/T-cell areas of the lymph (Rot *et al*., 2004). This upregulation of CCR7 on mature DCs brings them into close contact with lymphocytes, specifically T cells, to present antigen via MHC class I or II (Ebert *et al*., 2005). This is the typical process of DC activation, LN migration and presentation of antigen to T cells, and DC vaccines must be capable of this migration in order to effectively stimulate antitumor T cell responses.

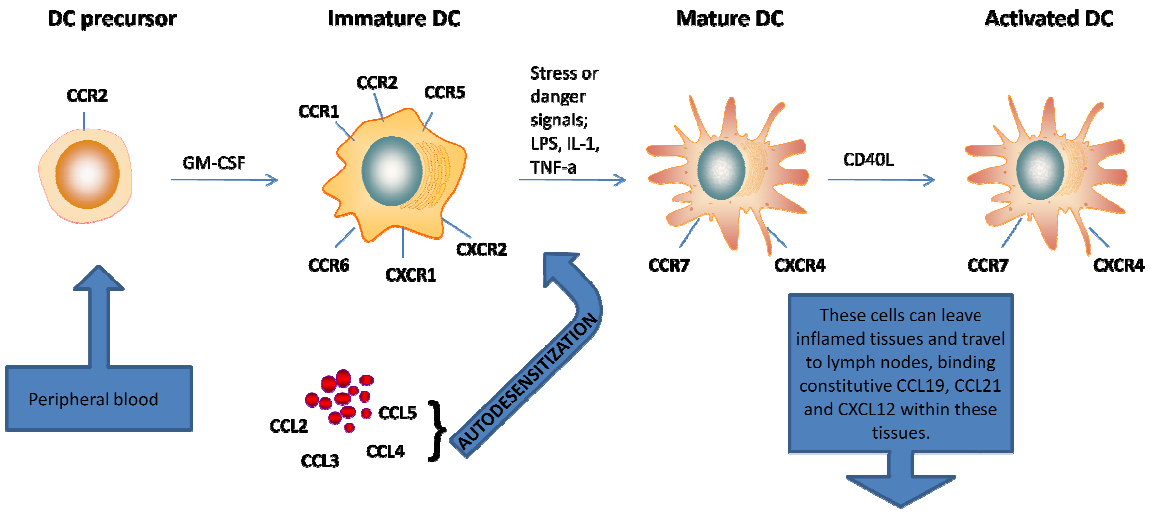


Figure 1.2. **Chemokine receptor expression from pre-DC to activated DC.** Dendritic cell precursors in peripheral blood express CCR2. These cells, once stimulated with GM-CSF, IL-4, Flt-3L or other stimulants, differentiate into immature DCs expressing inflammatory chemokine receptors CCR1, CCR2, CCR5, CCR6, CXCR1 and CXCR2. These chemokine receptors allow recruitment of immature DCs to sites of inflammation, following the inflammatory chemokine gradient. Once arriving at inflamed tissues, danger signals and inflammatory cytokines permit DC maturation. Maturing DCs undergo autodesensitization of inflammatory chemokine receptors, with concomitant expression of inflammatory chemokines. Shortly thereafter, mature DCs express CCR7 and CXCR4, which allow for their departure from inflamed tissues (since they no longer express inflammatory chemokine receptors), and recruitment to secondary lymph tissues.

1.6. Toll-like Receptors (TLRs)

The immune system is divided into two modes of protection: innate immunity and adaptive or acquired immunity. The innate branch was long thought to induce non-specific immunity, while the adaptive branch generated specific immune reactions via T- and B-lymphocytes. The 'nonspecific' definition of the innate immune system is being reconsidered as it is becoming increasingly more apparent that there is quite a deal of specificity involved. This modification of our understanding of innate immunity has been primarily driven by the expanding knowledge of pattern-recognition receptors (PRRs) and their ligation with pathogen-associated molecular patterns (PAMPs), as well as the continued discovery of novel PRRs and PAMPs. PRRs are a broad class of receptors that bind stress signals including microbial and synthetic components. They include TLRs, CD14, NOD-like receptors (NLRs), RIG-like receptors (RLRs), complement receptors, and C-type lectins (Palm *et al*., 2009). The subgroup TLRs has probably received the most attention. TLRs have been highly conserved throughout evolution, attributing to their biological importance. They are a subfamily of the larger superfamily including IL-1Rs. Both IL-1Rs and TLRs have cytoplasmic TIR (Toll/IL-1R) domains containing three signaling motifs. However, their extracellular (EC) regions differ: IL-1Rs have three immunoglobulin-like domains and EC TLR regions consist of stacks of leucine-rich repeats (LRRs) that are arranged into a horseshoe structure (Iwasaki *et al*., 2004; Akira *et al*. 2004). To date there are ten TLRs identified in humans and thirteen in mice. TLRs 1-10 are similar between human and mouse; however, the functionality of murine TLR8 is questioned (Gorden *et al*., 2006). TLR1, -2, -4, -5 and -6

are membrane-bound and TLR3, -7, -8 and -9 are located on endosomes within the cell, which is expected since they recognize nucleic acid structures (most often viral). Here we will focus on murine TLRs.

1.6.1. Dendritic Cells and TLRs

Dendritic cells are important mediators of TLR signaling since they are poised at the interface of innate and adaptive immunity. TLR expression on DCs greatly depends on the DC subset. $CD4^+$ DCs express all murine TLRs except TLR3; $CD8^+$ DCs lack TLR5 and TLR7; $CD4^-CD8^-$ DCs express TLRs1-9 (Edwards *et al*., 2003; Iwasaki *et al*., 2004); and BMDCs express all TLRs except TLR3 and TLR7 (Dearman *et al*., 2009). Interestingly, plasmacytoid DCs lack TLR3 which is one of the TLRs most prominent in IFN-β production. It makes sense that different DC subsets have different TLR profiles since they will encounter particular pathogens depending on their anatomical location. This is yet another way the immune system regulates tailored immune responses.

1.6.2. Tailored Immune Responses by TLRs

It is becoming increasingly evident that the complexity of the innate immune system may reach far beyond that of the adaptive. Each of the TLRs, although similar, induces slightly different combinations of cytokine, chemokine and other immune mediator profiles. Yet another level of regulation involves refined signaling with different combinations of TLR ligands. These responses may also vary depending on the type of cell they induce. One such way of regulating TLR responses is the recruitment of different adaptor proteins to the TLR cascade (Re *et al*., 2004). Even within one TLR ligand, there can be subtle differences that call for quite different responses. For

example, it was recently discovered that three different forms of polyriboinosinic-polyribocytidylic acids (poly I:C) elicit three different gene profiles in phenotypically mature DCs (Avril *et al*., 2009). Simultaneous activation of different TLRs can enhance or even inhibit TLR signaling pathways. It has been shown that TLR8 inhibits TLR7 and -9 and TLR9 inhibits TLR7 in HEK293 cells (Wang *et al*., 2006); this, however, has not been confirmed *in vivo*. In essence, different combinations of TLR expression on a particular cell type, the number of different signaling pathways that can be induced by each TLR, and the synergistic or antagonistic effects of multiple TLR ligand induction may induce millions of different gene profile combinations. Indeed, the complexity of innate immunity intensifies as our knowledge of the field expands.

1.7. Inflammation and Cancer

There has been long standing evidence for the involvement of inflammation and cancer. Infectious diseases causing chronic inflammation account for approximately one-fourth of all cancers in developed countries (Balkwill *et al*., 2001). *Helicobacter pylori*, Hepatitis B virus, Hepatitis C virus, and Epstein-Barr virus infections are all major contributing risk factors for gastric cancer, hepatocellular carcinoma, and lymphoproliferative disorders, correspondingly (Coussens, 2002). It was suggested that these microbes encouraged oncogene activation, but it has since been recognized that chronic inflammation is a major player in increased risk factors for certain cancers (Coussens, 2002). Most common cancers associated with chronic inflammation include the aforementioned as well as cervical (Castle *et al*., 2001), lung (Lee *et al*., 2009),

bladder (Michaud, 2007), esophageal (Deans *et al*., 2006), pancreatic (Farrow *et al*., 2002) and prostate cancers (Palapattu *et al*., 2005).

Along with the correlation between chronic inflammation and cancer, there is also an association of decreased risk for certain cancers with the prolonged used of NSAIDs (non-steroidal anti-inflammatory drugs), which is linked to the inhibitory effect on NFκB transcription factors (Garber, 2003). Aspirin, the archetype of NSAIDs, has been demonstrated to play a protective role against colorecal cancer (Dube *et al*., 2007), esophageal and gastric cancers (Gonzalez-Perez *et al*., 2003), and breast cancer (Zhao *et al*., 2009). Karin and Greten pointed out that not only does long-term use of NSAIDs correlate with decreased risk for certain cancers, but the use of ginseng, green tea, resveratrol and curcumin also benefit the reduction of cancer occurrence (Karin *et al*., 2005). These compounds all share inhibitory activities on the NFκB family of transcription factors that are responsible for inducing many of the inflammatory cytokines and chemokines (Bharti *et al*., 2002). So it seems as though inhibiting inflammation via downregulation of NFκB may be the key to decreasing risk factors for inflammation-associated cancers.

NFκB represents a family of five transcription factors including NFκB1 (p105/p50), NFκB2 (p100/p52), RelA (p65), RelB and c-Rel. The latter three are all capable of binding DNA, however, p105 and p100 must be cleaved from NFκB1 and NFκB2, respectively, to release the corresponding DNA binding subunits p50 and p52 (Caamano *et al*., 2002). Different combinations of NFκB subunits dimerize to achieve tailored responses to specific stimuli. All five members, p50, p52, RelA, RelB and c-Rel,

contain Rel homology domains (RHD) that are responsible for dimerization and DNA binding; and the RHD is also where inhibitory IκB family members interact with NFκB subunits (Ghosh *et al*., 1998). NFκB dimers are held inactive in the cytoplasm by IκB proteins. The primary inhibitory proteins include IκBα, IκBβ, IκBε, p100 and p105. In order for the NFκB dimers to travel to the nucleus, they must be released from the IκBs. Upon appropriate stimulation, IκB kinases (IKKs) are activated which in turn phosphorylate IκBs. There are three main subunits of the IKK complex, IKKα, IKKβ and IKKγ (NEMO). Typically, IKKβ is involved with inflammatory cascades, whereas IKKα is primarily implicated in morphogenic signaling. NEMO is the regulatory subunit of this complex in that its activation is required for IKKα and IKKβ phosphorylation of IκBs (Rothwarf *et al*., 1998). IKKs phosphorylate IκBs making them targets for ubiquitination and subsequent degradation, releasing the NFκB subunits and allowing their nuclear translocation.

Tollip, an adaptor protein first discovered in the IL-1R pathway (Burns *et al*., 2000), has since been identified as an inhibitory protein for certain TLR pathways (Zhang *et al*., 2002). Induction of IL-1R and most TLRs stimulates pro-inflammatory mediators, hence Tollip is involved in minimizing or suppressing the induction of inflammation. It does not act via direct NFκB inhibition, but rather acts upstream to inhibit IRAK1 (Zhang *et al*., 2002). Upon stimulation of TLR ligands, particularly TLR2 and TLR4, IRAK4 is brought into close proximity with IRAK1 and phosphorylates IRAK1 causing the release

of Tollip and downstream activation of the TLR pathway. It acts in a similar manner in IL-1R signaling, to suppress IL-1 induced signaling (Burns *et al*., 2000).

NFκB inhibitors are constantly being researched in efforts to develop drugs to treat such diseases as asthma, autoimmune, arthritis and certain inflammation-associated cancers. Many plant-based compounds have been identified to interfere with the inflammatory pathway by specifically inhibiting NFκB activation. These include many antioxidants such as curcumin, quercetin, epigallocatechin 3-gallate (EGCG), fungal products and many other compounds (Nam, 2006). Curcumin has been shown to inhibit NFκB in a dose-dependent manner, and its mechanism of action may by hinged to the induced expression of HSP70 (Dunsmore *et al*., 2001). Besides its inhibition of NFκB signaling, quercitin has also been shown to reduce constitutive NFκB activation in human prostate cancer cells (Nam, 2006). EGCG, the most reputable biologically active component of green tea, inhibits NFκB by inhibiting degradation of IκB via inhibition of IKK activity in both cancer cells and normal cells (Yang *et al*., 2001). Three fungal products, cycloepocydon, gliotoxin and panepoxydone, are known NFκB inhibitors. Umezawa and colleagues tested these three NFκB inhibitors for their potential to reverse the constitutive NFκB-induced protection from apoptosis in tumor cells, and their results were promising for cancer therapies (Umezawa *et al*., 2000). These compounds show potential for the future development of anti-inflammatory drugs as well as chemopreventive agents.

1.8. *Morinda citrifolia* (Noni)

Morinda citrifolia (Noni) has been used for centuries to treat an array of maladies. It is a small evergreen tree native to South Asia, with elongated leaves, white tubular flower clusters, and greenish fruits that ripen to whitish-yellow. Its popularity has been on the rise particularly since the relaxed FDA guidelines for dietary supplements (Wang *et al*., 2002) and the advent of Tahitian Noni International. Because of this, scientific research on Noni has expanded greatly over the last decade. The main objectives are to ensure its safety as a food product and to identify components responsible for its purported effects. Although its oldest uses primarily involved topical application of leaves and roots, the fruit has been more popular for modern usage (Pawlus *et al*., 2007). Fermented Noni is the most traditional method of consumption. Its fruits are harvested, collected into glass jars and allowed to ferment for several hours up to several weeks (Dixon *et al*., 1999). The documented, scientifically researched effects of Noni include anti-inflammatory, antiangiogenic, anticancer, antibacterial and antioxidant. Here we will focus on the applications and components of Noni fruits and their aforementioned effects.

Noni has been reported to modulate immune cells and has been implicated in enhancing the adaptive immune response by activating T and B cells. There is growing concern about the administration of antibiotics to production animals, therefore, new methods for boosting immune systems of livestock has been hotly pursued. Since Noni has long standing reports of being an immunomodulator, it is not surprising that it is being considered for the treatment of neonatal and newborn calves. In one such study,

Noni was used as a bactericidal supplement for calves and delivered promising results with enhanced killing of *Escherichia coli* (Schafer *et al*., 2008). There is also a patent on Noni formulations for immunomodulation of T cells in neonatal stock animals, an important mediator for resisting bovine RSV, a common cause for death of newborn calves (Darien *et al*., 2007). A study in 2008 revealed the immunomodulation of B cells as a result of direct DC stimulation with fermented Noni exudate. Murine DCs were treated with Noni for 24 hours, washed, then cocultured with splenocytes. Interestingly, the population of proliferative splenocytes was primarily B cells; and not only were B cells stimulated to divide, but they also underwent differentiation and Ig class switching (Zhang *et al*., 2009). Thus, Noni has obvious effects on the adaptive branch of immunity and may play an important role in future livestock management.

Traditional uses for Noni have included treatment for sprains, menstrual difficulties, arthritis, asthma and general swelling (Wang *et al*. 2002), all of which are associated with inflammation. One possible mechanism of the anti-inflammatory effects of Noni has been explained by Palu and colleagues. Here it was reported that Noni activates cannabinoid receptors, specifically CB_2, in a dose-dependent manner (Palu *et al*., 2008). CB_2 activation is associated with anti-inflammation. Also, *Morinda morindoides*, a close cousin of Noni, was shown to inhibit complement factors, which substantiates its use for treating rheumatic pains (Cimanga *et al*., 2003). These two mechanisms are only part of Noni's arsenal for inducing anti-inflammatory effects; the others may be attributed also to its antioxidant properties.

Antioxidant activity of Noni has been credited to several compounds, namely coumarin derivatives, scopoletin, 7-hydroxycoumarin (7-HC) and 4-HC, a novel anthraquinone, two novel iridoid glucosides, and quercetin (Ikeda *et al*., 2009). Scopoletin is a well kown antioxidant and has been attributed to antimicrobial and anti-inflammatory activities (Deng *et al*., 2007). The novel anthraquinone (2-methoxy-1,3,6-trihydroxyanthraquinone) was found to be nontoxic at high doses and nearly 40 times more potent at reducing quinone than the positive control (Pawlus *et al*., 2005). A detailed study by Deng and colleagues revealed eight compounds of Noni that inhibited lipoxygenase. These included scopoletin and two novel lignans, a lactone and (+)-3,3'-bisdemethyltanegool (Deng *et al*., 2007). In 2005, a novel lignan, Americanin A, was identified in Noni and it was confirmed to be a potent antioxidant (Su *et al*., 2005). Based on the recent detailing of the chemical constituents of Noni and their antioxidant activities, science is able to start backing the claims held by Noni users for centuries.

Lastly, Noni has received much attention for its antiangiogenic and anticancer properties in both animal models and clinical settings; however, the mechanisms underlying this effect are largely unknown. The first attempt to identify the component of Noni responsible for its antitumor activities described a water-soluble, ethanol-precipitable, polysaccharide-rich substance from Noni fruit as the immunomodulator (Hirazumi *et al*., 1999). Importantly, this report was the first to note the enhanced chemotherapeutic activities of Noni. Shortly thereafter, Wang and Su identified Noni to inhibit DMBA-DNA adduct formation (Wang *et al*., 2001), implicating its protective role in chemically induced cancers. In 2003, Furusawa and Hirazumi expanded their research

to demonstrate the antitumor properties of Noni precipitate (ppt) were dependent on macrophages, NK cells and T cells and extended its synergistic effects with even more chemotherapies (Furusawa *et al*., 2003). Also in 2003, Noni was tested for antiangiogenic effects and was found to inhibit new vessel sprouts at 5% concentration and induce vessel degeneration at 10% concentration (Hornick *et al*., 2003). The most recent account of antitumor activity by Noni confirmed the necessity of NK cells and T cells for tumor eradication (Li *et al*., 2008). Noni was shown to be effective at both cancer prevention and treatment within S180 and Lewis Lung tumor models. It is worth noting, mice that cleared tumor burden were rechallenged after two months, and all rejected the tumors; five months later the same mice were challenged again and 15 of 16 mice rejected the tumors. The strong animal evidence for anticancer properties of Noni has encouraged its research, and with time we are beginning to understand its mechanisms of action.

2. FUSION INDUCED REVERSAL OF DENDRITIC CELL MATURATION: ALTERED EXPRESSION OF INFLAMMATORY CHEMOKINES AND CHEMOKINE RECEPTORS IN DENDRITOMAS

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2.1. Abstract

Dendritic cell-mediated cancer immunotherapy employs several ways to engage tumor antigens. We have demonstrated in both pre-clinical animal studies and early clinical trials that dendritomas, highly purified hybrids between dendritic cells (DC) and tumor cells, are superior activators of anti-tumor immunity. It has been argued, however, that DC vaccines may be dysfunctional in lymph node migration. In the present study we examined inflammatory chemokine and chemokine receptor expression as well as other maturation induced genes in dendritomas produced from either immature or mature DCs in order to shed light on their capacity to migrate from injection sites to draining lymph nodes and elicit an appropriate immune response. RNA microarray analysis was used to identify gene expression profiles for inflammatory chemokines and receptors and other maturation induced genes within dendritomas, lysate-pulsed dendritic cells, immature DCs and mature DCs. Gene regulation was confirmed with relative quantification, real-time RT-PCR in a separate experiment. We found that fusion of immature DCs to tumor cells initiates maturation with respect to inflammatory chemokines, chemokine receptors and other maturation induced genes in a similar pattern as LPS matured DCs. Interestingly, we saw a reversed gene profile when mature DCs were fused to tumor cells. LPS matured DCs displayed the chemokine repertoire expected with DC maturation; however, once fused to tumor cells, these chemokines and other maturation induced genes reverted to levels comparable to immature DCs. It appears that mature DCs used for dendritoma production result in a de-mature phenotype. Our results indicate that dendritomas from immature DC/tumor cell fusions may be more effective in migration

from injection site to draining lymph nodes and, therefore, would be more effective in stimulating anti-tumor immunity.

2.2. Introduction

Dendritic cells (DC) are professional antigen presenting cells, which play a vital role in stimulating immune responses against infections and tumor cells (Banchereau *et al*., 1998; Hart, 1997; Dunn *et al*., 2002). DC-mediated cancer immunotherapy is aimed at picking up where the host immune system failed by presenting tumor antigens to innate and adaptive effector cells, thus stimulating anti-tumor immunity for immediate therapy and latent protection (Ullrich *et al*., 2008; Banchereau *et al*., 2002; Steinman *et al*., 2006). Three basic approaches have been employed to engage DCs with tumor antigens: tumor antigen pulsing, genetic modification with tumor antigen genes or RNA, and DC/tumor fusion (Schuler *et al*., 2003; Svane *et al*., 2003). Although all three approaches have been widely utilized and have successfully increased tumor-antigen reactive T cells in periphery, the DC/tumor hybridoma vaccine has proved more effective since this strategy provides a broader diversity of known and unknown tumor antigens as well as MHC class I and MHC class II antigens to the immune system (Ward *et al*., 2002; Shimizu *et al*., 2004).

Most DC hybridoma studies have utilized fusion mixtures as a vaccine due to the lack of selective markers on fused DC/tumor cells to purify hybrids from the fusion mixture (Haigh *et al*., 1999). The immune response stimulated by this mixture is compromised due to the presence of large numbers of unfused cells or self/self fused

cells. In order to solve this problem, we developed a novel hybrid purification technology that instantly purifies DC/tumor hybrids from the mixture (Holmes *et al*., 2001). Animal studies demonstrated that highly purified DC/tumor hybrids, or dendritomas (DT), are superior activators to stimulate anti-tumor immunity compared with fusion mixtures (Li *et al*., 2001). Several clinical trials using dendritoma vaccines have been conducted; and data shows that DT vaccines stimulate anti-tumor immune responses in some patients and demonstrate observable clinical responses (Wei *et al*., 2006; Wei *et al*., 2007).

On the other hand, although most DC hybridoma vaccines were effective in preclinical animal studies, clinical trials have shown less encouraging results (Gong *et al*., 2008). Consequently, an important field in DC mediated cancer immunotherapy is to understand and solve the inconsistencies between animal studies and human clinical trials. The increase of regulatory T cells and tolerogenic DCs found in tumors after DC vaccine administration are two of the major factors suppressing anti-tumor immunity (Steinman *et al*., 2003; Li *et al*., 2007; Dannull *et al*., 2005). Others include DC procurement, route of administration, and tumor microenvironment (Melief *et al*., 2008; Grover *et al*., 2006). The overall belief is that DCs must be presented with maturation stimuli and tumor antigen, administered through an appropriate route for different cancers with mediators aiding their lymph node migration, and have the capacity to process and present both MHC I and II peptides in order to acquire therapeutic and long-term protective immunity (Ueno *et al*., 2006). Much research has been done to progress DC vaccination in most of these areas; however, information on the migratory capacity of DC

vaccines is still needs attention. It is imperative to understand the factors involved in the migration of dendritomas, and other DC vaccines, to the draining lymph nodes where they encounter and activate effector T cells.

Inflammatory chemokines, such as macrophage inflammatory protein 1alpha (MIP-1α) and RANTES (CCL3 and CCL5, respectively), are predominantly located at nonlymphatic sites of inflammation where they recruit immune cells to participate in antigen presentation and recognition to ultimately elicit a cell-mediated response to infection or tumor cells. Immature DCs (iDC) typically express inflammatory chemokine receptors CCR1, CCR2 and CCR5 which bring them into contact with antigens at inflammatory sites (Sozzani *et al*., 2000). Once antigen uptake has ensued, DCs rapidly increase production of inflammatory chemokines and lose responsiveness to these CCLs, a process called autodesensitization (Sallusto *et al*., 2000), allowing for reverse transmigration of activated, mature DCs (mDC) into secondary lymphoid tissues where they present antigen to effector cells. Clearly, the completion of this process is essential for effective DC vaccines. In the present study, in order to understand whether dendritomas are capable of effective migration to secondary lymphoid tissues, we examined the regulation of key chemokines and chemokine receptors along with several maturation induced genes. Our microarray and real-time RT-PCR results demonstrate that fusing tumor cells to iDCs matures them with respect to *ccr* and *ccl* expression, while fusing tumor cells to mDCs causes the reversal of *ccr* and *ccl* expression by mDCs. Our results implicate immature DCs as better choice for dendritoma production, and a

migratory mediator adjuvant may be needed when mature DCs are used for dendritoma production.

2.3. Materials and Methods

2.3.1. Mice and Tumor Cells

Female C57BL/6J mice at 6-8 weeks of age were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in our pathogen-free animal facilities. Animal experiments were carried out in accordance with both Guidelines for the Care and Use of Laboratory Animals (NIH Publication number 85-23) and institutional guidelines. Murine acute myeloid leukemia cell line C1498 and murine melanoma cell line B16F0, both C57BL/6J-derived, were maintained in complete DMEM (Gibco BRL, Grand Island, NY) supplemented with 10% FBS (Hyclone, Logan, UT) and 50 μg/ml gentamicin (Gibco BRL) at 37 °C in a humidified atmosphere of 5% CO_2.

2.3.2. Dendritic Cells

Bone marrow derived DCs were cultured as previously described (Lutz *et al.*, 1999). Briefly, bone marrow cells flushed from C57BL/6J mouse femurs and tibiae with RPMI-1640 (Gibco BRL) were filtered through 40-μm nylon cell strainers. After the removal of RBCs by ACK lysate (Lonza, Allendale, NJ), the remaining cells were resuspended in DC medium containing RPMI-1640 supplemented with 10% FBS, 50 μg/ml gentamicin, and 20 ng/ml rmGM-CSF (Sigma, St. Louis, MO) and plated at 4-5x10^6 cells/10 ml in a 100-mm tissue culture dish. On day 4, 10 ml fresh DC media was

added to each dish. On day 8, non-adherent and loosely adherent cells were harvested, washed with RPMI-1640 and replated in fresh DC medium containing 10 ng/ml rmGM-CSF with or without 100 ng/ml LPS (Sigma). On day 10, non-adherent and loosely adherent cells were collected for further studies.

2.3.3. Pulsing DCs with Tumor Lysate

B16F0 cells were collected and resuspended in a conical tube in 1x PBS at a concentration of $1x10^7$cells/mL. The tube with cell suspension was immersed in a dry-ice/methanol bath for approximately 3 minutes. Once frozen, the cells were placed in a 37°C water bath with gentle agitation and thawed completely. The process was repeated for a total of four freeze/thaw cycles. The cells were then centrifuged at 15,000 x g for 10 minutes at 20°C and supernatant was collected. Protein concentration was determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Either immature DCs (iDC) or LPS matured DCs (mDC) were incubated with 100µg/mL tumor protein lysate overnight. Pulsed DCs (LPiDC or LPmDC) were then centrifuged at 300 x g to collect cells but discard lysate in the supernatant. Cells were washed three times in 1x PBS prior to RNA extraction.

2.3.4. Cell Staining and Fusion

DCs and tumor cells were stained green and red, respectively, using PKH67-GL or PKH26-GL kits (Sigma) according to manufacturer's protocol. Stained cells were washed thrice to remove unbound dye and tumor cells were irradiated with 50 Gy. Tumor

cells and DCs were fused at a ratio of 1:1 or 1:2 using a 50% PEG 10% DMSO solution (Sigma). After fusion, cells were incubated overnight in DC medium.

2.3.5. FACS Sorting

The fusion mixtures were harvested (both adherent and non-adherent) and resuspended in PBS at a concentration of $1x10^7$ cells/ml. Cells were sorted on a BD FACSCalibur (Becton Dickinson, San Jose, CA) according to the dual fluorescent colors. Sorted cells, labelled as dendritomas (DT), were resuspended in DC medium and incubated overnight with or without 100 ng/ml LPS prior to RNA extraction. Dendritomas exhibited both green and red fluorescence and purity was greater than 95 percent.

2.3.6. Microarray

LPiDC, LPmDC, DT, DC, and tumor cell RNA was extracted using ArrayGradeTM Total RNA Isolation Kit (SABiosciences, Frederick, MD) and was sent for pathway-focused GEArray service using mouse Dendritic and Antigen Presenting Cell Oligo GEArray (SABiosciences). Analysis was performed using the GEArray Expression Analysis Suite software (SABiosciences).

2.3.7. Real-time PCR

Real-time one-step RT-PCR was performed on total RNA via an Eppendorf Mastercycler ep Realplex^2 (Eppendorf, Westbury, NY) using QuantiTect Primers optimized for QuantiTect SYBR Green RT-PCR kit (Qiagen, Valencia, CA). Results were normalized

to β-actin, which was chosen over gapdh and β2M as the housekeeping gene, since it was least affected by treatment. Data was analyzed by ΔΔCt calculations.

2.4. Results

2.4.1. Tumor lysate matures iDCs

Overnight incubation of iDC with B16F0 tumor cell lysate induced DC maturation with respect to inflammatory chemokine and chemokine receptors. iDCs express high levels of inflammatory receptors: *CCR2 and* CCR5 and low levels of inflammatory chemokines: CCL3, CCL5 and CCL22. Upon antigen uptake and processing, iDCs are induced to decrease levels of inflammatory receptors, while increasing inflammatory chemokine expression. Microarray analysis shows tumor lysate pulsed iDCs (LPiDCs) displayed a drastic reduction in *ccr2* and *ccr5* and an increase in inflammatory and inducible chemokines *ccl3*, *ccl5* and *ccl22*; their levels were nearly identical to DCs matured with LPS (Figure 2.1A).

DCs cultured from C57BL/6J mice were matured with LPS on day 8 of culture. On day 10, they were incubated with B16F0 tumor lysate and RNA was extracted after overnight incubation. RNA microarray shows that tumor lysate pulsing of mDCs (LPmDC) caused no change in expression of *ccr2*, *ccr5*, *ccl3*, *ccl5*, or *ccl22* compared to LPS matured DCs (mDC, Figure 2.1B).

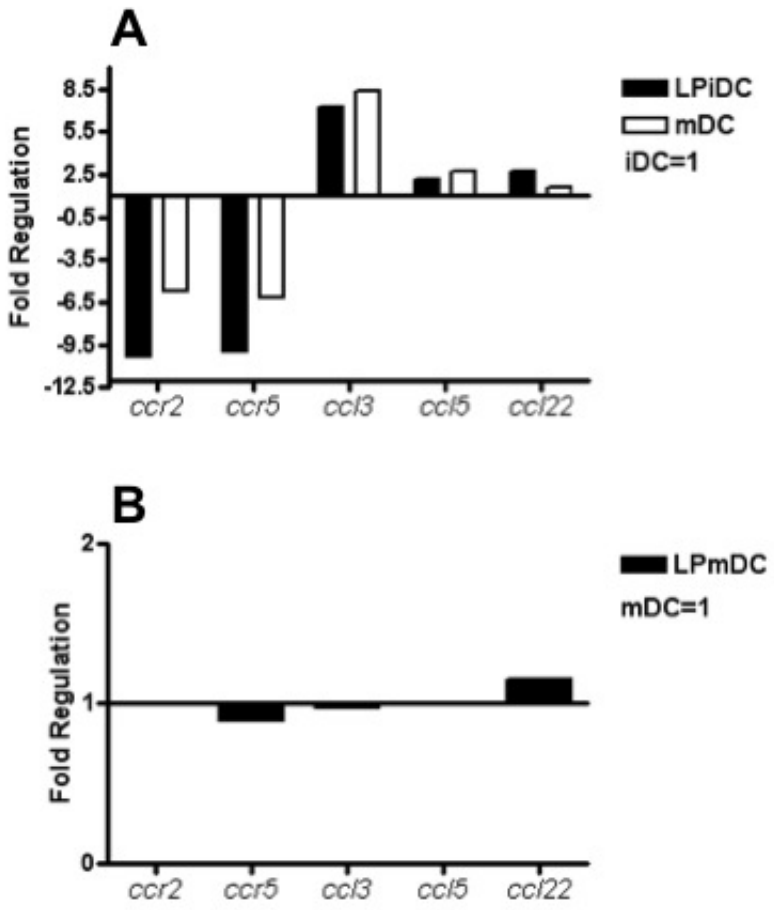

Figure 2.1: **Tumor lysate pulsing matures iDC, but causes no change in mDC**
Total RNA was extracted from iDC, LPiDC, mDC, and LPmDC and was analyzed by RNA microarray for the indicated chemokine and chemokine receptors. (A) Gene expression profiles of LPiDC and mDC compared to iDC. (B) Gene expression profiles of LPmDCs compared to mDC.

2.4.2. Fusion with tumor cells matures iDC

iDCs were fused with B16F0 tumor cells by PEG in a 2:1 ratio. The fused hybrids (immature dendritomas or iDT) were purified from the fusion mixture by dual fluorescent FACS sorting on day 11. RNA was extracted from iDTs following collection and used for RNA microarray. As shown in Figure 2.2A, iDTs dramatically decreased expression of *ccr2* and *ccr5*, but increased *ccl3*, *ccl5* and *ccl22* as compared to iDCs. This pattern is consistent with the expression of mDCs and LPiDCs (Figure 2.1A); therefore, fusion of iDC with tumor cells instigates maturation with respect to these inflammatory chemokines and receptors. To further confirm this finding, real-time RT-PCR was performed to measure the change of expression in *ccr2*, *ccr5*, *ccl3*, ccl5, and ccl22 in iDTs. The results, as shown in Figure 2.2B, demonstrate a similar pattern of expression: down-regulation of ccr genes and up-regulation of ccl genes.

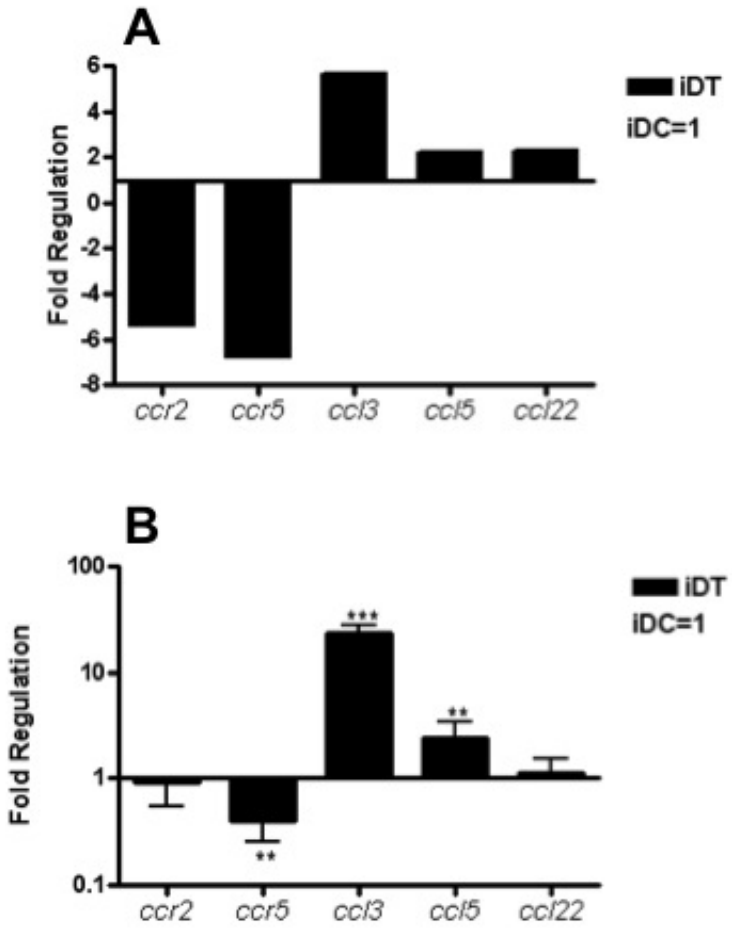

Figure 2.2: **Fusion with tumor cells matures dendritic cells.** iDCs were fused with C1498 or B16F0 tumor cells in a 1:1 or 2:1 ratio using PEG in two separate experiments. The fusion hybrids (iDT) were generated by FACS sorting based on the DT technology (see Materials and Methods). Total RNA was analyzed by RNA microarray and real-time RT-PCR for the indicated genes. (A) Microarray analysis of chemokine and chemokine receptor gene expression by iDTs, iDC = 1. (B) Real-time RT-PCR analysis of chemokine and chemokine receptor gene expression by iDTs, iDC = 1. (*$p<0.05$, **$p<0.01$ ***$p<0.001$)

Additional genes associated with LPS induced DC maturation were examined in two independent microarray experiments in iDTs made from either C1498 tumor cells or B16F0 tumor cells. As shown in Figure 2.3, acpp, atf4, clec4d, ifit1, il-1b, g1p2, and prg1 were upregulated (Figure 2.3A), while cd209a, f13a1, icosl, ifi30, ifngr1, rnase6, and s100a4 were downregulated (Figure 2.3B), indicating that fusion of iDCs and tumor cells yields a maturing hybridoma.

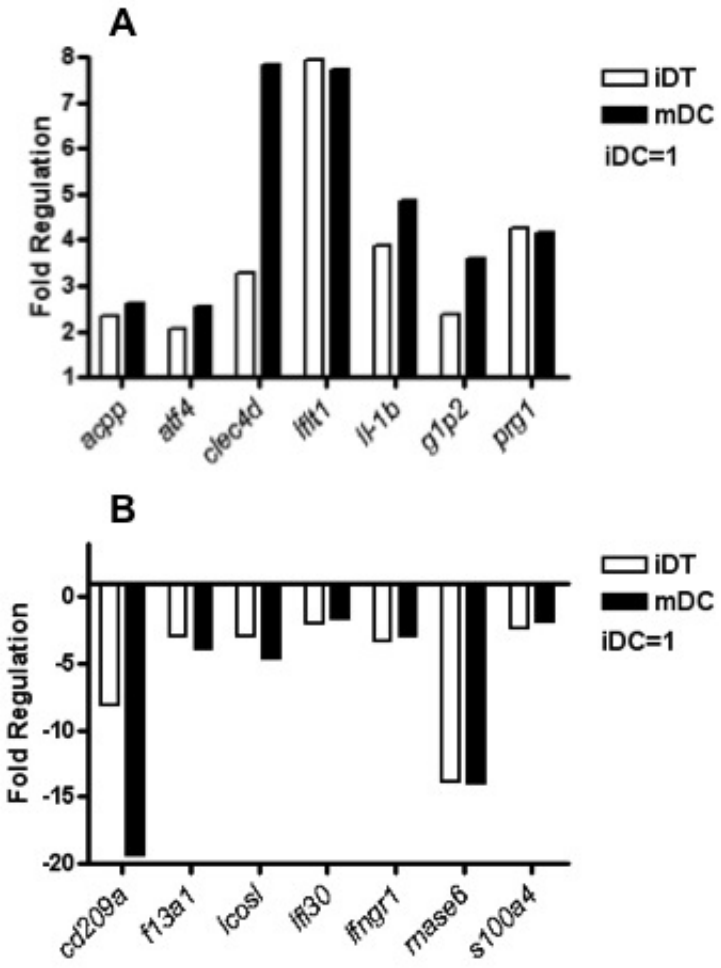

Figure 2.3: **Fusion of iDCs with tumor cells yields a similar gene profile to LPS matured DCs.** iDCs were fused with C1498 or B16F0 tumor cells in a 1:1 or 2:1 ratio using PEG in two separate experiments. The fusion hybrids (iDT) were generated by FACS sorting based on the DT technology (see Materials and Methods). Total RNA was analyzed by RNA microarray for the indicated genes. (A) Genes upregulated by both iDTs and LPS matured DCs compared to iDCs. (B) Genes downregulated by iDTs and LPS matured DCs compared to iDCs.

2.4.3. Fusion with tumor cells reverses the maturation for mDTs

DCs cultured for 8 days were matured with LPS for two days and fused with B16F0 tumor cells. The hybrids (mature dendritomas or mDT) were purified from the fusion mixture using the same technology described above. RNA microarray established the increased expression of *ccr2* and *ccr5*, while the expression of *ccl3*, *ccl5*, and *ccl22* decreased (Figure 2.4A): a clear pattern of DC de-maturation with respect to inflammatory ccl and ccr. This finding is in contrast to lysate pulsed mDCs where no significant change was observed (Figure 2.1B). Real-time RT-PCR also confirmed the change of expression in *ccr2, ccr5, ccl3, ccl5,* and *ccl22* in mDTs. The results, shown in Figure 2.4B, are consistent with microarray data: ccr genes are upregulated and ccl genes downregulated. Additional genes of interest analyzed by microarray of mDTs showed a similar pattern compared to iDCs. *Cd209a, f13a1, marcks, rpl13a, cd207, cdc42*, and *pfn1* were upregulated (Figure 2.5A), while *acpp, atf4, clec4d, il-1b, il-12b, cd36, sod2, cd80, pnrc1* and *tnfsf4* were downregulated (Figures 2.5B), further supporting de-maturation of mDTs.

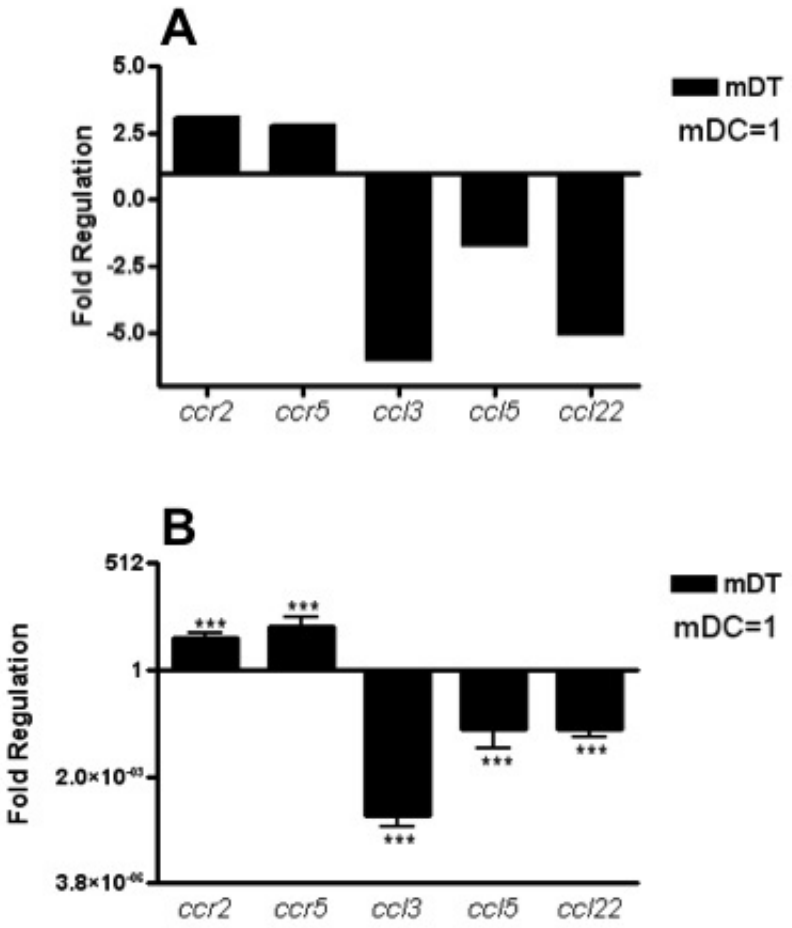

Figure 2.4: **Fusion of mDCs with tumor cells (mDT) yields an opposing chemokine/chemokine receptor pattern to mature DCs.** mDCs were fused with B16F0 tumor cells in a 2:1 ratio using PEG. The fusion hybrids, mDT, were generated by FACS sorting based on the DT technology (see Materials and Methods). Total RNA was analyzed by RNA microarray and real-time RT-PCR for the indicated genes. (A) Microarray analysis of chemokine and chemokine receptor gene expression by mDTs, mDC = 1. (B) Real-time RT-PCR analysis of chemokine and chemokine receptor gene expression by mDTs, mDC = 1. (*$p<0.05$, **$p<0.01$ ***$p<0.001$)

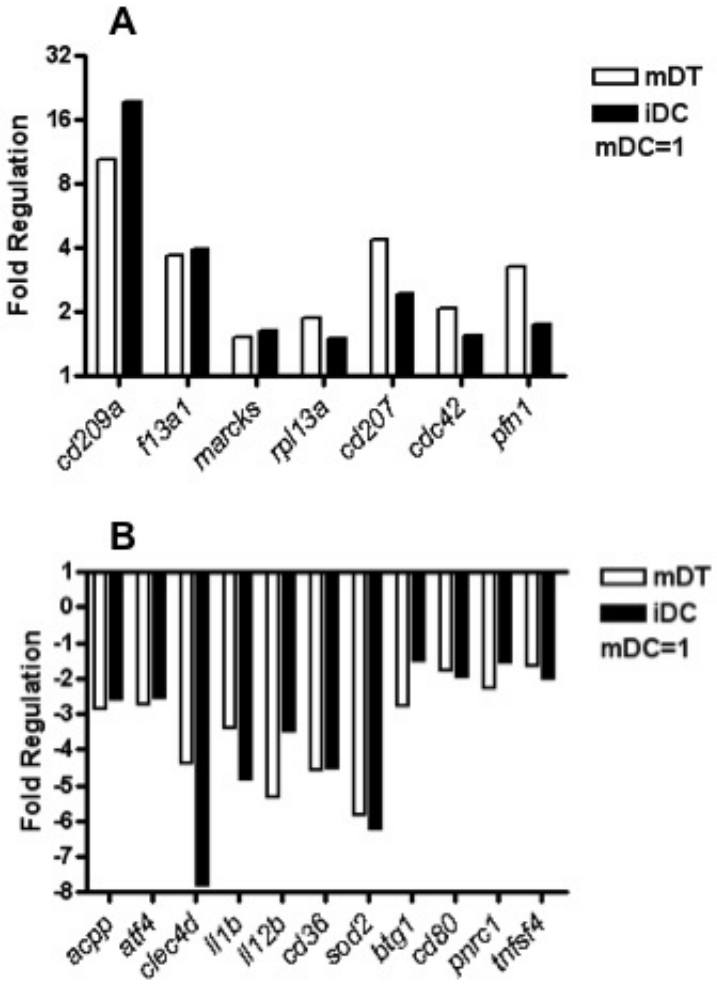

Figure 2.5: **Fusion of mDCs with tumor cells de-matures mDTs.** mDCs were fused with B16F0 tumor cells in a 2:1 ratio using PEG. The fusion hybrids, mDT, were generated by FACS sorting based on the DT technology (see Materials and Methods). Total RNA was analyzed by RNA microarray for the indicated genes. (A) Genes upregulated by both mDTs and iDCs compared to mDCs. (B) Genes downregulated by mDTs and iDCs compared to mDCs.

2.5. Discussion

Tumor lysate pulsed immature DCs (LPiDCs) are mature compared to iDCs. LPiDCs express increased levels of CD80, CD86, and CD40 costimulatory molecules as well as molecules involved in antigen presentation while decreasing those involved with antigen uptake (Paglia *et al*., 1996; Berard *et al*., 2000). For the first time, we show that LPiDCs are activated to elevate ccl levels and decrease ccr levels (Figure 2.1A), suggesting that antigen-pulsing results in efficient DC activation to mediate the chemokine receptor paradigm switch in the absence of other inflammatory stimuli or microbial products such as LPS.

We did not observe any significant change in the genes analyzed by microarray for tumor lysate pulsed, LPS matured DCs (LPmDCs, Figure 2.1B). This could be due to the notion that LPS matured DCs have downregulated antigen-uptake mechanisms, or compared to microbial danger signals, tumor lysates are weak DC maturation agents. Interestingly, it has been shown that coincubating mDCs with CCR7 ligands, CCL19/ELC or CCL21/SLC, re-stimulates endocytosis by previously matured DCs (Yanagawa *et al*., 2003); therefore, it seems plausible to induce mDC uptake of tumor lysate by concomitant incubation with a CCR7 ligand. Based on our data, it would be better to use iDCs to engage tumor antigens; nonetheless, it would be interesting to observe the effects of pulsing mDCs with tumor lysate combined with ELC or SLC.

iDTs, composed of tumor cells and iDCs, express increased levels of *ccl* genes and decreased *ccr* genes (Figure 2.2). This process is assumed to be mostly due to the

autocrine action of chemokines on their respective receptors expressed by DCs (Kikuchi *et al*., 2001; Sozzani *et al*., 1998); although it has been shown that C5a and fMPL do not have this autodesensitization effect on DCs (Sozzani *et al*., 2000). In addition to the *ccl* and *ccr* paradigm switch, iDT maturation is also accompanied with up-regulation of genes such as *acpp, atf4, clec4d, ifit1, il-1b, g1p2,* and *prg1* and down-regulation of *cd209a, f13a1, icosl, ifi30, ifngr1, rnase6,* and *s100a4* as confirmed in two separate microarray analyses and also observed in canonical DC maturation when iDCs are matured with LPS (Figure 2.3). Again, our data points to successful maturation when iDCs are fused to tumor cells (iDTs) which is important when designing DC vaccines. Interestingly, we observed that iDTs expressed high levels of *il-10* in two separate microarrays, while mDTs expressed non-detectable levels of *il-10* (data not shown), contradicting other reports where both iDC/tumor fusions and mDC/tumor fusions have elevated levels of IL-10 (Vasir *et al*., 2008). This may implicate the need for simultaneous addition of a danger signal to overcome IL-10 production when manufacturing iDTs. More studies are needed to clarify the role of IL-10 in this process.

Mature DCs used for fusion oddly de-mature, whereas iDCs are matured with fusion to tumor cells. Interestingly, the *ccl* and *ccr* expression of mDTs was completely reversed compared to iDTs and mDCs. There was a significant decrease in *ccl* expression with a compensating increase in *ccr* expression (Figure 2.4), closely mimicking the *ccr* and *ccl* expression of iDCs. This phenomenon may be due to complete washing of mDCs before fusion, which frees the supernatant of CCLs, no longer triggering autodesensitization. Taking this into account, mDTs may not be as

good as iDTs for migration from injection sites to draining lymph nodes. Not only did mDTs de-mature with respect to *ccr* and *ccl* expression, but they also upregulated *cd209a*, *f13a1*, *marcks*, *rpl13a*, *cd207*, *cdc42*, and *pfn1*, and downregulated *acpp*, *atf4*, *clec4d*, *il-1b*, *il-12b*, *cd36*, *sod2*, *cd80*, *pnrc1* and *tnfsf4* (Figure 2.5) in synchrony with iDCs. Our data suggest mDTs may need restimulation with CCLs or possibly other danger signals in order to resume proper migratory capacity and antigen presentation since they seem to have more genes in common with iDCs than mDCs. It is possible that stimulating dendritomas with microbial danger signals or adjuvants could overcome this problem as seen with pre-treatment and post-treatment with OK432 and CpG ODN increasing the effectiveness of DC fusion vaccines (Li *et al*., 2008; Koido *et al*., 2007a; Koido *et al*., 2007b).

In conclusion, our data unfolds a significant pattern of chemokine expression in dendritomas depending on the maturation state of DCs and demonstrates the plasticity of LPS matured DCs used for vaccine preparation. Although mature DCs are preferred to reduce tolerance or expansion of regulatory cells, we see here there may be problems with migration and induction of immune response. To circumvent this problem, either immature DCs should be used for fusion with simultaneous addition of a danger signal such as LPS, OK432, or CpG ODN to properly mature the DCs and abrogate production of IL-10; or if mature DCs are used for fusion, it must be investigated to see if they can be re-stimulated to secrete CCLs, down-regulate CCRs, and reclaim maturation in order to properly migrate and activate an immune response against presented tumor antigens.

2.6. Acknowledgements

We would like to thank Eric Holle and his staff for the professional care of mice used in this study, Xinhai Zhang for critical review and discussion of data, Leigh Theofanous and Hilary Bouton-Verville for acquisition of data, and Keri Nowend for help with statistical design. This study is partially supported by the GHS Oncology Endowment (all authors, EH, XZ, LT, HBV, KN) and the William K. and Frances J. Bryan New Hope Fund for Cancer Research (YW, TEW).

3. *MORINDA CITRIFOLIA* (NONI) AS AN IMMUNOMODULATOR OF DENDRITIC CELLS: INHIBITION OF TOLL-LIKE RECEPTOR AND NFκB PATHWAYS AND PROCUREMENT OF ANTI-INFMMATORY MEDIATORS

3.1. Abstract

Morinda citrifolia (Noni) has been used for centuries to treat a wide range of maladies. Its current growth as a dietary supplement to enhance the immune system has demanded more scientific research into its purported effects. Whereas most previous reports have been self-published, recent information has been described in peer-reviewed scientific journals on the anticancer, anti-inflammatory, and immunomodulatory effects of Noni. Also, fermented Noni exudate was shown to activate dendritic cells, one of the key players in innate and adaptive immunity. Noni fruit contains a wide variety of microorganisms, and upon fermentation all but one are killed, resulting in a large array of pathogen-associated molecular patterns (PAMPs), potentially activating pattern-recognition receptors. Since dendritic cells express a wide variety of Toll-like receptors (TLRs), we hypothesized that Noni-treated dendritic cells would alter the expression of inflammatory genes downstream of TLR pathways. The expression of *tnf*, *il-6*, *ptgs2*, *ccl3* and *ccl5* were examined in Noni-stimulated bone marrow-derived dendritic cells. Furthermore, in a second experiment, dendritic cells were treated with endotoxin-depleted Noni and 84 genes in the TLR pathway were evaluated. Both experiments were carried out by real-time RT-PCR with SYBR green chemistry. FACS analysis also revealed an intriguing shift from a CD11c+CD83+ dendritic cell population to a much larger Mac-3+ population. Overall, Noni induced low levels of inflammatory cytokines, significantly increased anti-inflammatory mediators, and inhibited elements of TLR

signaling, specifically NFκB. Gradually, Noni's molecular mechanisms of action on immune cells are being unraveled, and will hopefully clarify its antitumor and anti-inflammatory roles for possible drug development.

3.2. Introduction

Morinda citrifolia, commonly known as Noni in the US and Hawaii, is widespread throughout Polynesia, Southeast Asia, India, and Australia. It was brought to the Pacific Islands more than 2000 years ago as the ancient Polynesians second most popular medicinal herb. Cultures throughout the region have, for millennia, employed every part of the plant as a food staple and for an immense range of illnesses and ailments, including bacterial and viral infection, fungus, cancer, AIDS, high blood pressure, and much more. In all, there are 40 known and recorded uses for the plant in herbal remedies. In addition, the leaves of the plant have been used to produce dyes for clothing and other textiles (Wang, 2004; Pawlus *et al*., 2007).

Noni grows as a small evergreen tree. It reaches 12-15 feet in height, is decorated with large bright green elliptical leaves, columnar white flowers and lumpy yellow fruit, about the size of a potato. Its seeds have an air sac at one end, giving them the buoyancy they need to float across the pacific and take root in islands throughout the region, to earn the name "ocean-going Noni," as the plant has been called in some regions. Finally, the Noni fruit can be identified by its rancid odor and foul taste – justifying another popular name – the "cheese fruit" (Dixon *et al*., 1999; Wang *et al*., 2002)

Extensive research is ongoing to identify both known and novel active compounds of Noni. These include, but are not limited to, novel anthraquinones,

saccharide fatty acid esters, glycosides, iridoid glucosides and damnacanthal (Akihisa *et al*., 2007; Guangming *et al*., 2001; Su *et al*., 2005; Hiwasu *et al*., 1999; and Kamata *et al*., 2006). Currently, several potent antioxidants and anti-inflammatory agents have been extracted and identified (Pawlus *et al*., 2007; Deng *et al*., 2007; Pawlus *et al*., 2005; Ikeda *et al*., 2009; Su *et al*., 2005). Besides isolated compounds, different fractions of Noni have been studied for immunomodulatory effects. A polysaccharide-rich precipitate of Noni juice has been found to have antitumor potential (Hirazumi *et al*., 1999; Furusawa *et al*., 2003). Raw Noni, or Noni puree, shows antiangiogenic, cancer preventive and bactericidal properties (Wang *et al*., 2001; Wang *et al*., 2002; Wang *et al*., 2008; Hornick *et al*., 2003; Palu *et al*., 2007; Schafer *et al*., 2008). Wong reported two case studies involving patients with adenocarcinoma self medicating with fermented Noni. The first patient began drinking Noni on his deathbed after refusing medical treatment, and showed no evidence of disease a year after diagnosis. The second patient, who underwent surgery and had metastases in 17 of 28 lymph nodes tested, survived sixteen years disease-free with the Noni treatment (Wong *et al*., 2004). This is extremely significant since less than ten percent of patients survive even five years with this level of metastasis.

Recently, our lab has examined the immunomodulatory effects of fermented Noni exudate (fNE) in animal models. It was found that dendritic cells (DCs) cultured with fNE are capable of inducing B cell proliferation, differentiation and even Ig class switching (Zhang *et al*., 2009). Furthermore, fNE was found to be effective for both prevention and treatment of mice with S180 sarcomas and Lewis Lung carcinomas (Li *et*

al., 2008; Furusawa *et al*., 2003). These studies clearly demonstrated that both the innate and adaptive immune systems are involved in the antitumor effect induced by fNE.

Although fresh Noni fruit has a myriad of microflora, upon fermentation, only one fungus remains in fNE (personal communication, Marisa Wall, 2009). Our lab also found that fNE contains detectable levels of endotoxin (unpublished data). It was speculated that this novel strain of fungi along with endotoxin in fNE may be responsible for activating several innate pattern recognition receptors (PRRs), which could be responsible for its anti-inflammatory, anticancer and other immune-modulating properties. In this study, we tested DCs treated with fNE, endotoxin-removed fNE (E-fNE), or LPS for expression of genes involved with PRR signaling via real-time PCR. We show for the first time that fermented Noni, the most popular traditional method of Noni consumption, increases several inflammatory mediators at suppressed levels compared to LPS, but upon endotoxin removal, clearly promotes an anti-inflammatory response from DCs.

3.3. Materials and Methods

3.3.1. Animals

Female C57BL/6J mice, 6-8 weeks of age, were purchased from Jackson Laboratories (Bar Harbor, ME) and housed in our pathogen-free animal facilities. Animal experiments were carried out in accordance with both Guidelines for the Care and Use of Laboratory Animals (NIH Publication number 85-23) and institutional guidelines.

3.3.2. Dendritic Cells

Bone marrow-derived DCs were cultured as previously described (Lutz *et al*., 1999). Briefly, bone marrow cells flushed from C57BL/6J mouse femurs and tibiae with RPMI-1640 (Gibco BRL) were filtered through 40-μm nylon cell strainers. After lysing of RBCs with ACK lysing solution, the cell pellet was resuspended in DC medium containing RPMI-1640 (Gibco BRL) supplemented with 10% FBS (Hyclone), 50 μg/ml gentamicin (Gibco BRL), and 20 ng/ml rmGM-CSF (Sigma, St. Louis, MO) and plated at a density of 4-5x10^6 cells/10 ml in a 100-mm tissue culture dish. On day 4, 10 ml fresh DC media was added to each dish. On day 8, nonadherent and loosely adherent cells were harvested, washed with RPMI-1640 and replated into 6-well plates with fresh DC medium containing 10 ng/ml rmGM-CSF. These DC cultures were stimulated overnight with either filtered fNE supernatant at 1, 5 or 10% or LPS at 2.5, 12.5, 25 or 100ng/ml prior to RNA extraction.

3.3.3. Production of Fermented Noni Exudate (fNE)

Production of fNE was previously reported (Zhang *et al*., 2009). Briefly, ripe Noni (*Morinda citrifolia*) fruits were harvested from Kawaihae, Hawaii, thoroughly cleaned and placed in sterilized containers for fermentation at 24-30°C and humidity between 45-55%. Fermentation was terminated at 14 days and the seepage was collected in plastic bottles and stored at -80°C prior to its use. Before incubation with DCs, the liquid fermented Noni exudate (fNE) was pH adjusted to 7.0-7.4 with 50% NaOH, centrifuged to discard debris and sterilized by passing through a 0.22μm syringe filter. Endotoxin removal of Noni was performed by Detoxi-Gel™ Endotoxin Removing Gel (Thermo Scientific) according to manufacturer's instructions. Briefly, Detoxi-Gel

columns were regenerated with 1% sodium deoxycholate, then washed with pyrogen-free DPBS (Lonza) prior to fNE loading. Once fNE was loaded onto the column, it was allowed to sit at room temperature for one hour before washing through with an equal amount of DPBS. The endotoxin-depleted fNE flow-through was collected, passed through a 0.22µm filter, and used immediately in cell cultures.

3.3.4. Real-Time RT-PCR

Purified total RNA was isolated from stimulated or non-stimulated DCs using the RNeasy Plus Kit (Qiagen) according to the manufacturer's protocol and reverse transcribed with QuantiTect Reverse Transcription Kit (Qiagen). Nucleic acids were quantified using a Qubit fluorometer and appropriate Quant-it Assay Kits (Invitrogen). cDNA was analyzed for the expression of Tlr4, Tnf, Il-6, Ptgs2, Ccl3 and Ccl5 by SYBR green chemistry real-time PCR using the QuantiTect SYBR Green Kit (Qiagen). Real-time RT-PCR was carried out on the Mastercycler ep Realplex2 (Eppendorf) with PCR cycles recommended by the primer sets. Primer sequences were purchased from Real Time Primers, LLC and their sequences are available online at www.realtimeprimers.com.

3.3.5. PCR Array

Purified total RNA was isolated as described above for use in the PCR arrays; however, it was reverse-transcribed with RT^2 First-Strand cDNA Synthesis Kit (SABiosciences). Manufacturer's instructions were followed for running the PCRarray; briefly, cDNA templates were mixed with the provided PCR master mix and were

aliquoted into all 96-wells of the Toll-like Receptor Signaling Pathway PCRarray (SABiosciences). The PCR cycling program was set according to the PCRarray protocol.

3.3.6. Statistical Analysis

Fold change in gene expression for real-time RT-PCR data was calculated using the Pfaffl method, taking into account varying primer efficiencies. Statistical analyses of data were performed by analysis of variance (ANOVA) followed by Bartlett's test using Graph Pad Prism® 4 software. PCR arrays were analyzed with the complimentary RT^2 Profiler ^{TM} PCR Array Data Analysis (SABiosciences) which determines $\Delta\Delta C_t$ calculations normalized to at least 3-5 housekeeping genes, with pair-wise comparison between treated and untreated samples.

3.4. Results

3.4.1. Real-time RT-PCR

Immature DCs (iDCs) were treated with fNE or E-fNE at 1%, 5% or 10% total media concentration, or LPS at 2.5ng/ml, 12.5ng/ml and 25ng/ml for 2, 24, 48 or 72h. LPS concentrations were calculated to be equivalent to endotoxin levels of fNE. Total RNA was extracted and the expression of *Tlr4* and five downstream genes of the TLR4 pathway were assessed by real-time RT-PCR using ΔΔCt method of analysis.

3.4.1.1. Toll-like receptor 4 (tlr4)

fNE did not affect *tlr4* gene expression after 2h treatment. At 24 and 48 hours, 5% fNE significantly decreased *tlr4*, while surprisingly, 10% fNE did not change the gene expression in these time periods. After 72h treatment, a clear dose-dependent down-regulation of *tlr4* was observed (Figure 3.1A). Since fNE contains LPS (our un-

published data), we also tested the effect of LPS on *tlr4* expression by DCs. LPS in concentrations equivalent to the amount measured in fNE was used. LPS had no effect on *tlr4* at 2, 48 or 72h. Only at 24h was *tlr4* expression significantly downregulated by 12.5 and 25 ng/mL LPS, respectively (Figure 3.1B). Furthermore, since the down-regulation of *tlr4* gene expression induced by fNE was not completely repeated by LPS treatment, we tested the effect of endotoxin-removed fNE, or E-fNE, on iDCs. After 24 hours treatment, both 1% and 5% E-fNE significantly downregulated *tlr4*, while at other time points (2h, 48, or 72h), there was no effect (Figure 3.1C). These data suggest the down-regulation of *tlr4* gene expression by fNE induced DCs is not completely due to endotoxin.

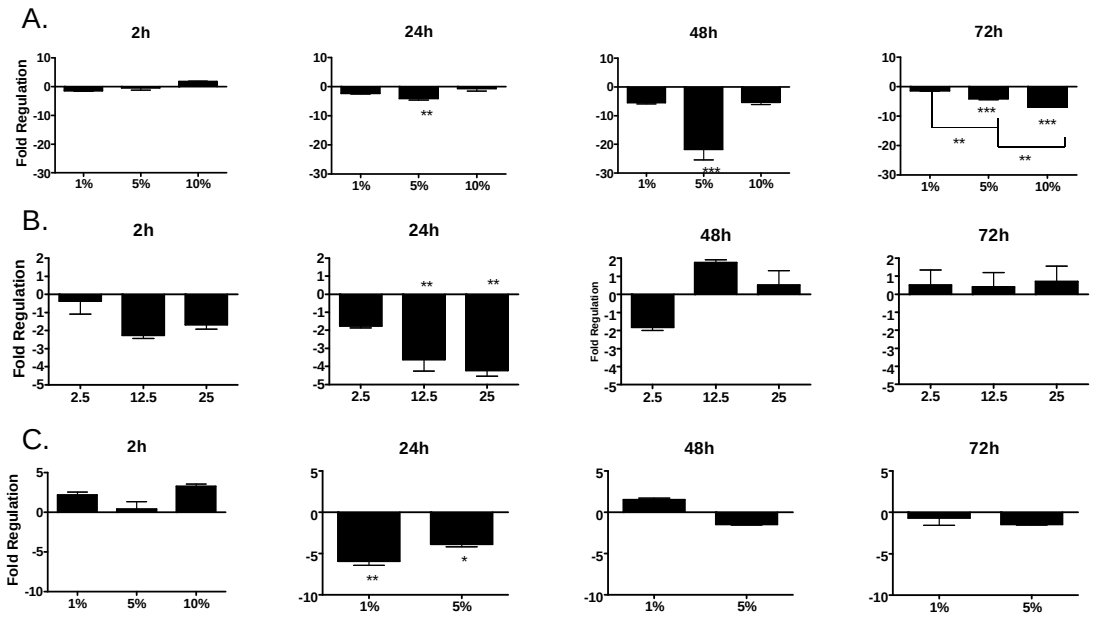

Figure 3.1. ***tlr4*** **expression by Noni- or endotoxin-depleted Noni-treated BMDCs compared to LPS treated BMDCs.** BMDCs were treated for 2, 24, 48 or 72h with Noni or endotoxin-depleted Noi at 1%, 5%, or 10% total media concentrations or with corresponding levels of endotoxin (2.5-, 12.5-, or 25ng/ml LPS). Real-time RT-PCR was performed on cDNA from the resulting samples. (A) fNE treatment of BMDCs; (B) LPS treatment of BMDCs; (C) E-fNE treatment of BMDCs. Experiments were perfomed in triplicate and data was analyzed by ANOVA followed by Bartlett's post-test. * $p<0.05$ ** $p<0.01$ *** $p<0.001$

3.4.1.2. Tumor necrosis factor-alpha (tnf)

DCs treated with fNE induced a dose-dependent increase of *tnf* gene expression at 2h, while at 24h and 48h, only 10% fNE caused a significant increase. At 72h, all three concentrations induced a significant, albeit small, increase (Figure 3.2A). Similarly, LPS treatment of DCs caused a strong, non-dose dependent increase in *tnf* at 2h. At 24h, only 25ng/mL LPS significantly altered the expression of *tnf*; and by 72h there was no significant change (Figure 3.2B). E-fNE treatment of DCs, on the other hand, induced a dose-dependent decrease in *tnf* expression at 2h. While 24h and 48h incubations did not cause a significant change, 72h treatment induced a very significant 6.6-fold decrease in *tnf* (Figure 3.2C). The increase in *tnf* by fNE-treated DCs seems to be attributed to the endotoxin in Noni, since upon endotoxin removal, not only is *tnf* production abrogated, but it is actually dose-dependently decreased. This fraction of Noni responsible for decreasing *tnf* is also observed in fNE-treated DCs since 5% fNE treatment only caused a 14-fold increase and equivalent endotoxin concentrations (12.5ng/mL LPS) induced 125-fold increase in *tnf* production; therefore there is some inhibition of *tnf* by Noni.

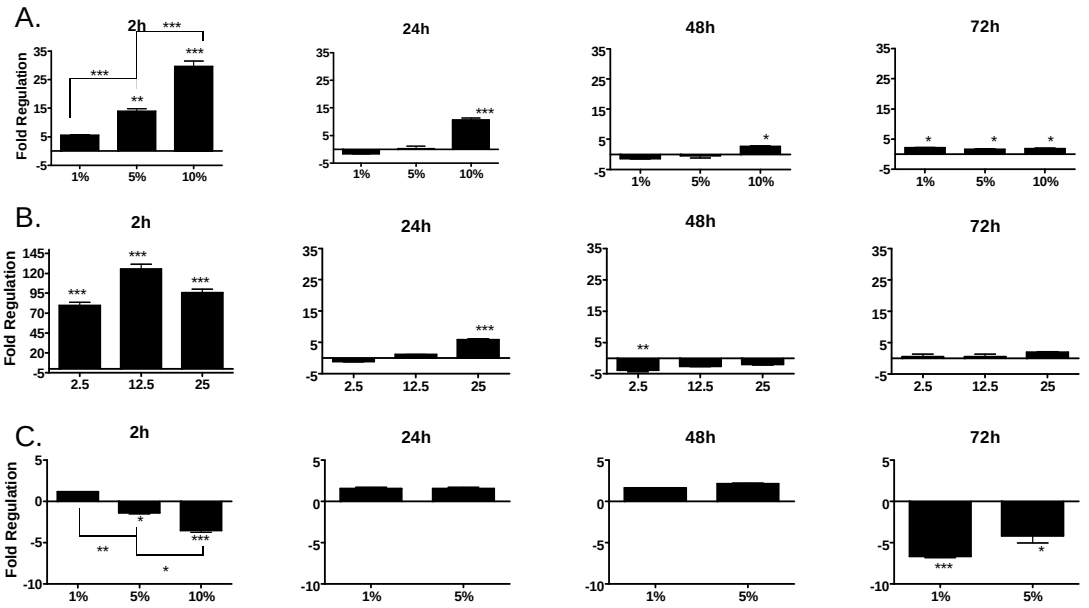

Figure 3.2: ***tnf* expression by Noni- or endotoxin-depleted Noni-treated BMDCs compared to LPS treated BMDCs.** BMDCs were treated for 2, 24, 48 or 72h with Noni or endotoxin-depleted Noi at 1%, 5%, or 10% total media concentrations or with corresponding levels of endotoxin (2.5-, 12.5-, or 25ng/ml LPS). Real-time RT-PCR was performed on cDNA from the resulting samples. (A) fNE treatment of BMDCs; (B) LPS treatment of BMDCs; (C) E-fNE treatment of BMDCs. Experiments were perfomed in triplicate and data was analyzed by ANOVA followed by Bartlett's post-test. * $p<0.05$ ** $p<0.01$ *** $p<0.001$

3.4.1.3. Interleukin-6 (il-6)

fNE treatment caused a dose-dependent increase in *il-6* production by iDCs at 2h; however, only 10% fNE remained effective through 72h, where its production peaked at 48h, 633.8-fold higher than untreated DCs (Figure 3.3A). LPS treatment also caused a non dose-dependent increase at 2h, where it peaked at 985.3-fold greater than the control. Lower dose-dependent responses were noted at 24h and 48h, with a drastic latent rebound at 72h where 12.5 and 25 ng/mL LPS treatment increased *il-6* expression 717- and 731-fold above untreated DCs (Figure 3.3B). E-fNE completely abolished the up-regulation of *il-6* induced by fNE. Furthermore, a very significant down-regulation of *il-6* occurred for 1% E-fNE, dropping 47-fold (Figure 3.3C). fNE significantly increased *il-6* expression in DCs, although not as high as LPS. Upon endotoxin removal, E-fNE ultimately decreases production of *il-6* at 72h. Again, endotoxin seems to be responsible for the increase in *il-6*, while E-fNE actually down-regulates *il-6* expression.

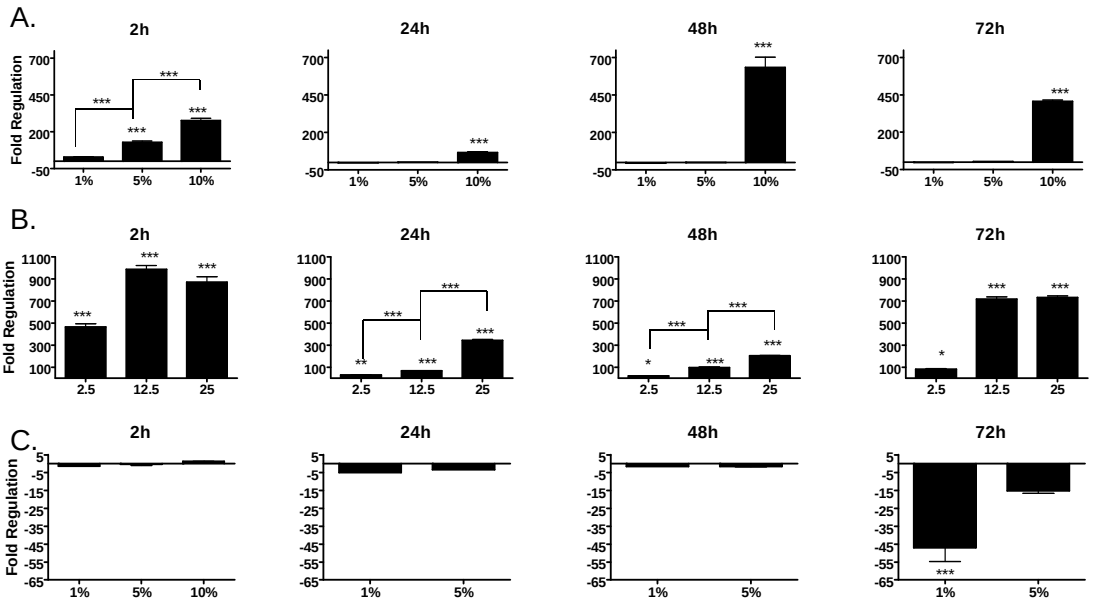

Figure 3.3: ***il-6* expression by Noni- or endotoxin-depleted Noni-treated BMDCs compared to LPS treated BMDCs.** BMDCs were treated for 2, 24, 48 or 72h with Noni or endotoxin-depleted Noi at 1%, 5%, or 10% total media concentrations or with corresponding levels of endotoxin (2.5-, 12.5-, or 25ng/ml LPS). Real-time RT-PCR was performed on cDNA from the resulting samples. (A) fNE treatment of BMDCs; (B) LPS treatment of BMDCs; (C) E-fNE treatment of BMDCs. Experiments were perfomed in triplicate and data was analyzed by ANOVA followed by Bartlett's post-test. * $p<0.05$ ** $p<0.01$ *** $p<0.001$

3.4.1.4. Prostaglandin-endoperoxide synthase 2 (Ptgs2)

Treatment of iDCs with fNE caused a dose-dependent increase in *ptgs2* at 2h, followed by a sustained up-regulation through 48h by 10% fNE. Although a lower fold increase, 5% and 10% fNE significantly upregulated *Ptgs2* expression at 72h (Figure 3.4A). LPS treatment also caused a dose-dependent increase at 2h, followed by an 854-fold peak at 24h. Levels decreased by 48h, but remained dose-dependently upregulated. Surprisingly, at 72h 2.5 ng/mL and 25 ng/mL LPS treatments upregulated *ptgs2* 635- and 584-fold, respectively, while 12.5ng/ml LPS did not show any effect. Of interest, at 2h the upper level of concentrations for both fNE and LPS treatments induced a nearly identical up-regluation of *ptgs2* at 453- and 445-fold, correspondingly (Figure 3.4B). E-fNE induced a much smaller scale up-regulation of *ptgs2*, peaking at merely 15.2-fold above the DC control. At 72h, it began to down-regulate expression, where 1% E-fNE decreased *ptgs2* 19-fold (Figure 3.4C). It is likely that some other component of fNE is responsible for up-regulating the production of *ptgs2*, since upon endotoxin removal, 2h treatment with E-fNE still induces a dose-dependent up-regulation, although a transient effect.

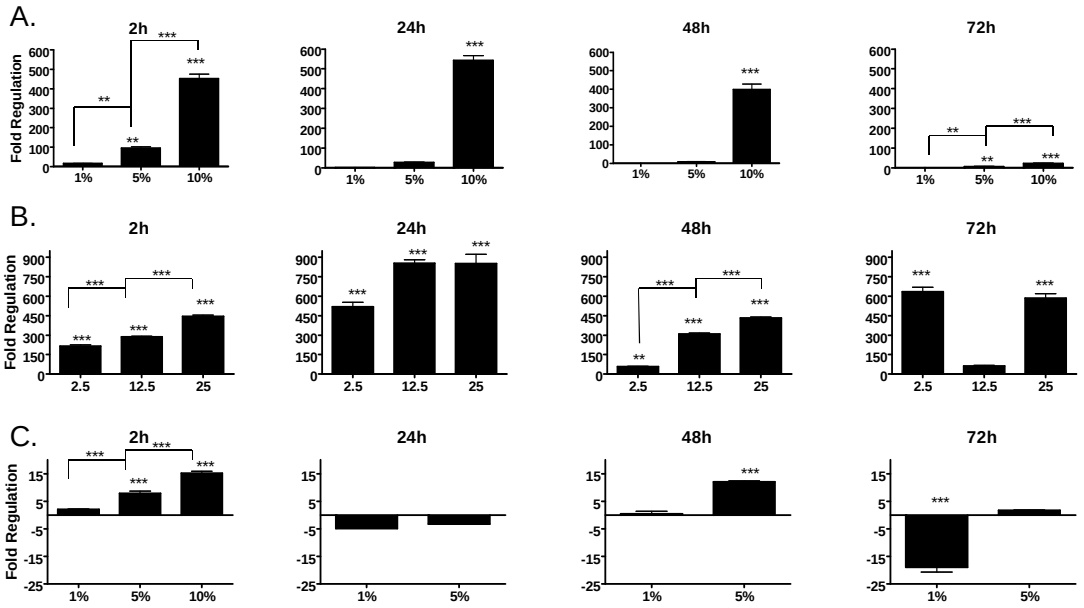

Figure 3.4: ***ptgs2* expression by Noni- or endotoxin-depleted Noni-treated BMDCs compared to LPS treated BMDCs.** BMDCs were treated for 2, 24, 48 or 72h with Noni or endotoxin-depleted Noi at 1%, 5%, or 10% total media concentrations or with corresponding levels of endotoxin (2.5-, 12.5-, or 25ng/ml LPS). Real-time RT-PCR was performed on cDNA from the resulting samples. (A) fNE treatment of BMDCs; (B) LPS treatment of BMDCs; (C) E-fNE treatment of BMDCs. Experiments were perfomed in triplicate and data was analyzed by ANOVA followed by Bartlett's post-test. * $p<0.05$ ** $p<0.01$ *** $p<0.001$

3.4.1.5. Chemokine ligand-3 (Ccl3)

At 2h, *ccl3* expression peaked with 5% fNE treatment. At 24h, 1% and 5% fNE did not significantly alter its expression, but 10% fNE induced the highest level of *ccl3* at 8.13-fold above control. By 48h, 1% and 5% fNE significantly downregulated *ccl3* (Figure 3.5A). LPS induced stronger *ccl3* expression at 2h than fNE, peaking at 26-fold above control for 12.5 ng/mL; however, no significant change occurred from 24-72h (Figure 3.5B). E-fNE abolished the effects of *ccl3* up-regulation by fNE and strongly downregulated its expression at 72h, where 1% E-fNE decreased *ccl3* expression as low as 27.5-fold below control (Figure 3.5C). Overall, fNE and LPS treatment quickly increased expression of *ccl3* in DCs, but this effect tapered off with time. Upon removal of endotoxin, E-fNE caused no significant change in *ccl3* expression from 2-48h, but a strong 27.5-fold down-regulation at 72h. Endotoxin appears responsible for *ccl3* expression by fNE-treated DCs.

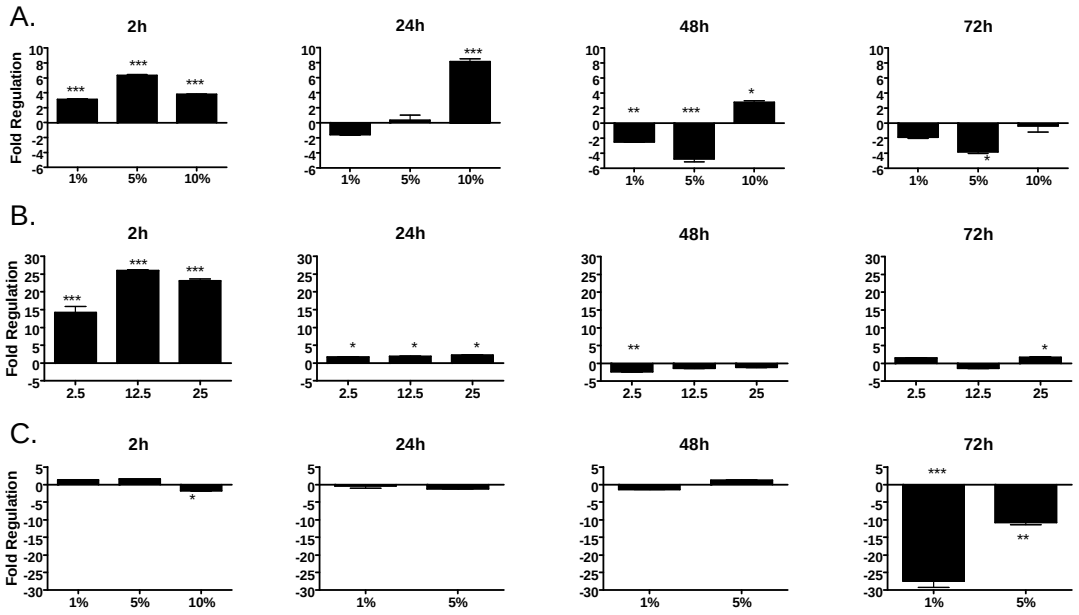

Figure 3.5: ***ccl3* expression by Noni- or endotoxin-depleted Noni-treated BMDCs compared to LPS treated BMDCs.** BMDCs were treated for 2, 24, 48 or 72h with Noni or endotoxin-depleted Noi at 1%, 5%, or 10% total media concentrations or with corresponding levels of endotoxin (2.5-, 12.5-, or 25ng/ml LPS). Real-time RT-PCR was performed on cDNA from the resulting samples. (A) fNE treatment of BMDCs; (B) LPS treatment of BMDCs; (C) E-fNE treatment of BMDCs. Experiments were perfomed in triplicate and data was analyzed by ANOVA followed by Bartlett's post-test. * $p<0.05$ ** $p<0.01$ *** $p<0.001$

3.4.1.6. Chemokine ligand-5 (Ccl5)

fNE treatment caused no significant change in *ccl5* production at 2h, but a dose-dependent increase was observed at 24h, 48h and 72h. Its production peaked at 72h for 10% fNE, where it was 62.5-fold higher than untreated DCs (Figure 3.6A). LPS at 12.5ng/ml and 25ng/ml induced minimal expression of *ccl5* at 2h, then a much greater expression from 24-72h, peaking at 78.4-fold at 24h. *Ccl3* production remained increased at relatively consistent levels for all concentrations of LPS from 24-72h (Figure 3.6B). E-fNE caused no significant change in *ccl3* expression from 2-48h, but again, induced down-regulation of the gene at 72h, where 1% E-fNE caused a 19.7-fold decrease, and 5% caused a 7.46-fold decrease (Figure 3.6C).

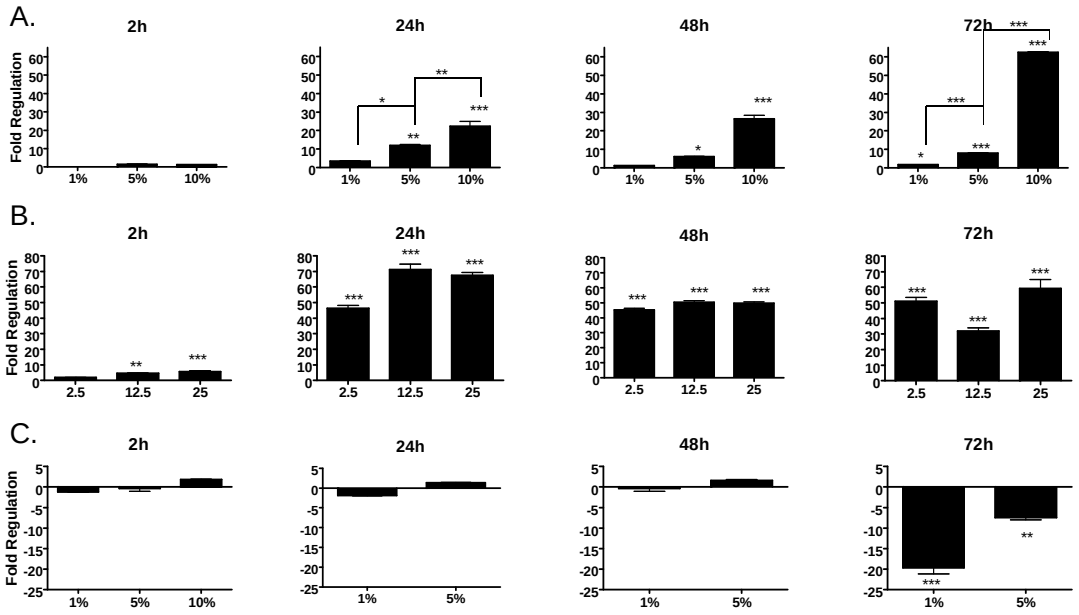

Figure 3.6: ***ccl5* expression by Noni- or endotoxin-depleted Noni-treated BMDCs compared to LPS treated BMDCs.** BMDCs were treated for 2, 24, 48 or 72h with Noni or endotoxin-depleted Noi at 1%, 5%, or 10% total media concentrations or with corresponding levels of endotoxin (2.5-, 12.5-, or 25ng/ml LPS). Real-time RT-PCR was performed on cDNA from the resulting samples. (A) fNE treatment of BMDCs; (B) LPS treatment of BMDCs; (C) E-fNE treatment of BMDCs. Experiments were perfomed in triplicate and data was analyzed by ANOVA followed by Bartlett's post-test. * $p<0.05$ ** $p<0.01$ *** $p<0.001$

3.4.2. RT-PCR Array

Based on the greater suppressive nature of E-fNE, we decided to further examine some other genes involved in signaling pathways and adaptor molecules affected by E-fNE. We performed real-time PCR array on 84 genes involved in TLR signaling and several downstream pathways (NFκB, JNK, IRF, etc). iDCs were incubated with 5% E-fNE for 2, 24 or 48h; total RNA was extracted, and real-time RT-PCR was performed to assess the regulation of genes related to TLR signaling. Upregulated and downregulated genes, compared to non-stimulated DC controls, are represented in Table 3.1 and Table 3.2, respectively.

	Upregulated Genes (Fold Regulation)				
Gene	2 hour	p	24 hour	p	48 hour
Tlr2	2.7	***			
Tlr5	2.3	*			
Cd14	1.7	**			
Hspa1a	2.1	**	32.8	***	560.3
Mapk8ip3	1.7	*			
Peli1	2.7	***			
Ripk2	2.3	*			
Tollip			1.6	**	
Traf6	2.6	*			
Ifnβ1	10.5	***	157.8	***	1596
Il-1a	16.6	***			
Il-1b	28.3	***			
Il-6	41.9	***	36.8	***	47.2
Il-10	18.7	***	15.7	***	1.6
Nfkb1	2	**			
Nfkb2	1.8	***			
Nfkbia	3.2	***			2.7
Nfkbib			1.8	*	
Nfkbil1	3.1	***			
Rel	4.7	***	2.3	*	
Tnfaip3	5.3	**			4
Jun	1.5	*	2		
Mapk8	1.8	*			
Cebpb			2		
Clec4e	3.4	**			
Ptgs2	83	***	267.8	***	2.4
Cxcl10	15.8	***	6	*	47.2
Ifnβ1	10.5	***	157.8	***	1596
Irf1	2.3	**			
Irf3	1.9	**			2.3
Tbk1	1.7				
Cd80	2.5	**	2.5	*	6.6
Cd86			3.7	**	3.3

Table 3.1: **Upregulated genes of the TLR signaling pathway by Noni treated DCs.** Non-stimulated BMDCs were treated with 5% endotoxin-removed fermented Noni exudate (E-fNE) for 2, 24 or 48h. Total RNA was extracted and real-time RT-PCR was performed on the 84 genes of the TLR pathway PCRarray from SABiosciences. Arrays were normalized to at least 2-5 housekeeping genes and compared to non-treated BMDCs. Triplitate PCRarrays were performed on 2h and 24h samples. Only one PCRarray was performed on the 48h E-fNE sample and one on the 48h non-treated DC control. The delta-delta Ct method was used to calculate fold change in gene expression.

	Downregulated Genes (Fold Regulation)				
Gene	2 hour	p	24 hour	p	48 hour
Tlr1			-2.6	*	-2.3
Tlr2			-4.2	***	-15.2
Tlr4			-5.3	**	
Tlr5					-10.6
Tlr7			-8.6	*	-4.2
Tlr8			-299	***	-228
Tlr9			-5.5	**	-6.7
Btk			-6.0	***	-5.3
Cd14					-9.1
Ly86			-22.8	***	-18.6
Ly96					-3.4
Myd88			-5.2	***	-9.5
Peli1					-2.4
Ticam2			-3	**	-6.5
Tirap	-2.4	*			-13.9
Casp8			-3.8	***	-10.3
Fadd			-3.1	**	-7.4
Irak1			-9.6	*	-3.5
Map3k7			-2.9	***	-4.3
Ube2n			-6.9	***	
Ube2v1			-2.3	**	
Ccl2			-2.2	***	-38.3
Ikbkb			-4.2	**	-10.6
Il-1a					-10.8
Il-1b					-76.6
Il-1r1			-4.6	**	-8.9
Map3k1			-2.7	*	
Rela			-10.3	*	
Tnf			-15.4	*	
Tnfrsf1a			-3.5	**	-3.4
Elk1			-2.4	*	
Fos			-5.9	**	-9.6
Map2k3			-3.6	***	-2
Map2k4			-2.5	*	-4.7
Map3k1			-2.8	*	
Map3k7					-4.3
Mapk9			-4	***	-6.5
Il-6ra			-7.6	**	-234

Table 3.2: **Downregulated genes of the TLR signaling pathway by Noni treated DCs.** Non-stimulated BMDCs were treated with 5% endotoxin-removed fermented Noni exudate (E-fNE) for 2, 24 or 48h. Total RNA was extracted and real-time RT-PCR was performed on the 84 genes of the TLR pathway PCRarray from SABiosciences. Arrays were normalized to at least 2-5 housekeeping genes and compared to non-treated BMDCs. Triplicate PCRarrays were performed on 2h and 24h samples. Only one PCRarray was performed on the 48h E-fNE sample and one on the 48h non-treated DC control. The delta-delta Ct method was used to calculate fold change in gene expression. Fold regulation gives magnitude and direction of regulated genes.

3.4.2.1. Toll-like receptors (TLRs)

Bone marrow-derived DCs expressed higher levels of genes for TLRs 2,4,8 and 9 than TLRs 1,5 and 7; however, *tlr3* was not expressed. *Tlr2* and *tlr5* were upregulated upon E-fNE stimulation after 2h. Then at 24h, *tlr(1,2,4,7,8,9)* were all downregulated with *tlr8* showing a striking 299-fold decrease. At 48h, *tlr(1,2,5,7,8,9)* all decreased with *tlr8* at 228-fold below untreated DCs.

3.4.2.2. TLR adaptors and interacting proteins

At 2h, several adaptor molecules commonly associated with TLR2 were upregulated (*ripk2*, *cd14*, *hspa1a*) (Zhang *et al*., 2009); while *tirap* and *ly96* (MD2), which are commonly associated with TLR4, were downregulated.

By 24h, only *hspa1a* remained upregulated, and was joined by *tollip*, known to diminish TLR2 and TLR4 responses in mammals. Many other adaptors were downregulated (*ly86* (MD1), *btk*, *myd88*, *tirap*, and *ticam2*). These genes were still downregulated at 48h and were joined by *cd14* and *ly96*. *Hspa1a* on the other hand, exponentially increased 560-fold.

3.4.2.3. Cytoplasmic effectors

The only upregulated gene in the effector group was *traf6*, which was upregulated at 2h. And by 24h, most effectors were downregulated including *casp8*, *fadd*, *irak1*, *map3k7*, *eif2ak2*, *ube2v1* and *ube2n*. At 48h, *casp8*, *fadd*, *irak1*, *map3k7*, and *eif2ak2* remain decreased.

3.4.2.4. NFκB pathway and target genes

At 2h, *nfkb1* and *nfkb2* were upregulated by E-fNE treated DCs, along with the inhibitors, *nfkbia*, *nfkbib*, *nfkbil1*, and *tnfaip3*. Expression of cytokines *il-6*, *il-1a*, *il-1b*, *il-10* and *ifnβ1* were also increased at 2h. *Ccl2* was the only gene significantly downregulated at 2h.

By 24h, *ifnβ1* increased 158-fold compared to untreated DCs, and *nfkbib*, *il-6*, *il-10*, and *rel* expression remained upregulated. Downregulated genes include *tnf* (15.4-fold reduction), *il-1r1*, *ikbkb* (IKK-β), *tnfrsf1a*, and *map3k1* (MEKK-1).

The only cytokines remaining increased at 48h include *ifnβ1*, which exponentially increased 1596-fold above untreated DCs, and *il-6*, which remained steady. *Tnfaip3* and *nfkbia* inhibitors were also upregulated at 48h. Several cytokines were downregulated (*il-1a*, *il-1b*, *ccl2*) along with *ikbkb* (IKK-β), *il-1r1* and *tnfrsf1a*.

3.4.2.5. JNK/p38 pathway

E-fNE treatment of DCs resulted in increased *mapk8* (JNK1) and *jun* expression by 2h. By 24h, *jun* remained upregulated, but most of the p38 pathway genes were downregulated including, *mapk9* (JNK2), *map2k3* (MKK-3), *map2k4* (MKK-4), *map3k1* (MEKK-1), *elk1* and *fos*). All but *map3k1* and *elk1* remained downregulated at 48h, but were replaced by *map3k7*.

3.4.2.6. NF/IL-6 pathway

The NF/IL-6 transcription factor is responsible for stimulating the promoter region of the COX-2 (*ptgs2*) gene. *Ptgs2* had the largest increase at 2h and 24h for all genes in this pathway (83- and 268-fold, correspondingly). *Clec4e* and *cebpb* (CRP2) were increased at 2 and 24h, respectively. And only *ptgs2* remained upregulated at 48h.

Il-6ra, on the other hand, was the only gene downregulated in this pathway with a 7.6-fold decrease at 24h and 234-fold at 48h.

3.4.2.7. Co-stimulatory markers

The gene for *cd80* was minimally increased at 2h, and both *cd80* and *cd86* were slightly induced from 24-48h.

3.4.3. FACS Analysis

When treating DCs with Noni, we observed a striking difference in cell adherence as well as the monolayer morphology compared to untreated DCs and DCs treated with LPS. These Noni-treated cells became very sticky even after only 2h treatment, and in most instances, cell scraping was required to collect enough cells for further experiments. This prompted further investigation to determine the phenotype of the cell population used in these studies. Non-stimulated DCs were harvested after 7 days of culture, resuspended in DC media and treated for 24h with fNE, E-fNE, LPS, E-fNE plus simultaneous addition of LPS, or LPS for 24h then fNE or E-fNE for an additional 24h. Fluorescent-labeled antibody staining was performed and analysis was carried out on the FACS Caliber. The results were confirmed in duplicate or triplicate runs.

3.4.3.1. Fermented Noni exudate (fNE)

Around 85% of the cells from all treatments were $CD11c^+$. DCs treated with fNE minimally increased $CD11c^+Mac\text{-}3^+$ cells by 6%, and a small increase was seen in the $CD11c^+CD62L^+$ population, compared to both DC and LPS controls (Figure 3.7). However, CD83 expression doubled, similar to the LPS control, and CD40 expression

quadrupled, although remaining 20% lower than LPS stimulated DCs. The $CD11c^{+}Gr1^{+}B220^{+}$ population nearly doubled, but was similar to the LPS control.

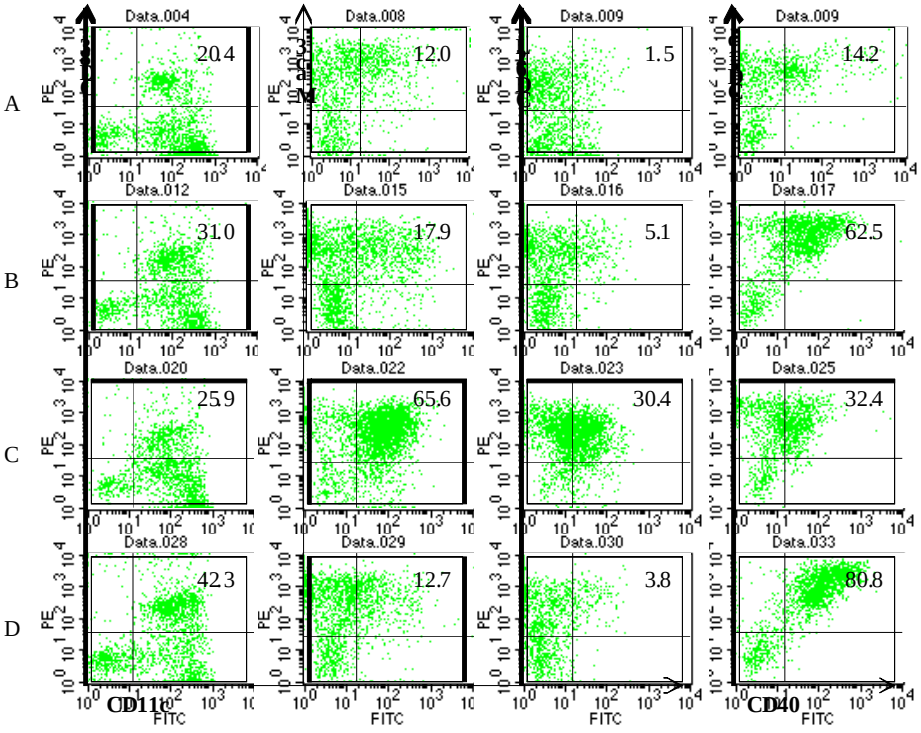

Figure 3.7: **Surface marker expression of dendritic cells treated with Noni or Entotoxin-depleted Noni compared to LPS.** BMDCs were treated with 5% Noni, 5% endotoxin-depleted Noni or 100ng/ml LPS for 24h, then stained with corresponding FITC- or PE-conjugated antibodies. (A) Non-stimulated CD11c+ BMDCs expressed low levels of CD83, Mac-3, CD62L and CD40. (B) Noni treated cells expressed slightly elevated levels of CD83, Mac-3, and CD62L, but a large increase in CD40. (C) Endotoxin-depleted Noni stimulated a large increase in Mac-3 and CD62L with only a moderate increase in CD40, and no change in CD83. (D) LPS stimulated the largest increase in CD83 and CD40, however there was no change in Mac-3, and a minimal increase in CD62L. All experiments were perfomed in duplicate or triplicate to confirm the resulting patterns.

3.4.3.2. Entotoxin-depleted fermented Noni exudate (E-fNE)

This visual phenomenon was observed for both fNE and endotoxin-removed fNE, so we examined the surface phenotype of E-fNE-treated DCs as well. An even larger proportion became CD11c+Mac-3+, a striking 66% of the total cell population. And the CD62L+ cells increased from 1.5% in the DC control (4% in the LPS control) to 30% (Figure 3.7). CD11c+CD83+ cells remained the same after treatment with E-fNE; however, CD40 expression doubled. The $CD11c^{+}Gr1^{+}B220^{+}$ population nearly doubled compared to the LPS control. Interestingly, E-fNE-treated cells slightly increased expression of MHC class I molecule.

3.4.3.3. E-fNE with LPS (E-fNE + LPS)

To determine if endotoxin removal was responsible for the large increase in $Mac3^{+}$ or $CD62L^{+}$ cells, and to assess its requirement for CD40 stimulation, we incubated DCs simultaneously with E-fNE and LPS for 24h. This appeared to have no effect on $Mac\text{-}3^{+}$ cells, and a very mild effect on $CD62L^{+}$ cells; however, CD40 expression was increased to nearly that of LPS treated cells (Figure 3.8). Hence, LPS is necessary for induction of CD40, but it does not significantly hinder the E-fNE induced differentiation to $Mac\text{-}3^{+}$ and $CD62L^{+}$ cells.

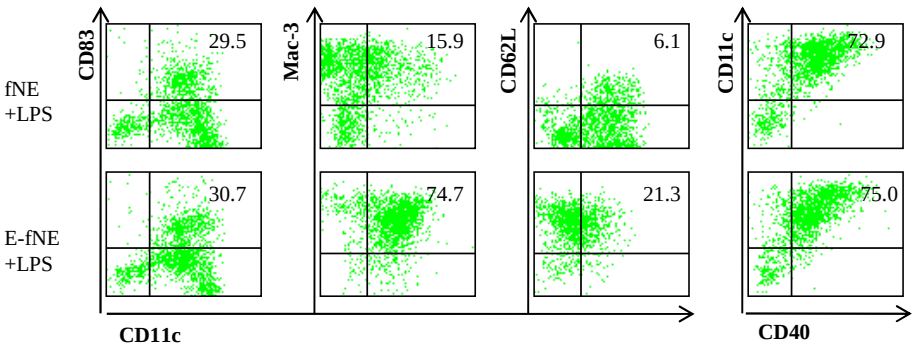

Figure 3.8: **Surface marker expression of dendritic cells treated with Noni or Endotoxin-depleted Noni and simultaneous addition of LPS.** BMDCs were treated with 5% Noni or 5% endotoxin-depleted Noni along with 100ng/ml LPS for 24h, then stained with corresponding FITC- or PE-conjugated antibodies. The addition of LPS was not sufficient to block the transformation to Mac-3+ cells in endotoxin-depleded Noni stimulated DCs. Although, it significantly increased expression of CD40 for both Noni and endotoxin-depleted Noni samples.

3.4.3.4. fNE post-LPS

Since immature DCs are known to have a high degree of plasticity, we decided to mature DCs with LPS for 24h, followed by a 24h fNE treatment to determine the strength of Noni's effect on matured DCs. Indeed, maturation of DCs with LPS was not sufficient in blocking the differentiation of DCs to Mac-3+ or CD62L+ cells by fNE, nor did it alter the expression of CD83+ cells (Figure 3.9). The only marker affected by LPS treatment prior to fNE treatment was CD40. Its expression increased to levels similar to the LPS control.

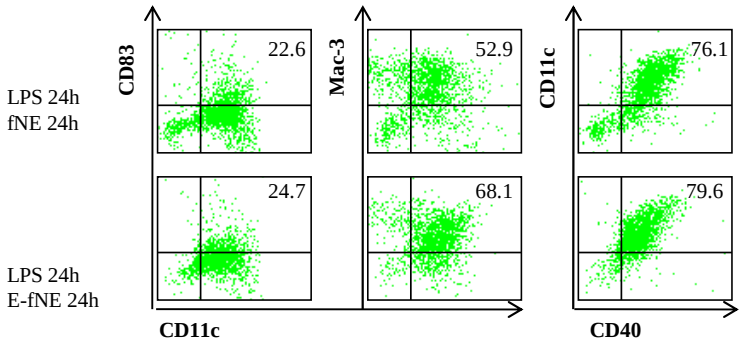

Figure 3.9: **Surface marker expression of dendritic cells treated with Noni or Entotoxin-depleted Noni, pre-treated with LPS.** BMDCs were treated with 100ng/ml LPS for 24h, then subsequently with 5% Noni or 5% endotoxin-depleted Noni for an additional 24h, and stained with corresponding FITC- or PE-conjugated antibodies. Pre-treatment of DCs with LPS was not sufficient to block the development of Mac-3+ cells for endotoxin-depleted Noni-treated DCs, and in fact, appeared to augment the expression of Mac-3 for Noni-treated DCs. LPS was responsible for the large increase in CD40.

3.4.3.5. E-fNE post-LPS

LPS pre-treatment did not hamper the ability of E-fNE to increase Mac-3+ or CD62L+ cells either. In fact, Mac-3+ cells actually increased compared to E-fNE treated DCs alone. CD83 and MHC-I expression remained unchanged compared to fNE treatment; however, the $CD11c^{+}Gr1^{+}B220^{+}$ population minimally increased to its highest, 35% of the total cell population. Again, CD40 expression was increased to levels near that of the LPS control, which were much higher than E-fNE-treated cells.

Here we show that LPS-matured DCs are not terminally differentiated, as they are affected by fNE or E-fNE treatment to trans-differentiate from CD11c+CD83hiMac-3lo population to CD11c+CD83loMac-3hi population with an increasing population of $CD11c^{+}Gr1^{+}B220^{+}$ cells.

3.5. Discussion

Overall BMDC treatment with Noni muted the level of inflammatory signaling compared to proportionate levels of LPS. By removing endotoxin from Noni (E-fNE), there was a shift from an early mild inflammatory response in BMDCs followed by a time-dependent increase in anti-inflammatory mediators. Endotoxin removal of Noni not only encumbered expression of *tnf*, *il-6*, *ptgs2*, *ccl3*, and *ccl5* initiated by fNE, but actually caused a decrease in their expression by at least 72h. This arrest of inflammatory factors was confirmed with real-time PCR arrays and revealed the eventual switch to anti-inflammatory mediators with time.

3.5.1. fNE and E-fNE decrease inflammatory mediators

In our experiments, fNE caused a decrease in *tnf*, *il-6*, *ptgs2*, *ccl3* and *ccl5* compared to LPS controls, and upon removal of endotoxin, this decrease was even more evident. It is possible this is achieved simply through a unique combination of pattern recognition receptor (PRR) signaling brought about by the plethora of microbial degradation compounds within fNE. Many microorganisms have been cultured from non-pasteurized Noni juice, including *E. coli*; however, upon fermentation there remains only one surviving microbe (personal communication). This plethora of microbial by-products may induce a combination of receptors to limit production of genes involved with inflammation.

Some of the most popular traditional uses of Noni targeted inflammation, where it was often applied topically to treat arthritis, inflammation due to sprains or broken bones, general pains, and even taken internally to treat asthma (Wang *et al*., 2002) (Pawlus *et al*., 2007). With its resurgence in popularity as an herbal tonic, more research has been conducted to identify components of Noni responsible for these effects. Constituents of Noni fruit have previously been implicated in reducing inflammatory cytokines and inhibiting 5-lipoxygenase activity (Wang *et al*., 2002) (Deng *et al*., 2007). Furthermore, Hiruzimi and Furusawa demonstrated that fermented Noni-ppt induced macrophage cells to produce lower levels of TNF-α and IL-1b than LPS-induced cells; and IL-10 production was consistent between the two groups. These Noni-induced macrophage cells were also responsible for the enhanced survival of mice with LLC tumors (Hirazumi *et al*., 1999). Our results similarly show that *tnf* is produced at much lower levels than

LPS stimulated BMDCs (Figure 3.1), and by 24h it is actually downregulated (*tnf* levels are below control levels); and *il-1a* and *il-1b* were stimulated early (Table 3.1) but by 48h were also below control levels (Table 3.2). More recently, Palu *et al* reported Tahitian Noni Juice precipitate was shown to activate cannabinoid receptors, specifically CB_2 (Palu *et al*., 2008). This receptor is found mainly in T cells, macrophages and B cells, as well as other hematopoietic cells and is responsible for mediating protective signals against inflammatory responses (Massa etl al., 2004). It has also been found that Echinacea and several other botanicals activate CB_2 receptors (Raduner *et al*., 2006; Woelkart *et al*., 2008). These data support our findings that Noni-stimulated BMDCs decrease inflammatory mediators. One mechanism of action may be their ability to activate CB_2 receptors.

3.5.2. Endotoxin-depleted Noni up-regulates anti-inflammatory mediators

Noni was observed not only to down-regulate inflammatory cytokines, but also to up-regulate anti-inflammatory mediators. With time, endotoxin-removed Noni increased levels of anti-inflammatory mediators in BMDCs such as *ifnβ1* and *hspa1a* (Table 3.1), while concomitantly decreasing inflammatory mediators (Table 3.2). Both IFN-β and HSP70 are implicated in treating chronic inflammatory maladies (Musch *et al*., 2007; Olofsson *et al*., 2007; Corr *et al*., 2009; Tao *et al*., 2009; Tanaka *et al*., 2007) (Pockley *et al*., 2008). Type I IFNs are capable of anti-proliferative effects and often mediate hematopoietic suppressive signals (Platanias *et al*., 2005). It has been shown that Type I IFNs increase NK cell activity, enhance phagocytic abilities of APCs, and promote cytotoxic properties of stimulated lymphocytes (Borden *et al*., 1998) (Sato *et al*., 2001).

HSP70 was also demonstrated to activate NK cells (Multhoff *et al*., 1997; Gross *et al*., 2003) via TLR2 and TLR4 (Asea *et al*., 2002). Recently, Li and colleagues reported the necessity of NK cells and T cells for the tumor killing and preventative properties of Noni (Li *et al*., 2008). Similarly, Furusawa *et al* attributed the anticancer properties of Noni-ppt to macrophages, NK cells and T cells (Furusawa *et al*., 2003). Our data, revealing Noni-induced BMDCs increase production of *ifnb1* and *hspa1a*, may shed light on the anti-cancer mechanisms of Noni observed by these studies.

Besides its NK-activating effects, HSP70 has been implicated as an anti-apoptotic and anti-inflammatory mediator (Hall *et al*., 1994) (Yenari *et al*., 2005) and there are several studies involving its use as an adjuvant for cancer therapies (Zhang *et al*., 2007i; Kumar *et al*., 2009; Wu *et al*., 2005). Recently it has been implicated in the protection from inflammatory-drug induced colon cancer in a mouse model for inflammatory bowel disease cancer (Tao *et al*., 2009). On a mechanistic level, HSP induction inhibits pro-inflammatory cytokines by inhibiting NFκB. This inhibition is through the stabilization of IkB; however the exact mechanism is still not clear (Yoo *et al*., 2000; Schell *et al*., 2005). *Hspa1a* levels increase drastically with time in endotoxin-removed Noni-treated DCs (Table 3.1), which may be partially responsible for the latent commitment to anti-inflammation. Since IkBa (*nfkbia*) levels are increased at 2 and 24h (Table 3.1), HSP70 may be essential for their stability and suppression of NFκB inflammatory responses. It was recently reported that the anti-inflammatory effects of HSP70 were critically dependent on IL-10 (Wieten *et al*., 2009). And our data revealed *il-10* gene expression was also elevated in BMDCs treated with endotoxin-removed Noni. Noni induction of

*hspa1*as a role in the inhibition of NFκB-mediated inflammation should be further investigated. The involvement of HSP70 in the stabilization of IkB and subsequent inhibition of NFκB should be examined.

3.5.3. E-fNE inhibition of NFκB

There has been long standing evidence for the association of inflammation and cancer, and NFκB transcription factors are responsible for the production of many inflammatory genes. TNF-α, IL-1 and IL-6 are programmed by the classical NFκB pathway and are associated with cancer progression (Karin *et al*., 2005). IL-6 in particular is associated with unfavorable prognosis in breast cancer recurrence (Zhang *et al*., 1999). These inflammatory mediators are hijacked by the tumor microenvironment to their own advantage, recruiting innate immune cells such as TAMs (tumor associated macrophages), tolerogenic DCs and regulatory T-cells to perpetuate this inflammatory environment, allowing for angiogenesis and metastasis. In congruence with the negative association of inflammation and cancer progression, long-term use of NSAIDs (non-steroidal anti-inflammatories), both manufactured drugs and natural remedies, have been linked to reduced incidence of certain inflammation-associated cancers (Jacobs *et al*., 2005; Fairfield *et al*., 2002; Corley *et al*., 2003). NSAIDs and botanicals such as green tea, resveratrol and curcumin all have been shown to inhibit NFκB (Siddiqui *et al*., 2008; Majumdar *et al*., 2009; Aggarwal *et al*., 2004; Aggarwal *et al*., 2003). Our results show that although fNE induces cytokine gene expression, its levels of *tnf, il-1* and *il-6* are much lower than LPS. Noni's immunomodulatory properties may regulate the immune system in a way that favors anti-tumor activity via inhibition of NFκB.

Our data show a clear trend in NFκB inhibition by E-fNE. Not only are NFκB inhibitors (*nfkbia, nfkbib, nfkbil1, tollip, tnfaip3*) increased, but *ikbkb* (IKK-β) is significantly downregulated. Tollip is an inhibitor of IRAK-1 activation, however, once TLR ligands are recognized and TLRs dimerize, Tollip is released and IRAK-4 is capable of activating IRAK-1 (Figure 3.10). Pellino is recruited with TRAF-6 to form a membrane complex (II) with TAB/TAK, then it causes poly-ubiquitination with subsequent degradation of IRAK-1, releasing Complex III into the cytosol. TAK1 then activates IKKs and MKKs. IKK-β is responsible for phosphorylating IκB and thus releasing NFκB dimers to enter the nucleus. As seen in Tables 1 and 2, many of these NFκB cascade genes are downregulated. This NFκB inhibition could be one explanation for anti-tumor and antiangiogenic properties of Noni, by limiting inflammatory immune cell proliferation and lifting the suppression of apoptosis. This may also explain why Noni enhances the effectiveness of certain chemotherapies.

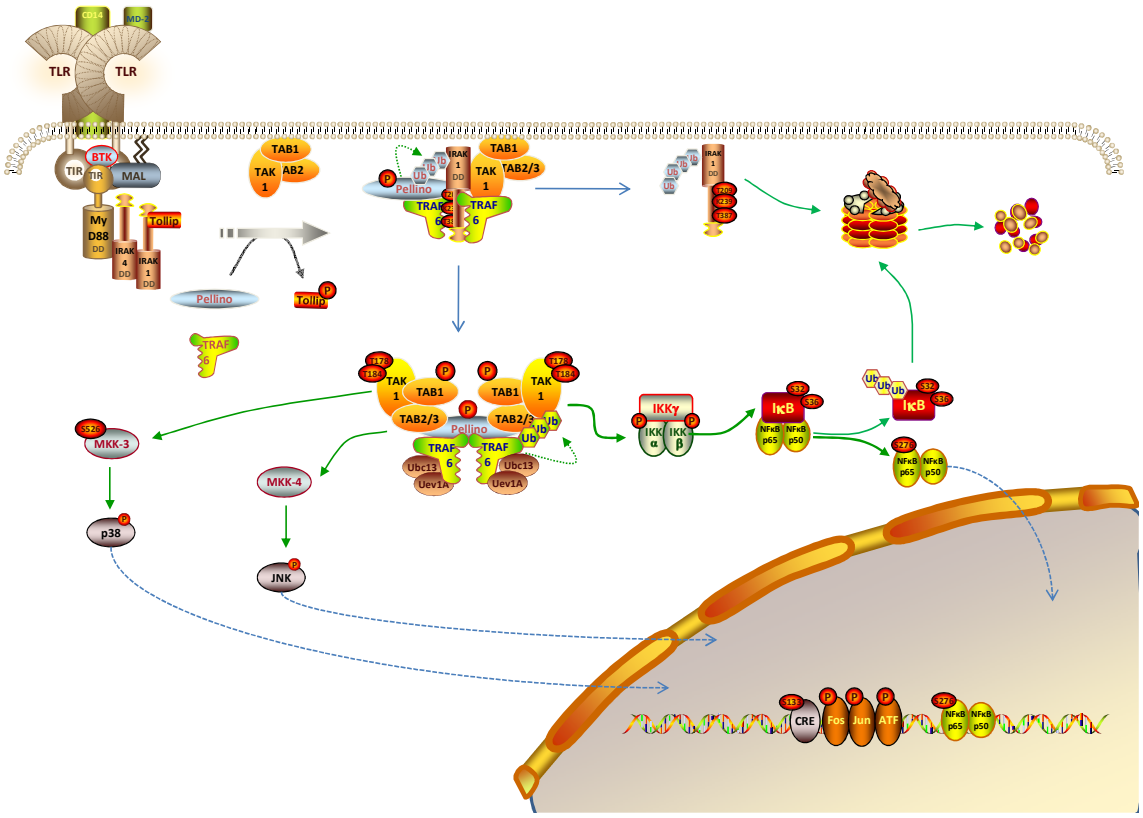

Figure 3.10: **Toll-like receptor signaling cascade.** Upon engagement of TLR ligand, receptors dimerize. This dimerization brings their cytoplasmic tails together where TIR domains can interact. IRAK4 and IRAK1 are brought together and IRAK4 phosphorylates IRAK1, allowing for the release of Tollip and its activation. Once IRAK1 is activated, it is released from the receptor tail and binds to the TAK/TAB membrane-bound complex. Pellino1 is a scaffold protein responsible for bringing TRAF6 to the IRAK1/TAK/TAB complex. Pellino ubiquitinates IRAK1, allowing for its proteasomal degradation and subsequent release of the TAK/TAB/TRAF6 complex. Upon complex formation, TAK1 is activated and phosphorylates IKKs, which in turn phosphorylate IκBs. Phosphorylated IκBs are targeted for proteasomal degradation, which releases the NFκB subunits and allows for their nuclear transmigration. Activated TAK can also phosphorylate MKKs, which can then activate the p38 and JNK pathways.

Experiments performed by Kawamur and colleagues presented antithetical properties of *Agaricus blazei*-treated BMDCs similar to the results we observed with Noni-treated BMDCs. *A. blazei*, an edible mushroom often consumed for purported health benefits, activated DC maturation with respect to co-stimulatory and MHC molecules; however, inflammatory cytokine production was reduced when DCs were treated with *A. blazei* prior to LPS. This phenomenon was related to its capacity to inhibit NFκB. Interestingly, *A. blazei*-treated DCs, although suppressing T_H1 cytokines (IL-12p40), increased T_H1 cell development and subsequent IFN-γ production in an allogenic mixed lymphocyte reaction assay (Kawamura *et al*., 2005). These results are similar to what we have observed with Noni-treated BMDCs and again point to the inhibition of NFκB as a major mechanism of immunomodulation.

3.5.4. Cell surface expression of Noni-treated BMDCs.

Interestingly, we observed a strange phenomenon with the morphology and cell adhesiveness of Noni-treated BMDCs. The underlying supportive monolayer in the cultured bone marrow cells became elongated and much denser than in LPS or untreated bone marrow cell cultures. When trying to collect these cells, they were much more difficult to harvest than typical non-stimulated BMDCs or even LPS-stimulated BMDCs. After staining for several bone marrow-derived cell markers, we learned that our Noni-treated cell population had an increasing amount of Mac-3^+ cells. This effect was most prominent in endotoxin-removed Noni-treated BMDCs. Simultaneous stimulation of BMDCs with endotoxin-removed Noni and LPS did not abrogate this differentiation to Mac-3^+ cells, but in fact slightly enhanced it. Since non-stimulated BMDCs are known to

be very plastic, we then incubated the cells with LPS for 24h prior to endotoxin-removed Noni treatment, but observed a nearly identical level of Mac-3^+ cells as the original endotoxin-removed Noni-treated BMDCs. The treatment groups highly positive for Mac-3 also had a large increase in $CD62L^+$ cells. Is it possible that endotoxin-removed Noni is capable of transforming DCs into $CD62L^+$ monocytes and allowing for their subsequent differentiation into Mac-3^+ cells? This marvel encourages more research to determine a more precise phenotype of these purported macrophage cells and to elicit the pathway by which Noni influences matured DCs to become macrophage-like cells.

3.6. Conclusion

With this study we hope to shed light on some of the anti-inflammatory and anticancer mechanisms of Noni, in order to better understand this product that has held long-standing native use for the treatment of many ailments and is receiving a resurgence in popularity with the advent of Tahitian Noni ® and its approval by the European Food and Safety Authority (European Food Safety Authority, 2009). Noni elicits an early mild inflammatory response in BMDCs, followed by an anti-inflammatory response triggered by high levels of *ifnβ1* and *hspa1a* production and NFκB inhibitors. This divergent occurrence of pro- and anti-inflammatory mediators may give a unique rise to T_H1 immunity while quenching the undesirable effects of prolonged inflammation. It also causes trans-differentiation of BMDCs into macrophage-like cells expressing Mac-3. Further research is needed to determine whether these anti-inflammatory mediators are indeed players in the anticancer effects of Noni. And further investigation into the known and unknown NFκB inhibitory compounds in Noni would be of great value.

SUMMARY

Since their discovery as mediators of innate and adaptive immunity, dendritic cells have been fervently researched. Their ability to enhance innate mediators such as NK cells, as well as process and present antigen to lymphocytes has led to their use in many immunotherapies. Scientists have put great effort into understanding their complex nature in order to harness their power for enhancing or even distinguishing immune responses. In this study, we have examined DCs in two different settings. First, we observed the influence of fusion with tumor cells; and second, we tested the effects of *Morinda citrifolia* on DCs.

In the first experiment, either immature or mature DCs were chemically fused with tumor cells and their expression of inflammatory chemokine (CCL), chemokine receptors (CCRs), and several other DC markers was evaluated. In immature DCs, fusion induced maturation with respect to CCLs, CCRs, and typical DC maturation markers; however, mature DCs used for fusion, which already expressed the aforementioned markers prior to fusion, proceeded to down-regulate CCLs, CCRs, and other markers of maturation. This peculiar phenomenon of 'de-maturation' of DCs clarifies the need for immature DCs utilized in tumor fusion procedures. This is essential in order to have the most effective DC-tumor hybridoma vaccine capable of migration to secondary lymph tissues where they will be most effective at eliciting cell-mediated responses against DC presented tumor antigens.

The second experiment was aimed at expanding the knowledge of Noni as an immunomodulator. Noni's popularity as a dietary supplement and immune enhancer is

growing, and it is important to understand the mechanisms behind its purported actions. It is a known activator of DCs, and consists of a wide variety of PAMPs from killed microflora after fermentation. So, we tested the ability of Noni to activate DCs via TLRs. By analyzing genes in the TLR signaling pathway via real-time RT-PCR, we observed that Noni stimulated production of anti-inflammatory mediators as well as inhibiting pathways downstream of TLRs, including NFκB. And an interesting find was that Noni-treated DCs may actually have trans-differentiated into macrophage cells.

Overall, DCs were shown to be activated by fusion to tumor cells and treatment with Noni. Further experiments to confirm these findings at the protein level would be advantageous. Chemotactic assays would benefit the first experiment. And further investigation of the Noni-induced trans-differentiation of DCs into macrophages would be of great interest. Lastly, since NFκB inhibitors are generating much attention with respect to anti-inflammation and anticancer therapies, the role of Noni and NFκB inhibition may lead to novel drug discoveries.

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