

**DIRECT EFFECTS OF MILK OLIGOSACCHARIDES ON
THE INFLAMMATORY RESPONSE IN RELATION TO
ALLERGY**

DIRECT EFFECTS OF MILK OLIGOSACCHARIDES ON THE INFLAMMATORY
RESPONSE IN RELATION TO ALLERGY

By

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ABSTRACT

Introduction: The incidence of food allergy has increased substantially in developed countries, with limited treatment and/or prevention options. Milk oligosaccharides have shown to modulate immune responses by serving as prebiotic substrates for the intestinal microbiota. However, some studies suggest that oligosaccharides may exert direct immunomodulatory effects, suggesting their therapeutic potential in preventing allergic diseases. We hypothesized that specific milk oligosaccharides including 6'sialyllactose, 2'fucosylactose, 3'sialyllactose and lacto-N-neotetraose may directly exert immunomodulatory effects on dendritic cells (DCs) and epithelial cells (ECs) by altering their phenotype and/or function *in vitro*.

Methods: The effects of milk oligosaccharides (MOs) on bone-marrow derived DCs and the T84 and MODE-K epithelial cell lines were studied via direct treatment, *in vitro*. The expression of immunomodulatory cytokines and maturation markers were assessed to measure the effect of MOs on DC phenotype. Pro- and anti-inflammatory cytokines as well as NFκB p65 activity were measured to assess the effect of MOs on DC and EC function. In addition, *in vitro* stimulation of CD23 with IgE-Antigen complexes were used to study the effects of MOs on ECs in relation to allergy. Lastly, inhibitory antibodies for Siglec-F and PPARγ were used to elucidate the mechanism used by specific MOs to exert their effects.

Results: Of the oligosaccharides studied, 6'sialyllactose has direct immunomodulatory effects on DC phenotype and on DC and EC function at high concentrations. 6'sialyllactose increased DC expression of IL-10 and HO-1; it also increased CpG- and LPS- induced IL-10 release and decreased IL-12p70 release. Blocking the PPARγ receptor with GW9662 resulted in attenuation of this latter effect on IL-12p70 release. 6'sialyllactose reduced TNF-α induced IL-

8 to a small but statistically significant extent and mKC to a great extent in T84 and MODE-K cells, respectively. In addition, 6'sialyllactose reduced IgE-Antigen stimulated release of IL-8 and CCL20, as well as NFκB p65 activity. Pre-treatment of cells with GW9662 resulted in attenuation of the effect of 6'SL on IL-8 release and p65 activation. In addition, 2'fucosylactose reduced CCL20 release and NFκB activity substantially, but these effects were not exerted via PPARγ.

Conclusion: Some oligosaccharides are able to directly modulate the inflammatory response in DCs and ECs, via pathways involving PPARγ activation and/or NFκB inhibition.

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ABBREVIATIONS

2'FL – 2'Fucosyllactose

3'SL – 2'Sialyllactose

6'SL – 6'Sialyllactose

Ag – Antigen

APCs – Antigen presenting cells

BMDC – Bone-marrow derived dendritic cells

BSA – Bovine serum albumin

CCL (17, 20 & 22) - Chemokine (C-C Motif) ligand

CCR (7 & 9) – Chemokine (C-C Motif) receptor

CD (11b, 11c, 23, 40, 80, 86, 103) – Cluster of differentiation

CMA – Cow's milk allergy

CpG – C—phosphate—G sites in DNA

CX₃CR^{high} – CX₃C chemokine receptor

DC – Dendritic cell

EC – Epithelial cell

FBS – Fetal bovine serum

FCeRI – High-affinity receptor for the Fc region of immunoglobulin E

FCeRII – Low-affinity receptor for the Fc region of immunoglobulin E

Foxp3 – Forkhead box P3

GI – Gastrointestinal

GALT – Gut-associated lymphoid tissue

GM-CSF – Granulocyte-macrophage colony-stimulating factor

HIEC – Human intestinal epithelial cells

IEC – Intestinal epithelial cells

IFN-gamma – interferon gamma

IgE – Immunoglobulin E

IL – Interleukin

iTregs – Induced T-regulatory cells

LNnT – Lacto-N-neotetraose

LP – Lamina propria

LPS - Lipopolysaccharide

MOs – Milk oligosaccharides

NFκB – Nuclear factor kappa beta

NP - Nitrophenol

P/S – Penicillin/Streptomycin

PBS – Phosphate-buffered saline

PMBC – Peripheral blood mononuclear cells

PolyI:C - Polyinosinic:polycytidylic acid

PPAR γ – Peroxisome proliferator-activated receptor gamma

PRR – Pathogen recognition receptor

RA – Retinoic acid

T₀ – Naïve T lymphocytes

TGF β – Tumour growth factor-beta

TH (1 & 2) – T helper lymphocytes

TLR – Toll-like receptor

TLRs – Toll-like receptors

TNF- α – Tumour-necrosis factor alpha

Tregs – T regulatory lymphocytes

TSLP – Thymic stromal lymphopoietin

BACKGROUND

The prevalence of food allergy

Food allergy is considered a “second wave” of allergic epidemic, with the global burden increasing dramatically in recent years, especially in infants and young children (Prescott & Allen, 2011). Food allergy affects between 4% to 8% of children and 1% to 2% of adults and its prevalence varies depending on geographical location (Bischoff, 2007). For example, while national peanut consumption in China and the United States is virtually identical, allergy to peanuts is relatively absent in China. Differences in dietary habits and food preparation account for the observed disparity. The practice of dry-roasting peanuts in the United States results in an increased allergenicity of peanut proteins through the Maillard reaction that forms advanced glycation end-products in roasted peanuts (Sampson, 2004), while the Chinese population consumes peanuts as boiled or fried.

In some populations, challenge-proven IgE-mediated food allergy has been reported in 1 in 10 children, with substantive increases in prevalence over the last 10 years (Poulos et al., 2007; Osbourne et al., 2011). Despite geographical differences, a meta-analysis conducted by the National Institute of Allergy and Infectious Disease estimates a global prevalence of 1% - 10% between all age groups (Prescott & Allen, 2011; Boyce et al., 2010).

Definition of food allergy

Food allergy is an abnormal and adverse reaction of the mucosal immune system to food particles. In certain cases, food allergy is acquired via non-oral routes such as pollen-food-related allergy, in which sensitization to pollen antigen occurs via the respiratory tract but culminates in a food allergy (Sampson, 2004). Normally, a vast array of foreign particles make their way to the gastrointestinal (GI) tract and although the task of the gut is to break down foreign particles, 2% substances routinely cross the gut barrier in an immunologically intact form (Sampson, 2004; Yu et al., 2011) but do not cause clinical symptoms. In such cases, the gut-associated lymphoid tissues (GALT) establish tolerance to harmless antigen, resulting in an immunosuppressive mucosal environment. However, in certain individuals, specific antigens that pass the epithelial barrier are falsely recognized as harmful foreign substances, and an immunologic memory is established to elicit an allergic response upon re-exposure to that antigen. Thus, food allergy is the result of a failure to establish oral tolerance to harmless particles, a concept that will be discussed in further detail below.

Food allergens

Food allergens that cause sensitization are generally water-soluble glycoproteins of 10 – 70 kDa in size, and are able to withstand heat, acidity and proteases commonly found in the gut lumen (Sampson, 2004). Hence, food allergens are proteins found in the normal diet of an individual, but avoid mechanisms of degradation and digestion, thus remaining intact upon reaching the GI tract (Bischoff, 2006). A few food products are responsible for

approximately 90% of all food-specific allergic responses (Bischoff, 2006) and according to the Canadian Food Inspection Agency the most common food allergens include cow's milk, eggs, peanuts, seafood, sesame, soy, and wheat (Canadian Food Inspection Agency, 2012). The prominence of specific allergens is dependent on various factors including age and geographic location. In children less than 5 years of age, the most common food allergens are cow's milk, eggs and peanuts, while in older children and adolescents tree nuts and seafood are additional to the list (Sampson, 2004). In westernized populations, cow's milk is the most predominant offender of food allergy among infants and its prevalence is shown to decrease over the first decade of life (Sampson et al., 2001). In fact, tolerance to cow's milk in allergic infants and young children is established by 5 years of age, with only 15% carrying it onto the second decade of life (Sampson, 2004). Alternatively, peanut and seafood allergy are more often seen to persist into adulthood (Fleischer et al., 2003).

Effects of food allergens in the body

The clinical manifestation of an allergic reaction can be categorized based on two factors: location and onset. Food hypersensitivity reactions can range from gastrointestinal symptoms of nausea, vomiting, cramps, colic, flatulence and diarrhoea, and although the site of sensitization is often localized to the intestine, the response can also be manifested on other mucosal tissues and organs such as the skin and respiratory tract. Skin manifestations include urticaria, angioedema (Sampson, 2001) and most commonly atopic dermatitis (Brant et al., 2003). Respiratory symptoms include rhinitis, wheezing and

asthma (Sampson et al., 2001; Yamaki & Yoshino, 2012). In severe cases of food hypersensitivity, such as peanut allergy, the clinical manifestations can be cardiovascular in nature, potentially resulting in anaphylactic shock (Sampson, 2004; Salazar & Ghaemmaghami, 2013). The onset of hypersensitivity reactions can be acute/sub-acute, or chronic/persistent. Location and onset of the response varies between individuals, food allergens, and the immunological response elicited by an allergen (IgE-mediated vs. cell-mediated).

IgE-mediated food allergy

The most clearly defined food allergy reactions are those that are mediated by immunoglobulin of the class E (IgE). In such reactions, immediate GI hypersensitivity occurs, as antigen-specific IgE binds the high-affinity receptor for the Fc region of immunoglobulin E (FcεRI) present on mast cells and basophils, which then release mediators (histamine, prostaglandins, leukotrienes), resulting in vasodilation, mucous secretion, smooth muscle contraction and an influx of inflammatory cells to the site of infection (Robbie-Ryan & Brown, 2002). IgE-mediated allergic reactions are generally rapid and acute, lasting from minutes to a few hours, and are rarely isolated to the GI tract (Sampson, 2001; Sampson 2004). In fact, less than 50% of IgE-mediated food allergic responses are manifested within the GI tract (Ortolani & Pastorello, 2006), with alternative locations of manifestations being the skin and respiratory tract (Sampson, 2001).

Cell-mediated food allergy

Cell-mediated food allergy, or non-IgE-mediated food allergy is a consequence of the profile of cytokines secreted by antigen presenting cells (APCs) or T cells upon recognition of particular food protein. The *in vitro* stimulation of T cells harvested from children with cow's milk allergy (CMA) with cow's milk protein resulted in a higher concentration of tumor necrosis factor alpha (TNF- α), an inflammatory cytokine that alters epithelial barrier function (Heyman et al., 1994). Chung et al. (2002) found that in duodenal biopsies of children suffering from food protein-induced enterocolitis, peripheral blood mononuclear cells (PBMCs) express increased amounts of TNF- α and decreased staining for the receptor of transforming growth factor beta 1 (TGF- β 1), which is a regulatory cytokine. A deficiency in TGF- β 1 responses and increase in TNF- α may account for the immunopathophysiology present in cell-mediated food allergic diseases (Sicherer & Sampson, 2010). Symptoms of cell-mediated food allergy are chronic, becoming apparent hours to days post allergen ingestion (Sampson, 2004).

The mucosal immune system

The gastrointestinal tract is the largest reservoir of immune cells in the mammalian body and within hours of birth, it is confronted by a large influx of foreign particles ((Berin & Sampson, 2013; Sampson, 1999). The mucosal immune system within the GI is separated from the external environment via a single layer of columnar epithelial cells and consists of physiological and immunological barriers that confer protection to the host (Artis et al., 2008). The intestinal epithelial cells (IECs) are joined by tight junction proteins that restrict paracellular entry of particles into mucosal sites (Sampson, 1999). In addition, the IEC

layer is coated with glycocalyx, which are glycoprotein and mucins that trap particles. The microvillus organization of the IEC lining serves to prevent penetration by foreign substances, and peristalsis in the gut helps flush out trapped particles. In addition to these functional barriers, the GI tract, including the stomach and upper small intestine, produces a vast array of enzymes and molecular mediators that serve to break down ingested antigens (Sampson, 1999). These include but are not limited to: extreme pHs, salivary amylases, trefoil factors that contribute to barrier restoration, gastric acid, pepsins, pancreatic and luminal brush border intestinal enzymes, bile salts, and lysozymal activity of IECs, all of which serve to destroy pathogens, break down protein and render antigens nonimmunogenic (Sampson, 1999; Sampson, 2004).

In addition to innate physiological barriers, the mucosal immune system is equipped with an advanced immunological barrier. Just beneath the epithelium in the lamina propria is a vast amount and variation of immune cells including resident and migratory mononuclear phagocytes such as dendritic cells (DCs) and macrophages, intraepithelial and lamina propria lymphocytes, natural killer T lymphocytes, basophils, eosinophils and mast cells (Sampson 2004, Sampson 1999). These immune cells are also found in organized lymphoid organs called Peyer's patches and the mesenteric lymph nodes, which play specific and vital roles in antigen sampling and the establishment of oral tolerance (Varol et al., 2009), as discussed below.

Oral tolerance

The average human diet consists of more than 100 grams of foreign protein per day, and the IEC lining is densely populated with commensal microbes collectively referred to as the gut microbiota, with more than 10^{12} microbes per gram of gut content in the colon (Pabst & Mowat, 2012). The mucosal immune system thus has the challenging task of discriminating between harmful antigens to induce protection and immunity or harmless antigens to induce what is known as oral tolerance.

Oral tolerance is described as the body's systemic non-responsiveness to harmless antigens that it encounters via oral administration (Sicherer & Sampson, 2010). Oral tolerance plays a central role in immune homeostasis and is responsible for the body's lack of immune responses against self-antigens under normal conditions. In addition, a state of oral tolerance attenuates a broad range of immune responses such as systemic delayed-type hypersensitivity to antigen, T cell proliferation and cytokine production, antigen-specific IgE production, T_H1 -dependent IgG2a production and IgA responses (Pabst & Mowat, 2012). This lack of clinical reactivity to foreign antigens and better yet to trillions of commensal microbes is achieved by a specialized milieu of synergistic immunoregulatory components.

Antigen uptake occurs at organized lymphoid organs such as Peyer's patches, mesenteric lymph nodes, or the lamina propria; the nature of the antigen determines its route of uptake

(Varol et al., 2009; Pabst & Mowat, 2012). While particulate antigens such as bacteria and viruses are taken up by specialized Microfold (M) cells lining gut-associated lymphoid tissues including Peyer's patches and mLNs, soluble antigens are primarily taken up in the lamina propria (Sampson, 1999; Pabst & Mowat, 2012). The lamina propria is abundantly populated with DCs that bear the integrin chain α_E , more commonly known as migratory CD103+ DCs (Pabst & Mowat, 2012; Coombes & Powrie, 2008). The phenotype and functionality of such DCs is heavily impacted by overlying epithelial cells, which produce cytokines such as TGF- β and thymic stromal lymphoprotein (TSLP), as well as retinoic acid (RA), all of which imprint a tolerogenic phenotype in CD103+ DCs (Pabst & Mowat, 2012; Ruitter & Shreffler, 2012). Upon antigen encounter, CD103+ DCs uptake antigen, process and display antigenic epitopes on major histocompatibility complex class II (MHC II) molecules, and are chemotactically transported via the afferent lymph to the mesenteric lymph nodes in a CCR7-dependent manner for T cell activation (Wijk & Knippels, 2007). CD103+ DCs express high levels of retinal dehydrogenase 2 (RALDH2), which converts retinoids (such as Vitamin A) to retinoic acid (RA). The production of RA and immunosuppressive cytokines such as TGF- β induces naïve T cell differentiation into Foxp3+ T regulatory cells (Tregs) (Pabst & Mowat, 2012). The presence of RA also includes gut-homing molecules CCR9 and $\alpha_4\beta_7$ integrin on T cells, and drives the TGF- β mediated conversion of CD4+ T cells into Foxp3+ cells (Pabst & Mowat, 2012). Tregs are chemotactically driven to the lamina propria via the efferent lymph, where they undergo secondary expansion. CD4+Foxp3+ Tregs create a homeostatic environment within the lamina propria and are maintained in their regulatory state by CX₃CR^{high} and CD11b+ DCs

that produce an abundance of IL-10 (Wijk & Knippels, 2007; Pabst & Mowat, 2012). In this way, tolerance to harmless substances is established and maintained within the gut.

Although immunosuppression is the dominant response of the GALT in response to foreign antigen, in specific individuals certain antigens bypass this suppressive mechanism and instead cause sensitization within the host, as discussed below.

Immunologic response to an allergen

1) Sensitization

Of the 2% of immunogenic particles that cross the mucosal epithelium in an intact form, a few food proteins lead to the development of food allergy in some individuals (Sampson, 1999; Sampson, 2004). Upon crossing the gut barrier, immunogenic protein is taken up by professional APCs such as DCs and small peptide fragments called epitopes are presented on MHC II molecules. These DCs then travel via the afferent lymph to lymphatic tissues such as PPs and mLNs to interact with naïve T cells (T_0 cells). T_0 cells bearing the appropriate complementary receptor bind to the peptide-MHC II complexes on DCs, serving as the first signal for T cell proliferation. Optimal T cell activation requires a second costimulatory signal, which is achieved by cell surface molecules on DCs and T

cells. The costimulatory pathways known to predominate T₀ activation are the CD40-CD154 and CD28/CTLA-4-/CD86 interactions (Wijk & Knippels, 2007).

Costimulatory pathway activation as well as the secretion of cytokines by the DCs promotes differentiation of T₀ cells into Th2 cells. Th2 cells secrete IL-4, IL-5 and IL-13 while interacting with B cells causing immunoglobulin isotype switching (Wijk & Knippels, 2007; Ruiter & Shreffler, 2012). Hence B cells begin producing and secreting IgE antibodies specific for the antigen in question. These allergen-specific IgE molecules travel throughout the body and are loaded onto the naturally occurring high affinity Fc epsilon receptors (FcεRI) on resident mast cells and basophils (Salazar & Ghaemmaghami, 2013; Berger, 2000) triggering Ca²⁺ uptake and priming the immune system to have an allergic response at the next encounter with the same antigen (Robbie-Ryan & Brown 2002; Bischoff, 2009). At this point, the individual or host is considered “sensitized” to the antigen.

Although oral tolerance is established via the oral route in the gut, sensitization to antigens can take place at any mucosal site within the body. For example, it is often the case that children experience their first allergic response to peanut without ever having been orally exposed to peanut protein. Since oral tolerance is achieved by the oral administration of food protein, extensive delay of oral exposure delays the development of tolerance, creating opportunity for cutaneous/topical sensitization, especially in individuals experiencing atopic dermatitis, in which the skin epithelium is dysfunctional (Berin & Sampson, 2013). Delayed allergen avoidance in infants and children is therefore not

recommended as a food allergy prevention strategy (Berin & Sampson, 2013; Sicherer & Sampson, 2010).

2) Re-exposure

As explained previously, the IgE-mediated type I hypersensitivity reaction is characterized by the synthesis of allergen-specific IgE in mucosal tissues (Bischoff, 2007; Berger, 2000). An allergic reaction manifests when allergens cross the intestinal epithelial barrier that is maintained through epithelial cell tight-junctions (Salazar & Ghaemmaghami, 2013; Li et al., 2007) via either transepithelial transport through endosomes, or paracellularly (Sampson, 1999).

Antigens to which sensitization has taken place can attach to FcεRI-bound antigen-specific IgE antibodies, causing IgE cross-linking. This process triggers Ca^{2+} intake and initiates activation of mast cells and basophils bearing the IgE-Ag complexes (Robbie-Ryan & Brown 2002; Bischoff, 2009). A cascade of signal transduction results in cellular degranulation and release of many molecular mediators that contribute to inflammation and allergic reactions in the mucosa, classified as the “early reaction” (Bischoff, 2009). The immediate release of soluble molecular mediators includes heparin, histamine, proteases and prostaglandins, among others (Robbie-Ryan & Brown 2002; Bischoff, 2009). These mediators produce local responses characteristic of an allergic reaction, such as increased permeability of blood vessels, inflammation and increased mucus production. Mast cells produce $TNF-\alpha$, IL-5, IL-3 and IL-4, which attract and activate pro-inflammatory cells

such as neutrophils, T_H2 cells and eosinophils, culminating in a “late-phase” response (Robbie-Ryan & Brown 2002; Bischoff, 2009).

Furthermore, antigen recognition by other immune cells such as dendritic cells (DCs), remains ongoing, resulting in the influx of other pro-inflammatory cells such as macrophages, neutrophils, and T cells to the site of injury (Li et al., 2007; Akbari & Umetsu 2005). Upon recruitment of these cells, epithelial barrier function is lost and inflammation and bacterial infiltration into the mucosa ensues (Bischoff, 2009). Increased intestinal permeability leads to increased exposure to intact protein, promoting possible sensitization to other harmful antigens, and also enhances the severity of the allergic response by promoting further infiltration of the offending allergen (Bischoff, 2009).

The detection of food allergens by the antigen-specific FcεRI-bound IgE antibodies is translated as a threat, leading to a secretory and propulsive response in the intestine. This is expressed in the GI as common symptoms of food allergy such as abdominal pain, cramping, diarrhea and fecal urgency (Demaude et al., 2007).

Role of dendritic cells in allergy

Dendritic cells are mononuclear phagocytes found in organized lymphoid organs such as Peyer’s patches, MLNs and the lamina propria, among other distant sites within the body (Varol et al., 2009). Dendritic cells (DCs) are sentinels of the immune system, acting as professional APCs and play an imminent role in the body’s decision to establish tolerance

or sensitization, by controlling naïve T cell differentiation (Wijk & Knippels, 2007). As APCs, DCs carrying out the very important function of antigen presentation to T lymphocytes (Li et al., 2007; Akbari & Umetsu 2005), which generates specific T cell subsets, and ultimately a specific physiological response ensues. DC-induced T cell differentiation however is an outcome of DC subset and environmental stimulation – more specifically, the local cytokine milieu and immune stimuli. Innate immune stimuli such as pro-inflammatory cytokines and TLR-signalling are able to induce mature and activated DCs that are capable of T cell priming. For example, the production of TSLP by IECs activates neighbouring DCs and leads to subsequent DC-induced T cell production of pro-allergic cytokines such as IL-4, IL-5 and IL-13 (Ruiter & Shreffler, 2012; Watanabe et al., 2015).

DC subsets

The lamina propria is most commonly abundant in non-follicular bone-marrow derived DCs under the influence of Flt-3 ligand, GM-CSF and other cytokines (Varol et al., 2009). In addition to the lamina propria, such DCs can be found in the lymphatic tissues such as T cell regions of draining lymph nodes, non-lymphoid tissues (such as the interstitium of internal organs) or at the epidermis and dermis of the skin (Langerhans cells and dermal DCs, respectively) (Kelsall et al., 1996).

Two main lamina propria dendritic cells exist: CD103⁺ CX₃CR1⁻ DCs arise via a GM-CSF receptor-dependent manner (Berin & Sampson, 2013; Bogunovic et al., 2009; Schulz

et al., 2009) and CD11b⁺ CD14⁺ CX3CR1⁺ DCs, which are exclusively derived from Ly6C^{hi} but not Ly6C^{lo} monocytes in a GM-CSF-controlled manner (Varol et al., 2009)

In addition, a specific subset of DCs can extend dendrites between IECs and capture bacteria or sample antigen directly. These are CD11c⁺CX₃CR1⁺ cells, in which CX₃CR1 is a chemokine receptor required for the transepithelial extension of dendrites into the lumen (Berin & Sampson, 2013; Pabst & Mowat, 2012). These DCs are non-migratory as they lack CCR7 expression, and are more closely related in function to tissue macrophages than DCs.

In the context of antigen presentation, the DC subset predominantly involved is CD103⁺ CXCR1⁻ DCs. In addition to antigen presentation, these cells bear the ability to migrate to MLNs in a CCR7-dependent manner to drive T₀ differentiation and subsequent T cell activation (Schulz et al., 2009). These cells are therefore major determinants of oral tolerance and immunity to specific antigens.

DC functions and T-cell interactions

Innate immune stimuli such as TLR-signalling and pro-inflammatory cytokines are able to induce mature and activated DCs that are capable of T cell priming. DCs exhibit a diverse set of surface receptors such as pathogen recognition receptors (PRRs), toll-like receptors (TLRs) and C-type lectin receptors that are able to recognize conserved motifs on antigens (Robinson & Moehle, 2014). For example, TLR4 found on DCs is able to recognize lipopolysaccharide (LPS) on bacterial cell wall, while TLR9 is able to recognize unmethylated bacterial DNA sequences including CpG (Akira et al., 2001; Campeau et al.,

2012). In addition, DCs exhibit various types of sialic acid-binding immunoglobulin-type lectins (Siglec) receptors, which are capable of recognizing sialic acid containing glycans and glycoproteins (Macauley et al., 2014).

In response to direct external stimuli or indirect sensing of inflammation or infection via cytokine secretion (such as TNF- α), DCs are prompted for activation and maturation into immunostimulatory DCs. DC maturation involves the loss of endocytic and phagocytic receptors, upregulation of co-stimulatory receptors such as CD40, CD80 and CD86, increased expression of MHC II molecules for antigen displaying and CCR7 for migration to MLNs as well as extension of dendrites to increase surface area for T-cell interaction (Banchereau et al., 2000; Steinman et al., 2003).

The large contact surface of DCs in comparison to cell volume facilitates their function as the most important APCs for B and T lymphocytes (Akbari & Umetsu, 2005; Steinman et al., 2003). In an adaptive immune response, DCs process antigens and present them to T lymphocytes found in either the lamina propria or MLNs, leading to T cell differentiation into T cell subsets, namely T_H1, T_H2 and T regulatory cells (Tregs) (Steinman et al., 2003). CD4⁺ T cells activated by DCs release cytokines such as IL-4, IL-5 and IL-13 which stimulate B cells to form and release specific antibodies including IgE (Madigan et al., 2009; Eiwegger et al., 2004) and increased IgE levels are closely related to levels of IL-13 (Bischoff et al., 2009). This process is subtype-specific as CD4⁺ T_H2 cells promote IgE and eosinophilic responses while T_H1 cells reduce atopy (Berger et al., 2000; Romagnani, 1995). DCs are strong determinants of subsequent T and B cell responses; in fact, DC from spleen and Peyer's patches of food allergic mice are able to induce antigen-specific IgE

responses in naïve mice in the absence of immunization or allergen challenge (Chambers et al., 2004). Allergy is therefore regarded as a T_H2 -weighted imbalance and redirection in favour of the T_H1 response is thought to be therapeutic (Akbari & Umetsu, 2005).

On the other hand, DC production of cytokines such as IL-12 promote T_H1 differentiation and those mice with deficient production of IL-12 produce higher specific IgE responses and more severe allergic symptoms to allergens; blocking of IL-12 increases susceptibility to food allergy (Ruiter & Shreffler, 2012). In addition, IL-10 release by DCs indicates a regulatory and/or tolerogenic DC function, and promotes Treg differentiation. Thus, the cytokines and molecular mediators released by DCs determine T-cell skewing, which dictates whether a response will be regulatory (Tregs), or inflammatory (T_H1/T_H2 imbalance) (Steinman et al., 2003).

Mucosal DCs also contribute to the establishment and maintenance of a tolerogenic environment. Tolerogenic DCs release immunosuppressive cytokines such as IL-10, which causes naïve T cells to differentiate into Foxp3⁺Tregs (Ruiter & Shreffler, 2012). During an allergic type T_H2 response, DCs also produce IL-10, which functions as an anti-inflammatory cytokine that induces Tregs that ultimately reduce the inflammatory effects of Th2 cells (Akbari & Umetsu, 2005). Additionally, heme oxygenase-1 is now believed to be an important enzyme that drives immune suppression by DCs, and DCs expressing HO-1 promote Foxp3⁺ Treg function (Karimi et al., 2012; George et al., 2008).

Role of epithelial cells in allergy

At the interface of the external-internal environment, IECs serve as the first line of defense against antigens in the gut, including food allergens. A balance between crypt stem cell proliferation and intestinal surface cell shedding maintains the epithelium; new cells quickly differentiate into absorptive or secretive types of ECs (Yu et al., 2011). As discussed previously, the IEC lining is equipped with a plethora of defense mechanisms that are normally sufficient at preventing entry of immunogenic material into the body. However, properties that contribute to the penetration of a small subset of allergens include a) stability to heat and digestion, b) numerous IgE binding sites on the allergen, c) internalized IgE binding sites that are unreachable by digestible enzymes, and in some cases, d) the sheer abundance of allergenic protein in a food substance, such as peanut (11 peanut allergens known to date) (Price et al., 2013). Although some antigens are able to penetrate the IEC lining directly, such as milk allergens (Roth-Walter et al., 2008), and egg allergens (Mine et al., 2003), others are insoluble protein often aggregated, which can only permeate the epithelium via specialized IECs called M cells found in the follicle-associated epithelium of Peyer's patches (Price et al., 2013).

In the gut, resident DCs are neighbours to intestinal ECs, the first cells to encounter allergen upon enteral entry into the body. Through production of various cytokines, epithelial cells possess the capacity to educate and prime mucosal DCs. For example, IECs of various types have shown to produce TSLP, which stimulates DCs to produce chemokines CCL17 and CCL22 (Ruiter & Shreffler, 2012). TSLP-activated DCs prime

naïve T cells to produce IL-4, IL-5 and IL-13, which are major cytokines in food allergic responses. Lung epithelial cells are promoted to produce other allergic cytokines such as IL-25 and IL-33, as well as GM-CSF and TSLP (Bartemes et al., 2012) in certain airway allergic diseases. These molecules all serve to recruit and activate DCs at the site of allergen entry and through these immunological messengers IECs prime DCs to produce a specific immunological response. In allergic responses, DCs cause T cell differentiation to T_H2 ultimately initiating the type I hypersensitivity reaction, culminating in the symptoms characteristic of food allergy (Ruiter & Shreffler, 2012).

Gut permeability and CD23

In addition to their ability to relay responses to DCs, IECs play a significant and rate-limiting factor in determining gut permeability. Two modes of antigen transport are possible via the gut barrier: transcellular transport (apical-to-basolateral movement of material through epithelial cells), and paracellular transport (transfer between adjacent epithelial cells) (Price et al., 2013; Yu et al., 2001). Normally, transcellular transport occurs through endocytosis of luminal contents, but such content is sorted into lysosomal compartments for degradation. Tight junctional proteins on apico-lateral membranes of IECs maintain cell-to-cell contact, preventing paracellular transport (Yu et al., 2011). Despite such mechanisms, 2% of intact antigenic material makes way into the lamina propria (Sampson, 1999; Sampson, 2004).

Various studies on human and mice demonstrate that intestinal permeability is increased upon antigen-challenge in food allergic subjects (Dupont et al., 1989; Troncone et al., 1994; Perrier & Corthesy, 2011). Pituzzi et al., (2011) show that *in vitro* exposure of small intestinal biopsy specimens to food allergens decreases expression of tight junction proteins occludin, claudin-1 and ZO-1 in patients with food allergy. In addition, in the presence of T_H2 cytokines such as IL-4 and IL-13, *in vitro* studies demonstrate an increase in intestinal permeability, represented by a) a decrease in transepithelial resistance (TEER), b) increased luminal-to-basal movement of horse-radish peroxidase (HRP) through trans- and paracellular pathways (Berin et al., 1999; Ceponis et al., 2000; Leo et al., 2002), and c) increased expression of a pore-forming tight junction protein called claudin-2 (Wisner et al., 2008; Heller et al., 2008).

Other *in vitro* studies of transepithelial antigen transport demonstrate an increase in antigen transport even prior to mast cell activation, and the onset of a response (Berin et al., 1997). This suggests that an alternative antigen recognition mechanism exists at the epithelial cell level.

More recently, a low affinity IgE receptor has been found on epithelial cell membranes, which contributes to antigen recognition and uptake in allergic animals. This receptor is the low-affinity FcεRII or CD23 (Li et al., 2007). Research demonstrates the role of CD23 in gut barrier maintenance, as neutralizing antibodies to CD23 prevent increased transepithelial uptake in allergic mice, and sensitized CD23^{-/-} mice do not experience enhanced antigen uptake by enterocytes (Linda et al., 2003). In addition, CD23 translocates from the cell surface to allergen containing endosomes during the process of endocytosis,

bringing persevered immunogenic forms of protein into the lamina propria (Bevilacqua et al., 2004). Hence in sensitized individuals, food allergens bind antigen-specific IgE, which binds to CD23; this interaction causes enhanced epithelial antigen transport, which later contributes to mast cell activation and the subsequent allergic response explained previously (Yu et al., 2011).

In addition, CD23 activation leads to secretion of pro-inflammatory chemokines such as CCL20 and IL-8, which are capable of recruiting cells of the innate and adaptive immune system to the site of entry (Li et al., 2007). While IL-8 serves as a chemoattractant for neutrophils and eosinophils, increased secretion of CCL20 causes the recruitment of DCs, T cells and B cells to sub-epithelial regions (Li et al., 2007). CD23 therefore serves as a critical receptor in the initiation of an allergic response in the gut.

Therapy

Currently, the only proven method of therapy for food allergic individuals is strict allergen avoidance. This includes the implementation of safe practices such as education on food allergen avoidance, availability and education on self-injectable epinephrine (Epi-pens), label-reading and avoidance of cross-contact (Prescott, 2013; Sampson, 2001; Sampson, 2004). Antihistamines are used to relieve the symptoms associated with oral allergy syndrome as well as IgE-mediated skin symptoms (Bindslev-Jensen et al., 1991).

More recently, novel forms of immunotherapy are being explored. Leung et al. (2003) tested the effect of injected anti-IgE antibodies in patients with peanut allergy, and found that treated individuals required a significantly greater amount of peanut allergens to induce an allergic response. Another therapeutic intervention currently under study is oral immunotherapy (OIT), which involves the administration of small amounts of allergen in a highly controlled clinical setting. Although shown to be effective in certain cases, OIT has limitations in its usefulness as in some types of food allergy even trace amounts of the allergen can result in fatal physiological responses such as anaphylactic shock (Price et al., 2013). Other therapeutic approaches involve the modulation of immune cell functions to disrupt T_H2-skewing ability (Mosmann & Coffman, 1989).

As diet is a major risk factor for development of food allergy, dietary intervention is perhaps a therapeutic avenue. A growing body of evidence suggests a protective role of breastfeeding on the development of allergic diseases. In a meta-analysis of 132 studies on infant breastfeeding in relation to food allergy, the authors concluded that breastfeeding has protective effects in atopic disease development (Odiijk et al., 2003). It is, however, unclear whether this protective effect is the result of exposure to antigen during pregnancy and/or breastfeeding, or an effect induced by breast milk constituents upon feeding. More recently, the immunomodulatory effects of dietary constituents found in human and animal milk is an interesting and emerging field of research as these prebiotic substances play different roles in anti-allergic responses.

Milk Oligosaccharides

Early in research of prebiotic oligosaccharides, Schonfeld (1926) found that a growth promoting “bifidus factor” for bifidobacterium exists in the human gut, which was later confirmed to be milk oligosaccharides (MOs) (Gauhe et al., 1954; György et al., 1954).

Structure and abundance

Milk oligosaccharides (MOs) are unconjugated glycans, which constitute the third largest proportion of the nutritious ingredients present in human milk, after lactose and lipids (Chichlowski et al., 2011; Kuntz et al., 2008; Caicedo et al., 2005). In fact, one litre of human colostrum contains between 20-23 g of oligosaccharides, while mature milk contains between 12-14 g/L (Arslanoglu et al., 2008). Although found in the milk of several mammals, the sheer abundance and structural and functional diversity is known to be the greatest in human milk (Bode, 2012; Kunz et al., 2000).

The structure of milk oligosaccharides consists of a combination of three to five of the following monosaccharides: glucose, L-fucose, galactose, *N*-acetylglucosamine and sialic acid. All MOs have lactose (glucose + galactose) at their reducing end and are elongated via β 1-3- or β 1-6- linkages (Bode, 2012). To date, more than one hundred oligosaccharides have been isolated and classified (Kobata, 2010; Bode, 2012). Interestingly, the amount and subset of oligosaccharides produced differs between women and even changes upon lactational periods (Chaturvedi et al., 2001). One major determinant of MO composition is blood group characteristics of the individual woman (Stahl et al., 2001; Thurl et al., 2010).

The structural diversity of MOs translates into structure-specific biological effects (van Hoffen et al., 2009).

2' fucosyllactose (2'FL) is a neutral oligosaccharide and is the most abundant oligosaccharide in human milk with the exception of lactose (Bode, 2012). The production of 2'FL is controlled by the enzyme FUT, which is responsible for fucosylation. Gene expression of FUT, and other enzymes responsible for the biosynthesis of these glycans varies between women, explaining the difference in MO quantities and profiles in different women (Kumazaki & Yoshida, 1984; Thurl et al., 1997). 6' sialyllactose (6'SL) is an acidic oligosaccharide, abundant in both human and mouse milk and is considered a good bifidofactor (Bode, 2012). Interestingly, mouse milk only contains 6'SL and 3'sialyllactose (3'SL) but not the elongated, branched or fucosylated oligosaccharides found in human milk (Fuhrer et al., 2010). 3'SL is also an acidic oligosaccharide, shown to modulate epithelial cell responses by altering cell surface expression of specific glycans, which prevents pathogen entry (Angeloni et al., 2005).

Prebiotic effects of MOs

More recently, MOs are classified as prebiotics, which are defined as “selectively fermented ingredients that allow specific changes, both in the composition and/or activity in the gastrointestinal microflora, that confer benefits upon host well-being and health” (Gibson et al., 2004). Upon ingestion, MOs are found intact and at high concentrations in

the small intestine and colon, thus meeting the criteria that constitute a prebiotic: resistance to gastric acidity, hydrolysis by host enzymes and gastrointestinal absorption (Bode, 2012).

The prebiotic effects of milk oligosaccharides have been shown in many *in vivo* and *in vitro* models. Via the oral route, milk oligosaccharides make way to the gut microflora, for which they act as beneficial substrates that enhance the growth of commensal bacteria such as *bifidobacterium infantis* (*B. infantis*) (Gibson & Wang, 1994). This dominance of commensals indirectly prevents harmful growth of pathogenic bacteria by outcompeting for limited nutrient supplies (Gibson & Wang, 1994).

More recently, Marcobal et al. (2011) showed that Lacto-*N*-neotetraose (LNnT) provides a significant growth advantage for *B. infantis* over *Bacteroides thetaiotaomicron* in germ-free mice. Introduction of LNnT in drinking water increased the relative abundance of *B. infantis* from 2% to 40%. With respect to their prebiotic effects in food allergy, Castillo et al. (2015) recently showed that the oral administration of 2'FL and 6'SL attenuates food allergic symptoms in a mouse model of ovalbumin-induced food allergy. These MOs were found to induce IL-10 expression in Tregs and cause an indirect stabilization of mast cells, culminating in the reduced symptoms of food allergy including reduced diarrhea, rectal temperature and serum mast cell protease 1 (mMCP-1) (Castillo et al., 2015). In addition, 6'SL directly inhibited IgE-mediated mast cell activation *in-vitro* at relatively high concentrations (1 mg/ml) but this effect was not present for 2' FL, suggesting that 6'SL is capable of attenuating the allergic response (Castillo et al., 2015).

The prebiotic role of MOs is also shown by their ability to act as anti-adhesive anti-microbials. Pathogenic bacteria must first bind to mucosal surfaces to gain entry; MOs have shown to prevent microbial infections by interrupting this process (Kunz et al., 2006; Newburg et al., 2005). Lectin-glycan interactions are a predominant form of pathogen adhesion; *Escherichia coli* (*E. coli*) with type 1 fimbriae must bind to mannose-containing glycans to gain entry into the host (Firon et al., 1983). As unconjugated glycans, MOs mimic cell surface glycans, serving as soluble decoys that interfere with pathogenic lectin-glycan interactions, therefore reducing infections (Bode, 2012).

Various studies report the anti-adhesive anti-microbial effect of MOs *in vitro*. Ruiz-Palacios et al. (2003) found that the addition of α 1-2-fucosylated MOs blocks binding of *Campylobacter jejuni* (*C. jejuni*) to cultured epithelial cells and reduces *C. jejuni* colonization in mice. In fact, infants of mothers whose milk contained high concentrations of 2'FL had significantly less occurrence of *C. jejuni* induced diarrhea (Morrow et al., 2004).

Additionally, Lin et al. (2013) found that MOs antagonize specific uropathogenic *E. coli* (UPEC)-induced host intracellular signalling to protect human bladder epithelial cells from UPEC invasion and cytotoxicity. MOs prevented exfoliation of epithelial cells by protecting several focal adhesion molecules such as paxillin, B-integrin 1 and Dsc2/3 from UPEC-mediated degradation. This protection against exfoliation and subsequent cell detachment and death correlates with augmentation of UPEC-suppressed NF- κ B activity. This research demonstrates that MOs are able to impact the expression of proteins by modulating common signaling pathways such as the NF κ B pathway.

Direct effects of MOs

In addition to their role as prebiotics, there is evidence that MOs can directly interact with immune cells to have cell cycle and immunomodulatory effects. Kuntz et al. (2008) have shown that both neutral and acidic oligosaccharides reduce proliferation in the HIEC, Caco-2 and HT-29 cell lines. Furthermore, using alkaline phosphatase activity as a marker for differentiation, they found that at high concentrations (15 mg/mL) both neutral and acidic MOs are able to stimulate differentiation in HIEC and HT-29 cells. Illustrated by marked increases in caspase-3 activity, Kuntz et al. (2008) also showed that at such concentrations of neutral sugars, HIEC, Caco-2 and HT-29 cells start to undergo apoptosis. These findings illustrate the direct effects of MOs on the maturational and cell cycle changes that result in the renewal of intestinal epithelial cells.

A growing body of evidence demonstrates the role of MOs as direct immune modulators. Eiwegger et al. (2004) showed that upon treatment of cord blood T cells with acidic MOs, there is a marked increase in interferon-gamma (IFN- γ) producing CD3+CD4+ and CD3+CD8+ T cells, with an increase in IL-13-producing CD3+CD8+ cells (Eiwegger et al., 2004). These findings support the notion that MOs are able to modulate immune responses by promoting Th1/Th2 cell balance. Furthermore, a reduction of IL-4 in lymphocytes of patients with peanut allergy (Eiwegger et al., 2010) as well as the virtual absence of IgE in B cells stimulated with neutral and acidic MOs (Eiwegger et al., 2004) suggests that MOs may play a preventative role in allergy. In addition, Atochina & Harn

(2005) showed that fucosylated MOs are able to stimulate macrophages and to increase levels of prostaglandin E₂, IL-10 and TNF- α *in-vitro*, further exemplifying their role as direct immune modulators. Kurakevich et al. (2013) demonstrated that 3'SL plays a role in colitis onset and development as IL-10 deficient mice that are also deficient for the gene responsible for 3'SL biosynthesis (St3gal4) have delayed onset and progression. In fact, oral administration of the sugar led to increased colitis severity. Furthermore, it was demonstrated that 3'SL activates intestinal CD11c⁺ cells through TLR4 signalling (Kurakevich et al., 2013). Interestingly, a separate study showed that fucosylated oligosaccharides such as 2'FL and 3'FL are able to reduce colon motor contractions *ex vivo*, and thus this MO may exert its effects onto the enteric nervous system (ENS), which controls gut motility (Bienenstock et al., 2013). Together, these results suggest that while mechanisms used by sialylated MOs in alleviating allergic symptoms remain to be elucidated, the effects of dietary 2'FL may be mediated via the ENS.

MOs stabilize the Th1/Th2 balance *in vivo* by producing Th1 proinflammatory cytokines such as IL-1, IFN- γ , and IL-12, as well as anti-inflammatory cytokines such as IL-4 and IL-10. Although largely unclear, a few studies aim to elucidate the mechanism of action used by MOs at the molecular level. Zenhom et al. (2011) found that 3'SL reduces proinflammatory cytokines IL-8 and IL-12 in Caco-2 cells via activation of the peroxisome proliferator-activated receptor gamma (PPAR γ) and peptidoglycan recognition protein 3 (PGly3). The involvement of transcription factors in this regulation of proinflammatory cytokines remains to be elucidated.

The above findings shed light onto some mechanisms MOs use to directly modulate immune responses such as allergic inflammation. The following research serves as preliminary work to further determine the direct effects of MOs on immunological pathways in both dendritic cells and epithelial cells. This research will help us further understand whether MOs can directly influence regulation of the immune system, and if so, what mechanisms are used by specific MOs to exert such effects.

HYPOTHESIS

Certain milk oligosaccharides are able to directly exert immunomodulatory effects on epithelial cells and dendritic cells to attenuate the inflammatory response in relation to allergy.

OBJECTIVES OF THIS STUDY

1. To investigate the direct effects of prebiotic milk oligosaccharides, 2'FL, 6'SL, 3'SL and LNnT on epithelial cell and dendritic cell phenotype and function related to allergy.

2. To elucidate the possible mechanism(s) by which prebiotic milk oligosaccharides directly exert immunomodulatory effects on immune cells.

METHODS

Milk oligosaccharides

Milk oligosaccharides were obtained as a gift from Abbott Nutrition (Columbus, OH, USA). MO "purity" was established by high performance ion chromatography with pulsed amperometric detection (IC-PAD) using relative peak area comparisons. Moisture content was determined separately using the Karl Fischer method for moisture determination. 6'Sialyllactose (6'SL) and 2'Fucosylactose (2'FL) 3'Sialyllactose (3'SL) and lacto-N-neotetraose (LNnT) were derived from bacterial synthesis. Endotoxin levels were estimated by limulus assay (Limulus Amebocyte Lysate (LAL) QCL-1000, Lonza catalogue number 50-647U, Wilmington, MA, USA) and lipopolysaccharide (LPS) 500,000 EU/mg from Sigma (catalogue number L2637, St. Louis, MO, USA) (Table S1).

Preparation of chimaeric human anti-NP-IgE-Antigen complexes

Chimaeric human IgE anti-NP monoclonal antibody was obtained from AbD Serotec (MCA333S). To remove sodium azide, dialysis of the antibody was performed as suggested by AbD Serotec using a Slide-A-Lyzer® dialysis cassette (AbD Serotec EQU003). Dialysis was performed overnight at 4°C in PBS. Dialyzed antibody was aliquoted and stored at -20°C for later use.

NP-BSA was obtained from Biosearch Technologies (N-5050L-10) and prepared at working concentrations according to the manufacturer's instructions and stored at -20°C for later use.

Cell Cultures

Bone-Marrow Derived Dendritic Cells (BMDCs)

Monocytes were extracted from bone marrow of male balb/c mice, washed with PBS and grown in cell culture dishes at a concentration of 1×10^6 cells/mL, supplemented with 10 ng/mL murine recombinant granulocyte macrophage colony stimulating factor (GM-CSF) (Peprotech, 315-03) and cultured for a period of 7 days. 5 mL of BMDC media containing RPMI 1640 media, 10% heat inactivated fetal bovine serum (FBS) (Gibco, Grand Island, NY), 1% penicillin (100 units/mL)-streptomycin (100 ug/mL) (P/S), 1% L-Glutamine and 0.05% β -mercaptoethanol (Gibco, Grand Island, NY) was added on day 2 of culture. On day 6, half of the volume of cells was collected and centrifuged at 1500 rpm, 4°C for 10 minutes. Supernatant was decanted and fresh BMDC media was added. Cells were

reintroduced to the original culture dishes, along with further addition of 10 ng/mL GM-CSF. On day 7, cells were harvested and split at the desired concentration and ready to be used in experiments.

T84

The T84 colonic epithelial adenocarcinoma cell line was provided by the Ashkar Lab at McMaster University. T84 cells were grown in RPMI-1640 with 10% FBS, 1% P/S, 1% L-Glutamine and 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Cells were grown at a concentration of 5×10^5 cells/mL in a T-75 tissue culture flask and grown for 7-10 days. Media was changed every fourth day. Upon confluence, cells were washed with PBS to remove excess media, and were harvested by incubating with 3 mL of 0.25% trypsin for 2 minutes at 37°C, 95% O₂. Cells were centrifuged at 1300 RPM, 20°C and subsequently cultured in 24-well cell culture plates at a concentration of 1×10^5 for another 3 days (until confluence was reached). Upon confluence, media was changed and the cells were ready for experimentation.

MODE-K

MODE-K is an intestinal epithelial cell line derived from C3H/HeJ mice that was kindly provided by Dr. Firoz Mian (McMaster University, Hamilton, ON). MODE-K cells were grown in media consisting of 1:1 DMEM low glucose:F-12 supplemented with 10% heat-inactivated FBS, 1% P/S, 1% L-Glu and 1% HEPES. Cells were grown in a T-75 tissue culture flask at a concentration of 1×10^5 cells/mL for 1-3 days, until confluence was reached. Upon confluence, cells were harvested by incubating with 3 mL of 0.25% Trypsin

(company) for 2 minutes at 37°C, 95% O₂. Cells were collected, centrifuged and split at 1X10⁵ cells/mL in a 24-well cell culture plate. Cells were confluent within 12-24 hours, at which point they were ready for experimentation.

Stimulation Experiments

Testing the Direct Immunomodulatory Effects of MOs on Dendritic Cells *in vitro*

On day 7, cells were given sugars 6'SL, 2'FL, 3'SL and LNnT individually at concentrations of 1.0 mg/mL, 0.1 mg/mL and 0.01 mg/mL. Cells were stained for extracellular BMDC surface markers with CD11c-PerCp-Cy5.5 and MHCII-PE-Cy7 (BD Pharmingen, San Diego, CA, USA). Cells were then fixed and permeabilized by BD Cytotfix/Cytoperm reagent (BD Bioscience, Mississauga, ON, Canada) and stained for intracellular expression markers, IL-10-PE and HO-1-FITC (eBioscience, San Diego, CA, USA). Data were acquired with FACSCanto (Becton Dickinson, Oakville, ON, Canada) and analyzed by FlowJo software (TreeStar, Ashland, OR, USA).

Assessing the Direct Effect of MOs on Dendritic Cell Function *in vitro*

BMDCs were extracted and cultured as explained above. On day 7, cells were treated with sugars 6'SL and LnNT at concentrations of 1.0 mg/mL, 0.1 mg/mL, 0.01 mg/mL and 0.001 mg/mL (in some cases). One hour after treatment, cells were stimulated with 100 ng/mL LPS, 10 µg/mL CpG and 10 µL/mL PolyI:C, individually. Supernatants were collected after 24 hours and kept at -20°C. ELISAs were performed for various cytokines, including IL-10, IL-12p70 and TNF- α according to the manufacturer's guidelines.

Assessing the Direct Effect of MOs on Epithelial Cell Function *in vitro*

T84 cells were cultured onto 24-well plates as explained above at a concentration of 1×10^5 cells/mL. Upon confluence, cells were treated with 6'SL, 2'FL and Lactose at concentrations of 0.01 mg/mL, 0.1 mg/mL and 1.0 mg/mL. 12 hours post-treatment, cells were stimulated with 10 ng/mL of recombinant human (rh) TNF- α and incubated for an additional 12 hours. Supernatants were collected at t=0, t=12 and t=24 after TNF- α stimulation, and stored at -20°C. IL-8 from supernatants of T84 cells was measured by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Assessing the effects of MOs in a Co-culture system

MODE-K cells were grown as explained above. Once confluent, cells were split into cell culture inserts with a 0.4 µm pore size and polyethylene terephthalate membrane. (EMD Millipore, PIHT12R48), following the guidelines provided by Millipore in "Millicell

Hanging Cell Culture Inserts: Single and Preloaded Inserts”. Cells were plated on the apical sides at a concentration of 1×10^5 cells/cm³ and the suggested volume of MODE-K media was added to the apical and basolateral compartments, and allowed to grow until confluent. Upon confluence, cells were treated with 2’FL, 6’SL, 3’SL, LNnT and Lactose (separately) at concentrations of 1.0 mg/mL, 0.1 mg/mL and 0.01 mg/mL, for 24 hrs.

BMDCs were extracted and cultured as explained above, such that day 6 of BMDC culture coincided with sugar treatment of MODE-K cells (above). On day 7 of BMDC culture, cells were split into 24-well plates at a concentration of 1×10^5 cells/mL. Immediately, basolateral supernatants from sugar-treated MODE-K cells (above) were treated to BMDCs at a 1:1 BMDC-MODE-K media ratio. BMDCs were then stimulated with a high-dose of LPS (1 ug/mL), and incubated at 37°C, 95% O₂ for 24 hours. BMDCs were centrifuged for 10 minutes at 1500 RPM at 4 C, after which supernatants were collected and stored at 20 C. IL-10 and IL-12p70 from culture supernatants of BMDCs were assessed by ELISA according to the manufacturer’s instructions (eBioscience, San Diego, CA).

BMDCs that were centrifuged were harvested and stained for FACS analyses. Cells were first stained for cell surface markers surface markers including CD11c-PerCp-Cy5.5, and MHC II-PE-Cy7 (BD Pharmingen, San Diego, CA, USA). Cells were then stained for DC maturation markers CD80- and CD86-APC (company). Cells were then fixed and permeabilized by BD Cytotfix/Cytoperm reagent (BD Bioscience, Mississauga, ON, Canada) and stained for intracellular expression markers, IL-10-PE and HO-1-FITC (eBioscience, San Diego, CA, USA). Data were acquired with FACSCanto (AB

Dickinson, Oakville, ON, Canada) and analyzed by FlowJo software (TreeStar, Ashland, OR, USA).

Establishing an *in vitro* Epithelial Cell System to Study Allergy

T84 cells were grown as explained above. Upon confluence, cells were split into 24-well plates at a concentration of 1×10^5 cells/mL, and allowed to reach confluence again (approximately 3 days). Once confluent, cells were stimulated with 10 $\mu\text{g/mL}$, 5 $\mu\text{g/mL}$ and 1 $\mu\text{g/mL}$ of chimaeric human IgE Anti-NP:NP-BSA complexes (Bio-Rad AbD Serotec Ltd, UK). Complexes were incubated for 1-hour prior to use, as explained above. Supernatants were collected 24 hours after stimulation, and ELISA was performed for IL-8. rh-TNF- α was used as a positive control at concentrations of 10 ng/mL.

Testing the Effects of MOs on IgE-Ag stimulated T84 cells *in vitro*

Using the IgE-Ag stimulation model explained above, the effect of MOs on T84 cells was tested. Upon stimulation with IgE Anti-NP:NP-BSA complexes, cells were immediately treated with 6'SL and 2'FL at concentrations of 0.001 mg/mL, 0.01 mg/mL, 0.1 mg/mL, 1.0 mg/mL and 10 mg/mL for 24 hours, after which supernatants were collected and stored at -20°C . IL-8, CCL-20 and IL-33 (R&D Systems, Minneapolis, MN, USA) and TSLP (eBioscience, San Diego, CA) from culture supernatants of T84 cells were measured by ELISA according to the manufacturer's instructions.

Cells were harvested and nuclear protein was extracted using a Nuclear Extraction Kit (Abcam, ab113474). Nuclear and cytoplasmic lysates were collected and stored at -20°C for later detection of NFκB p65 activation and protein measurement of IκB.

Inhibition Experiments

Testing the effect of Siglec F neutralizing antibody on DCs

To test whether the effect of sugars on BMDCs was mediated completely or in part by the sialic-acid binding receptor Siglec-F, a mouse neutralizing Siglec-F antibody was used (R&D Systems MAB17061) to block Siglec-F on DCs. BMDCs were cultured as explained above. On day 7, cells were split onto 24-well plates at a concentration of 1×10^6 cells/mL and treated with 1 µg/mL of Siglec-F Ab for 1 hr at 37°C, 95% O₂. BMDCs were then treated with 6'SL and 2'FL at concentrations of 0.01 mg/mL, 0.1 mg/mL, 1.0 mg/mL and 10 mg/mL. Cells were harvested stained for extracellular markers CD11c-FITC and CD86-APC (BD Pharmingen, San Diego, CA, USA) and intracellular markers IL-10-PE and HO-1-PerCp (eBioscience, San Diego, CA, USA). Data were acquired with FACSCanto (Becton Dickinson, Oakville, ON, Canada) and analyzed by FlowJo software (TreeStar, Ashland, OR, USA).

In separate experiments, the effect of Siglec-F neutralizing Ab on TLR-ligand stimulated DCs was also assessed. BMDCs were treated with Siglec-F as explained in this section, followed by treatment of sugars at the given concentrations. Cells were incubated for 12 hrs after which they were stimulated with LPS and CpG at standard concentrations listed previously. Cells were incubated for another 12 hours, after which supernatants were collected and stored at -20°C. IL-10 and IL-12p70 from culture supernatants of BMDCs were assessed by ELISA according to the manufacturer's instructions (eBioscience, San Diego, CA).

Testing the effect of PPAR γ inhibitor GW9662 on DCs and T84 cells

To test whether the effect of sugars on BMDCs was mediated completely or in part by the PPAR γ receptor, as shown previously by Lin et al. (2014) for 3'SL, the PPAR γ antagonist GW9662 (Sigma-Aldrich M6191) was used on BMDCs and T-84 cells separately.

BMDCs were treated with 0.1 $\mu\text{mol/mL}$ of GW9662 for 1 hr prior to treatment with 6'SL and 2'FL and the remaining BMDC TLR-ligand stimulation protocol was followed as explained above. Supernatants were collected and stored at -20°C and IL-10 and IL-12p70 from culture supernatants were measured by ELISA according to the manufacturer's instructions (eBioscience, San Diego, CA).

T84 cells were treated with 0.1 $\mu\text{mol/mL}$ of GW9662 for 1 hour prior to stimulation with IgE Anti-NP:NP-BSA complexes. Cells were immediately treated with 6'SL and 2'FL at

concentrations listed above. Supernatants were collected after 24 hours and stored at -20°C. IL-8, CCL-20 and IL-33 (R&D Systems, Minneapolis, MN, USA) and TSLP (eBioscience, San Diego, CA) from culture supernatants of T84 cells were measured by ELISA according to the manufacturer's instructions.

Nuclear Transcription Factor Activity

NFκB p65 activity was measured for T84 IgE-Ag Stimulation experiments using the sandwich-ELISA style NFκB p65 Transcription Factor Assay Kit (Abcam ab133112), according to the manufacturer's instructions (Abcam, Cambridge, UK). Assay was performed using DDT (Thermo-Scientific, R0861) when required.

Cytokine Measurement

Measurement of all cytokines was performed on supernatants with a 1:2 or 1:4 dilution (depending on experiment), using Ready-Set-Go ELISA kits for IL-10, IL-12, TSLP and TNF-α (eBioscience, San Diego, CA) and the Duo-Set Kit for IL-8, CCL20, and IL-33 (R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions.

Flow Cytometry

Cells were first stained for appropriate surface markers (depending on experiment) and then fixed and permeabilized by BD Cytotfix/Cytoperm reagent (BD Bioscience, Mississauga, ON, Canada) and stained for intracellular expression markers, as needed. Data were acquired with FACSCanto (Becton Dickinson, Oakville, ON, Canada) and analyzed by FlowJo software (TreeStar, Ashland, OR, USA).

Statistical analysis

Statistical analysis of the data was done using One-way Analysis of Variance (ANOVA) (GraphPad Prism 5.0, GraphPad, San Diego, CA), followed by Dunnett's Multiple Comparison Test for comparing all experimental groups to control groups. Significant differences between two groups were determined using the unpaired Student's *t* test. A *p* value of less than 0.05 was considered statistically significant. Experimental results are expressed as means \pm the standard errors of the means (SEM). A statistical software package (GraphPad PRISM™ version 5.0) was used for the analysis.

RESULTS

Anti-inflammatory effects of MOs on DCs and ECs, *in vitro*

Dendritic cells

Direct treatment of BMDCs with 6'SL led to marked increases in the expression of IL-10 and HO-1 (Fig. 1A, 1B, respectively) at the highest dose of 1.0 mg/mL from $17.21 \pm 1.62\%$ to $26.40 \pm 2.07\%$ ($p= 0.0012$) for IL-10 and from $20.08 \pm 2.141\%$ to $30.07 \pm 3.336\%$ for HO-1 ($p= 0.0149$). On the contrary, 2'FL, 3'SL and LNnT did not significantly alter the expression levels of IL-10 and HO-1 under the same conditions.

6'SL also induced marked increases in IL-10 in response to TLR-ligand stimulation. Of the TLR ligands tested, 6'SL (1.0 mg/mL) had the greatest effect on CpG- induced IL-10 release (103.5 ± 21.53 pg/mL to 548.9 ± 52.85 pg/mL ($p=0.0001$)) (Figure 2A) while LPS-induced IL-10 release was increased from 250.5 ± 25.68 pg/mL to 774.5 ± 60.00 pg/mL ($p<0.0001$). The enhanced release of IL-10 was dose-dependent, as increased concentration of the oligosaccharide amplified the amount of cytokine produced (Figure 2A). 6'SL also induced a gradual decrease in CpG-induced BMDC release of IL-12p70 with 1.0 mg/mL of 6'SL decreasing it from 169.8 ± 3.003 to 60.63 ± 10.59 ($p<0.0001$) and LPS-induced IL-12p70 decreasing from 64.90 ± 2.71 pg/mL to 16.66 ± 0.728 pg/mL ($p<0.0001$). On the contrary, the neutral oligosaccharide LNnT did not alter the release of either cytokine under any of the three stimulatory conditions. Lastly, BMDC release of TNF- α increased upon stimulation with TLR-ligands, but remained unaltered as a result of 6'SL (Figure 2E) or LNnT (Figure 2F) at varying concentrations. These data suggest the establishment of an anti-inflammatory or regulatory phenotype and functionality for BMDCs upon direct treatment with 6'SL

Epithelial cells

Treatment of cells with 6'SL at all tested concentrations, and subsequent stimulation by (rh)TNF- α yielded a relatively small but statistically significant dose-dependent decrease in IL-8 release, with 1.0 mg/mL of 6'SL decreasing IL-8 secretion from 919.1 ± 35.03 pg/mL to 733.3 ± 15.95 pg/mL ($p=0.0002$) (Figure 3A). Decreased secretion of IL-8 was present

for LNT and lactose as well but at low doses of the sugars (0.1 mg/mL and 0.01 mg/mL, respectively) (Figure 3D & 3E, respectively). On the contrary, 2'FL and 3'SL did not alter the release of TNF- α -induced IL-8 by T84 cells.

Next, to verify that the anti-inflammatory effect of 6'SL was not restricted to the T84 cell line, its effect was measured on the mouse duodenal epithelial cell line, MODE-K.

Treatment of MODE-K cells with 6'SL led to marked decreases in TNF- α induced mKC/CXCL8, the murine homologue for IL-8. The highest dose of 6'SL decreased mKC release from 5440 ± 468.9 pg/mL to 2818 ± 307.5 pg/mL ($p=0.0045$) (Figure 4A). On the contrary, 2'FL did not alter TNF- α -induced mKC production under the same conditions.

These data suggest that 6'SL may exert an anti-inflammatory effect onto intestinal epithelial cells, rendering them less responsive to pro-inflammatory stimulation.

The establishment of tolerance involves complex molecular intercom between IECs and mucosal DCs. Having determined that specific MOs such as 6'SL directly exert anti-inflammatory effects on BMDCs and IECs separately *in vitro*, we next assessed the ability of MOs to regulate IEC-DC communication and their possible role in DC maturation. To do so, IECs were treated with 6'SL, 2'FL and Lactose separately, and supernatants from these cells were treated to DCs.

6'SL, 2'FL and Lactose-treated MODE-K supernatants did not significantly impact DC functionality or phenotype, represented by no significant changes in production of IL-10 (Figure 5A, 5E, 5I), and DC maturation markers MHC II (Figure 5B, 5F, 5J), CD80 (Figure 5C, 5G, 5K) and CD86 (Figure 5D, 5H, 5L). In addition, consistent with results

from flow cytometry, cytokine measurement of IL-10 production by BMDCs remained unchanged; however, 6'SL-primed MODE-K supernatant caused a small but statistically significant reduction in IL-12p70 production from 643.8 ± 27.76 pg/mL to 519.4 ± 28.33 pg/mL ($p=0.0106$) by BMDCs (Figure 6B) at 0.1 mg/mL of the sugar. IL-12p70 production was also reduced in BMDCs treated with 2'FL- and Lactose-primed MODE-K supernatants at low concentrations (0.01 mg/mL) ($p=0.0045$, $p=0.0080$, respectively).

Anti-inflammatory effect of MOs on DCs and IECs in relation to allergy

To assess the effect of MOs on *in vitro* IgE-Ag stimulated cells, we first elucidated the concentration and IgE:Ag ratio required for effective stimulation of T84 cells. Stimulation with 1 ug/mL of IgE-Ag complexes at a 1:1 ratio yielded the greatest increase in IL-8 production ($p=0.0105$) (Figure 7), and was therefore used as the standard stimulation for all subsequent experiments.

To accurately assess the effect of MOs in this system, raw cytokine measurement values (pg/mL) were converted into percent increase (%-increase) (Supplementary Table S2). Treatment of T84 cells with 6'SL led to marked inhibition of IgE-Ag-induced IL-8 release in a dose-dependent manner; incremental increase in 6'SL concentration lead to more pronounced inhibition of IL-8 release (Figure 8A) with it decreasing from 169.9 ± 8.923 %-increase with IgE-Ag stimulation alone to 112.3 ± 9.782 %-increase with IgE-Ag stimulation followed by treatment with 10 mg/mL of 6'SL ($p=0.0015$). On the contrary,

2'FL exerted no statistically significant alteration in IgE-Ag-induced IL-8 release (Figure 8B).

6'SL and 2'FL both inhibited IgE-Ag-induced CCL20 chemokine release at different concentrations. IgE-Ag-induced CCL20 inhibition became more pronounced with increasing concentration of 6'SL, with 10 mg/mL decreasing levels from 131.0 ± 3.827 %-increase, to almost baseline levels of 103.4 ± 3.708 %-increase ($p=0.0004$) (Figure 8C). Changes in CCL20 release by 2'FL did not follow a typical dose-dependent pattern. In fact, reduction in IgE-Ag stimulated CCL20 was most pronounced at 0.01 mg/mL of the sugar, decreasing it from $131.0 \pm 3.827\%$ to 100.9 ± 7.126 % ($p=0.004$) (Figure 8D).

The inhibitory effect of 6'SL and 2'FL on IgE-Ag induced IL-33 release was present but not statistically significant (Figure 8D, 8E, respectively). At 10 mg/mL of 6'SL, IL-33 release reduced from 157.7 ± 27.73 %-increase to 100.5 ± 20.33 %-increase ($p=0.1159$). At 1 mg/mL, 2'FL treatment caused to a pronounced reduction in IL-33 from 157.7 ± 27.73 to 109.3 ± 18.61 ($p=0.1668$).

Reduction of pro-inflammatory cytokines is mediated by PPAR γ

Pre-incubation of BMDCs with 1 ug/mL of a Siglec-F neutralizing antibody (Sig-F Ab) and subsequent treatment of cells with 6'SL and 2'FL did not yield significant changes in expression of IL-10 (Figure 9A, 9B) and HO-1 (Figure 9C, 9D). In fact, cells pre-incubated with Sig-F Ab and treated with 2'FL showed an increased IL-10 expression in a dose-dependent manner, but these results were not statistically significant (Figure 9C). In

addition, the Sig-F Ab used did not significantly alter IL-10 release for either 6'SL (Figure 9E, 9F) or 2'FL (Figure 9G, 9H) in TLR-ligand stimulated BMDCs. These data indicate that the effects exerted by these specific oligosaccharides onto BMDCs were likely not mediated by Siglec-F.

Pre-incubation of BMDCs with 0.1 $\mu\text{mol/mL}$ of GW9662, a potent PPAR γ inhibitor, and subsequent treatment with 6'SL resulted in insignificant changes in LPS- and CpG-induced IL-10 release (Figure 10A, 10B), but significant changes in IL-12p70 release (Figure 10E, 10F). IL-12p70 production decreased due to 6'SL treatment, and this decrease was attenuated with the addition of GW9662. The effect was most significant at 1 mg/mL of the sugar upon LPS stimulation, increasing IL-12p70 production to 22.20 ± 0.6191 pg/mL when treated with GW9662, compared to 16.66 ± 0.7275 when no inhibitor was added ($p=0.0012$) (Figure 10E). Similarly, CpG-induced cells also released significantly higher amounts of IL12-p70 when inhibited with GW9662 and treated with 1 mg/mL or 10 mg/mL of 6'SL ($p=0.0173$, $p=0.0204$, respectively) (Figure 10F).

To assess the role of PPAR γ in IgE-Ag stimulated cells, T84 cells were pre-incubated with GW9662, prior to both IgE-Ag stimulation and MO-treatment. As predicted from earlier experiments, 6'SL inhibited IgE-Ag-induced IL-8-release in a dose-dependent manner; the addition of GW9662 weakened this reduction. IgE-Ag stimulated cells experienced a 157.2 ± 10.25 %-increase in IL-10 production in comparison to unstimulated cells. With treatment of 10 mg/mL 6'SL, the increase in IL-10 was minimized to 104.8 ± 1.302 %-increase. However, with the addition of GW9662, the %-increase in IL-10 was ameliorated

to 133.6 ± 7.334 %-increase. Stated differently, GW9662-inhibited T84 cells experienced a smaller reduction in IL-10 production, in comparison to uninhibited controls at 1 mg/mL and 10 mg/mL of 6'SL. These data suggest that the inhibitory effect of 6'SL on IgE-Ag induced IL-8 release is mediated at least in part by PPAR γ .

Pre-incubation of T84 cells with GW9662 did not alter CCL20 (Figure 11C, 11D) or IL-33 release (Figure 11E, 11F) by either MO.

6'SL reduces NF κ B p65 activity through PPAR γ

As NF κ B is a central regulatory transcription factor responsible for immunomodulation in epithelial cells, including the production of IL-8, we assessed the effect of 6'SL and 2'FL on NF κ B p65 activation in IgE-Ag stimulated cells. Upon stimulation with IgE-Ag complexes alone, p65 activity increased significantly from 0.9468 ± 0.02404 to 1.494 ± 0.03903 , expressed as absorption at 450 nm ($p < 0.0001$) (Figure 12A). Upon treatment with 6'SL at even low concentrations (0.001 mg/mL), p65 activity reduced significantly ($p = 0.0326$). At the highest concentration of 6'SL (10 mg/mL), p65 activity was reduced dramatically to 1.050 ± 0.03619 ($p < 0.0001$).

The effect of 2'FL on NF κ B p65 activity was even more pronounced; at all concentrations of the sugar, p65 activity reduced significantly. While in IgE-Ag stimulated controls the absorbance was 1.494 ± 0.03903 , experimental wells treated with 10 mg/mL of 2'FL had mean absorbance of 1.196 ± 0.02608 ($p = 0.0005$).

We next investigated the involvement of PPAR γ on 6'SL- and 2'FL- mediated NF κ B p65 inhibition. Pre-treatment of cells with GW9662 and subsequent treatment with high doses of 6'SL (10 mg/mL) led to an increase in p65 activation from 0.9410 ± 0.03300 (6'SL alone) to 1.642 ± 0.01000 (GW9662 + 6'SL) ($p=0.0024$) (Figure 12C). On the contrary, GW9662 did not alter the inhibition of p65 that was found with 2'FL treatment at all concentrations.

For tabular summary of results, see Supplementary Table S3 and S4.

FIGURES

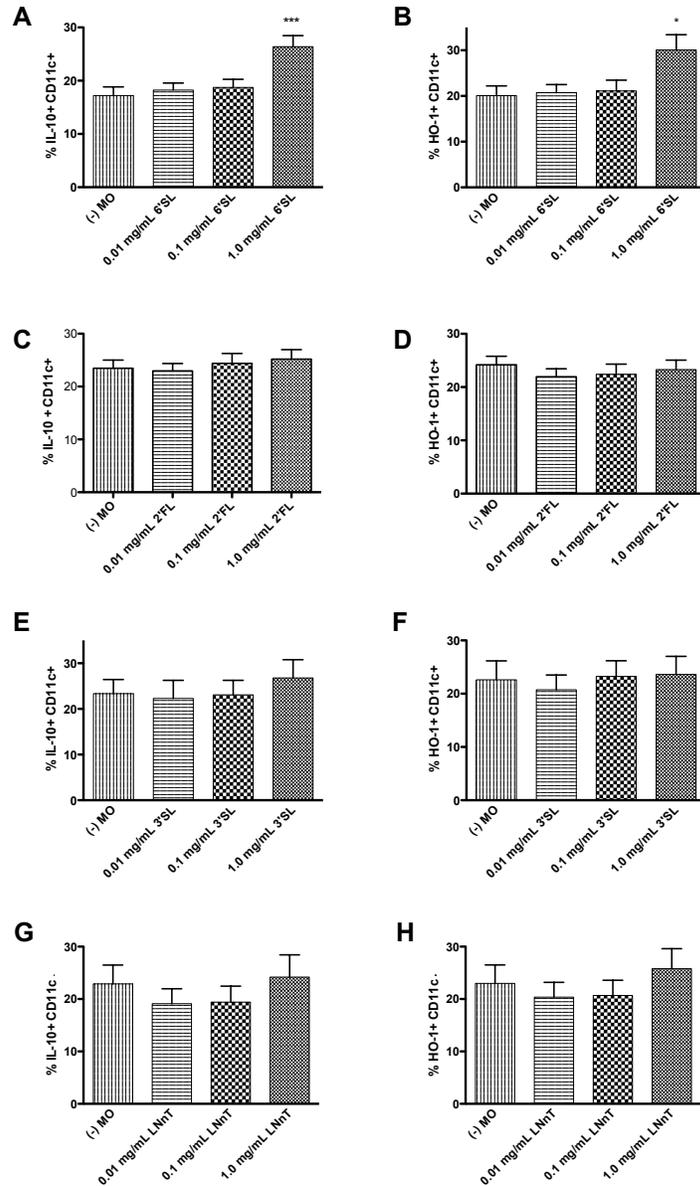


Figure 1: Effect of 24-hour treatment of DCs with 6'SL (A, B) 2'FL (C, D), 3'SL (E, F) and LNnT (G, H) on extracellular expression of IL-10 and HO-1 as measured by flow cytometry. Values expressed as mean \pm SEM with $9 \leq n \leq 21$. All means compared to negative control of untreated-BMDCs. (***= $p < 0.001$, *= $p < 0.05$). For full gating strategy, see Supplementary Figure S1.

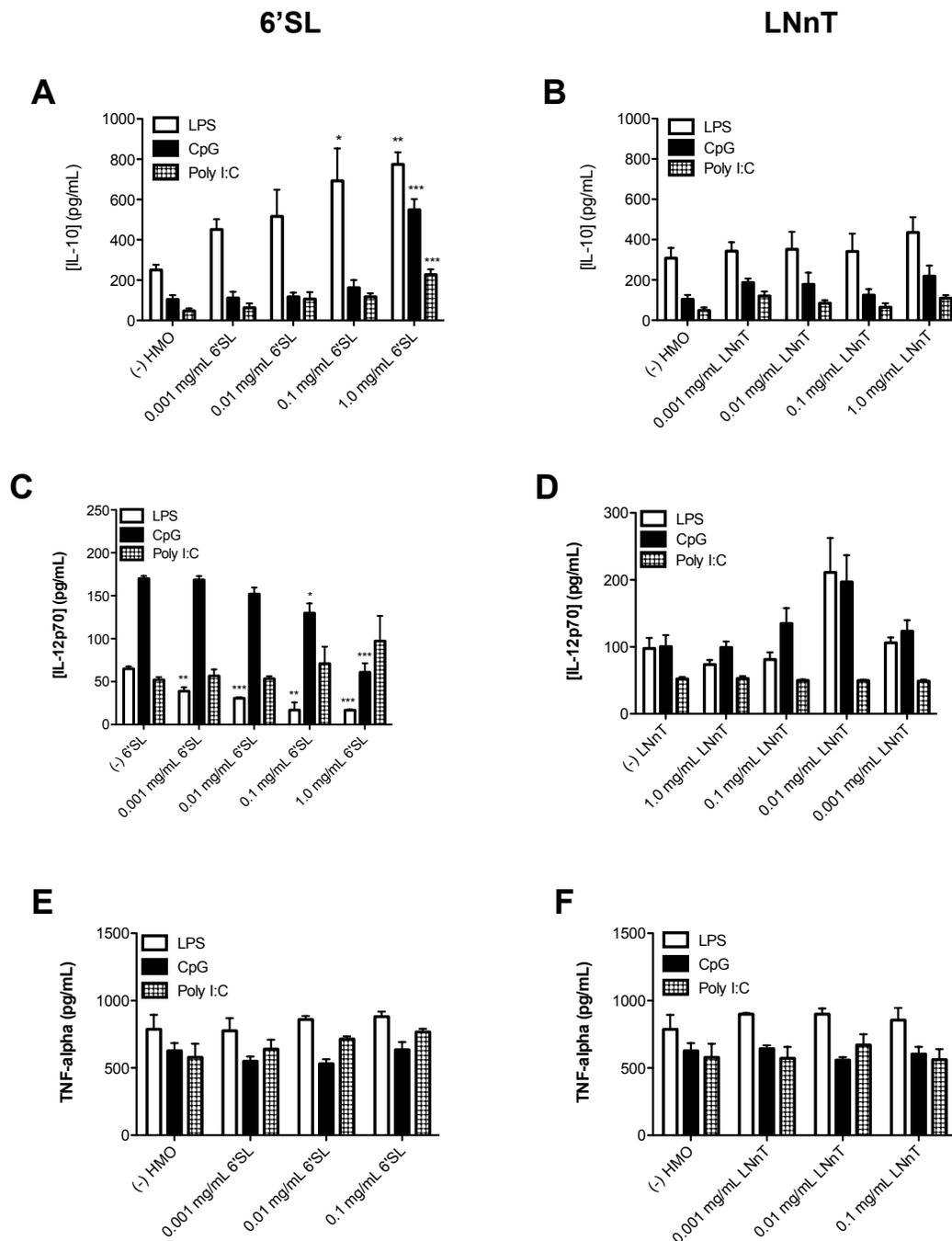


Figure 2: Effect of 1-hour treatment of BMDCs with 6'SL (A, C, E) and LNnT (B, D, F) followed by stimulation with TLR-ligands LPS, CpG and PolyI:C on secretion of IL-10, IL-12p70 and TNF- α . Cytokines measured 24 hours post-stimulation. Values expressed as mean \pm SEM with $n \geq 3$. All means compared to positive control of untreated-TLR-ligand stimulated-BMDCs. (**= $p < 0.01$, ***= $p < 0.001$, *= $p < 0.05$).

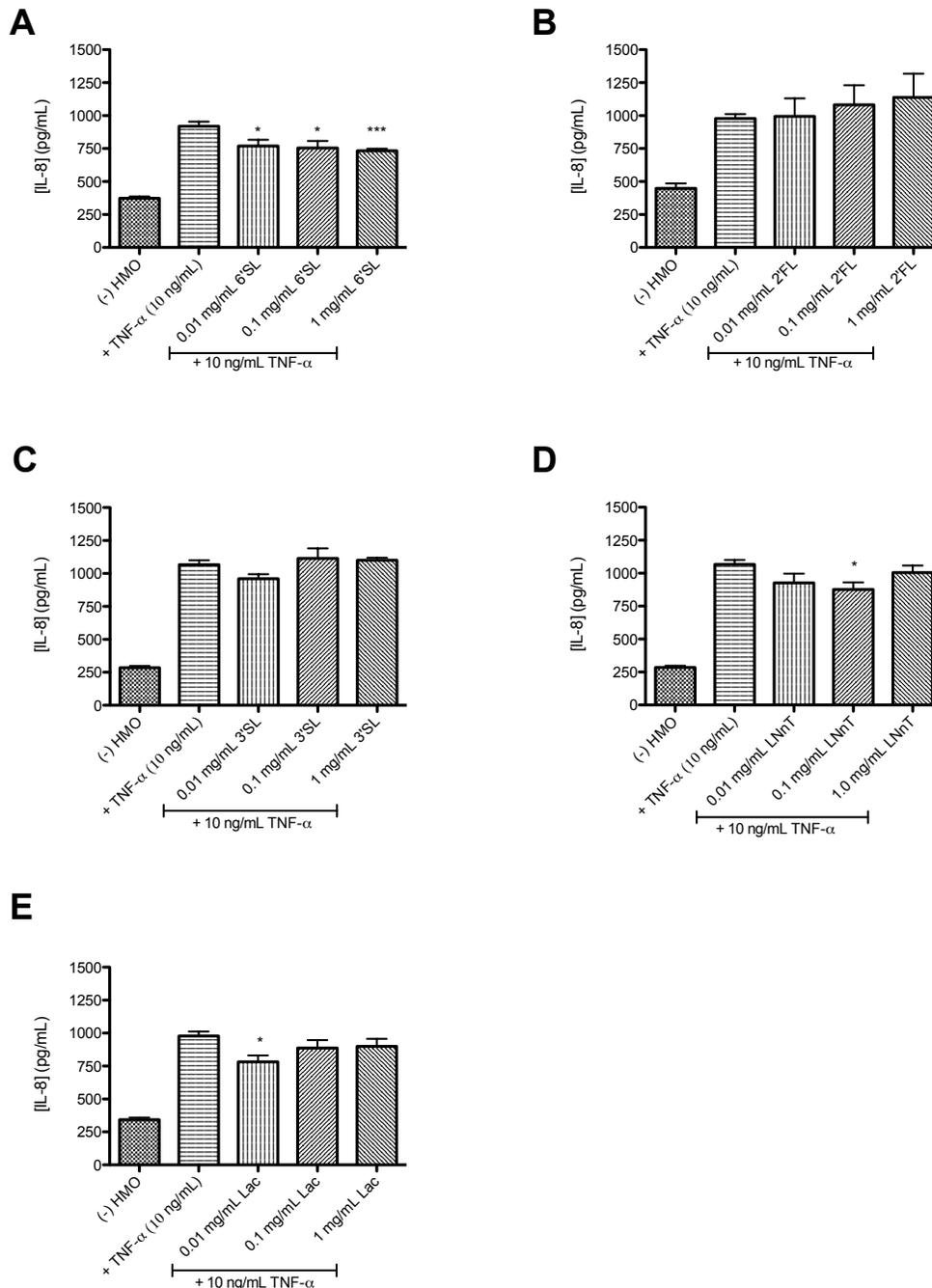


Figure 3: Effect of 12-hour treatment of T84 cells with 6'SL (A), 2'FL (B), 3'SL (C), LNnT (D) and Lactose (E), followed by stimulation with rh-TNF- α on production of IL-8. IL-8 measured 12 hours post-stimulation. Values expressed as mean \pm SEM with $4 \leq n \leq 12$. All means compared to positive control of rh-TNF- α stimulation. (**= $p < 0.0001$, *= $p < 0.05$).

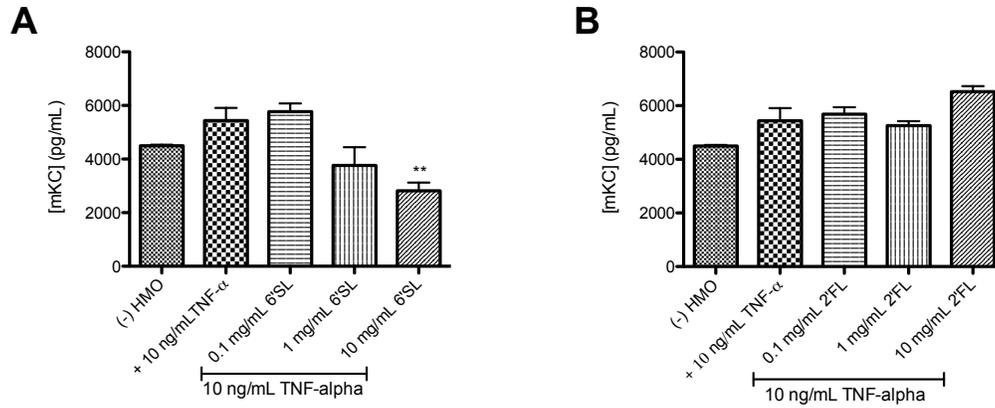
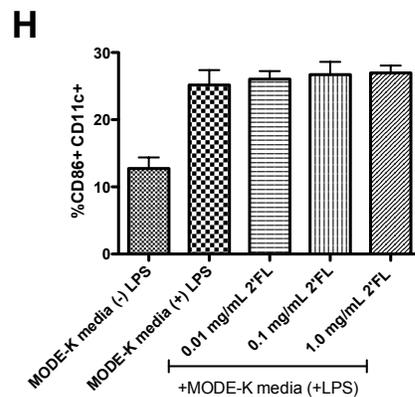
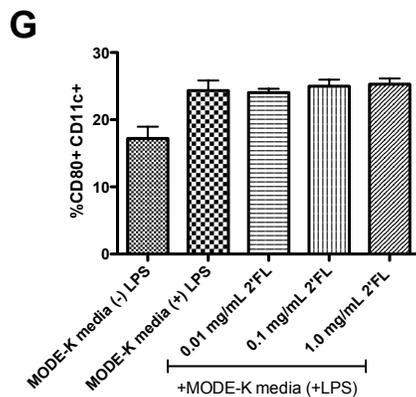
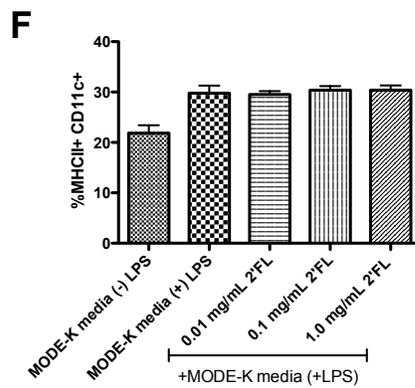
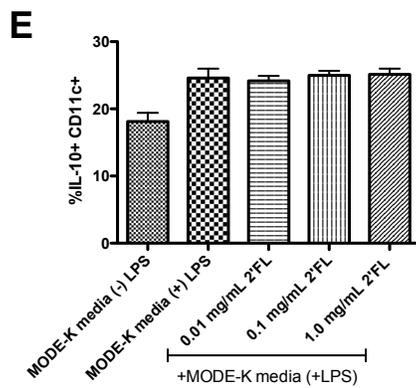
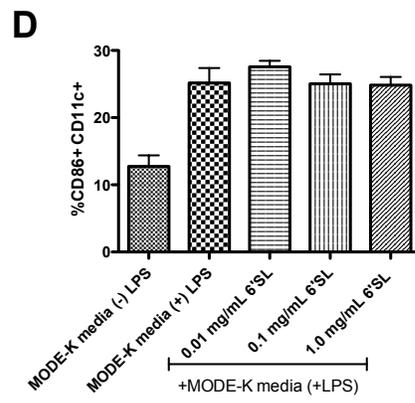
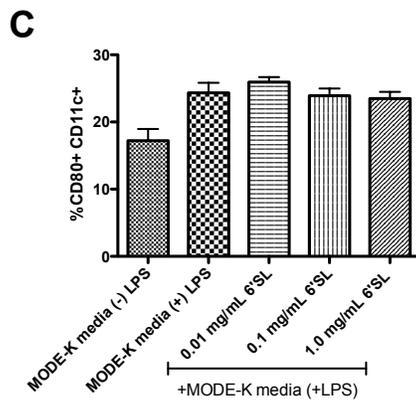
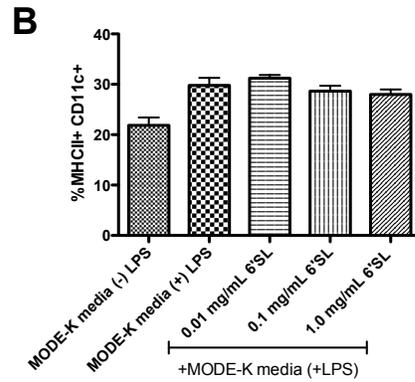
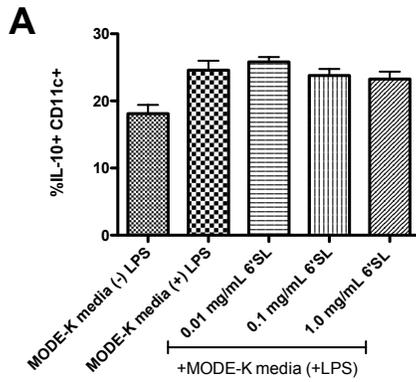


Figure 4: Effect of 12-hour treatment of MODE-K cells with 6'SL (A), and 2'FL (B), followed by stimulation with m-TNF- α on production of mKC/CXCL8. mKC/CXCL8 measured 18 hours post-stimulation. Values expressed as mean \pm SEM with $8 \leq n \leq 12$. All means compared to positive control of m-TNF- α stimulation. (**= $p < 0.01$).



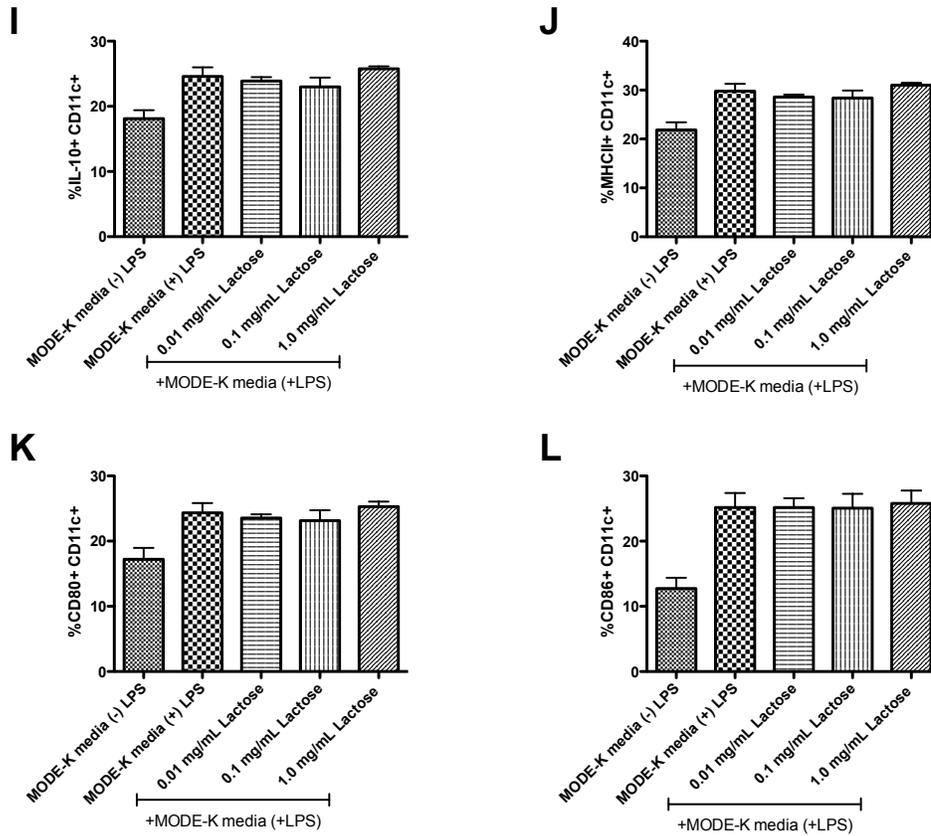


Figure 5: Effect of 24-hour treatment of BMDCs with cell-free supernatant collected from 6'SL-, 2'FL-, or Lactose-treated MODE-K cells at a 1:1 dilution, on BMDC expression of IL-10 (A, E, I), MHC II (B, F, J), CD80 (C, G, K) and CD86 (D, H, L). Values expressed as mean \pm SEM with $n \geq 4$. All means compared to positive control of 'untreated-MODE-K media+LPS stimulation'.

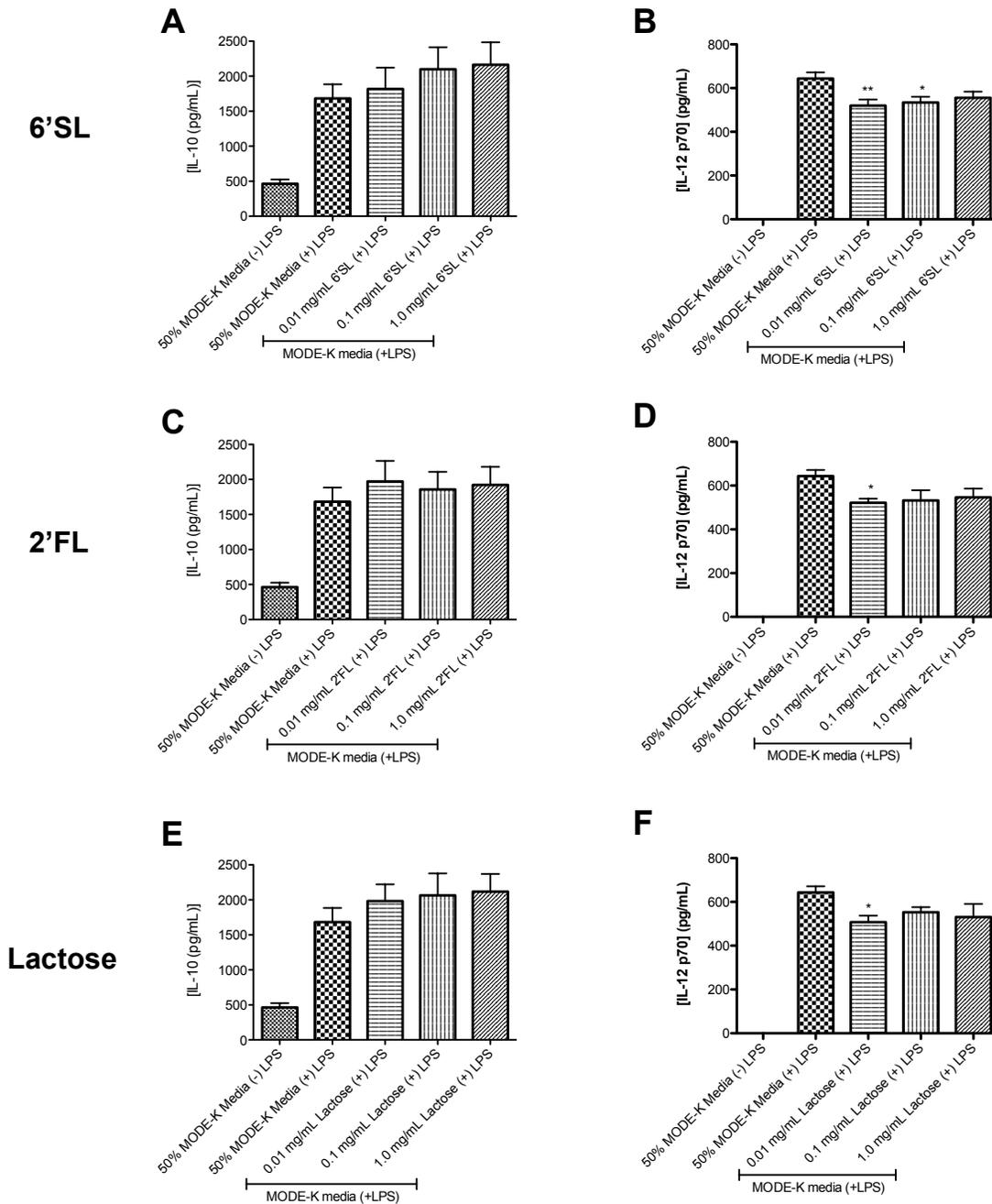


Figure 6: Effect of 24-hour treatment of BMDCs with cell-free supernatant collected from 6'SL-, 2'FL-, or Lactose-treated MODE-K cells at a 1:1 dilution, on BMDC production of IL-10 (A, C, E) and IL-12p70 (B, D, F). Values expressed as mean \pm SEM with $n \geq 5$. All

means compared to positive control of ‘untreated-MODE-K media +LPS stimulation’.
(**= $p < 0.01$, *= $p < 0.05$).

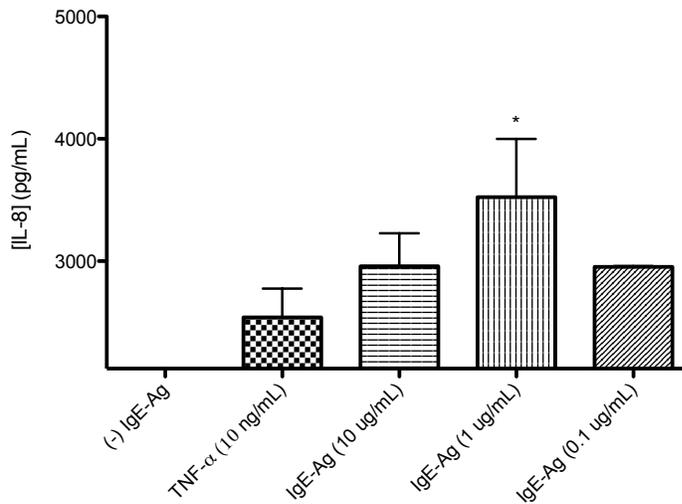


Figure 7: Effect of 24-hour stimulation of T84 cells with 1:1 ratio of IgE-Ag complexes (pre-incubated for 1 hour) at varying concentrations. Values expressed as mean \pm SEM with $n = 2$. All means compared to negative control of untreated T84 cells. (*= $p < 0.05$).

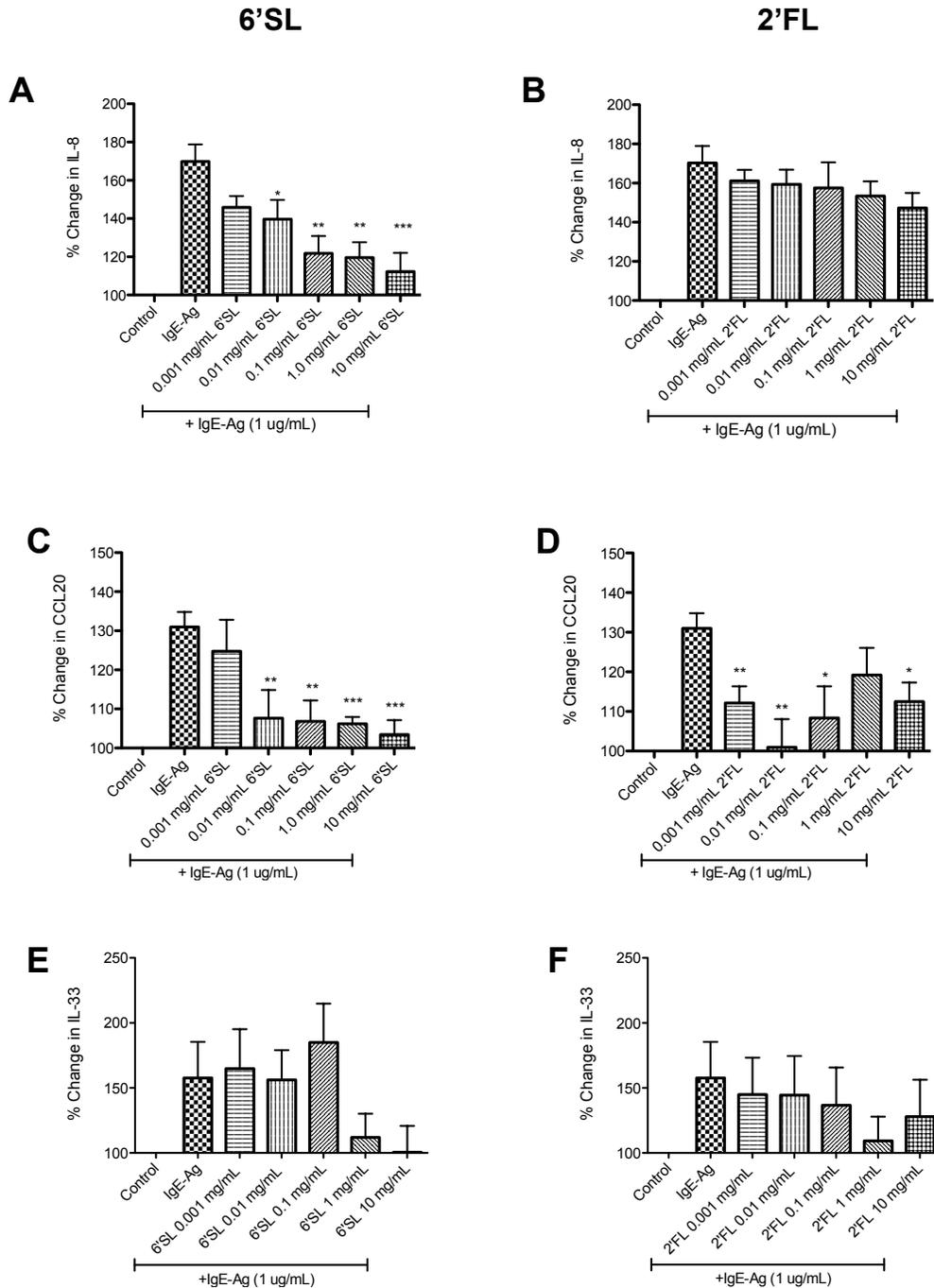


Figure 8: Effect of 24-hour treatment of IgE-Ag stimulated T84 cells with 6'SL (A, C, E) and 2'FL (B, D, F) on production of IL-8 (A, B), CCL-20 (C, D) and IL-33 (E, F). IL-8 and CCL-20 cytokine production represented as % change in comparison to unstimulated negative controls. IL-33 represented as cytokine production in pg/mL. Values expressed as mean \pm SEM with $n \geq 6$. All means compared to positive control of untreated-IgE-Ag stimulated T84 cells. (**= $p < 0.01$, *= $p < 0.05$).

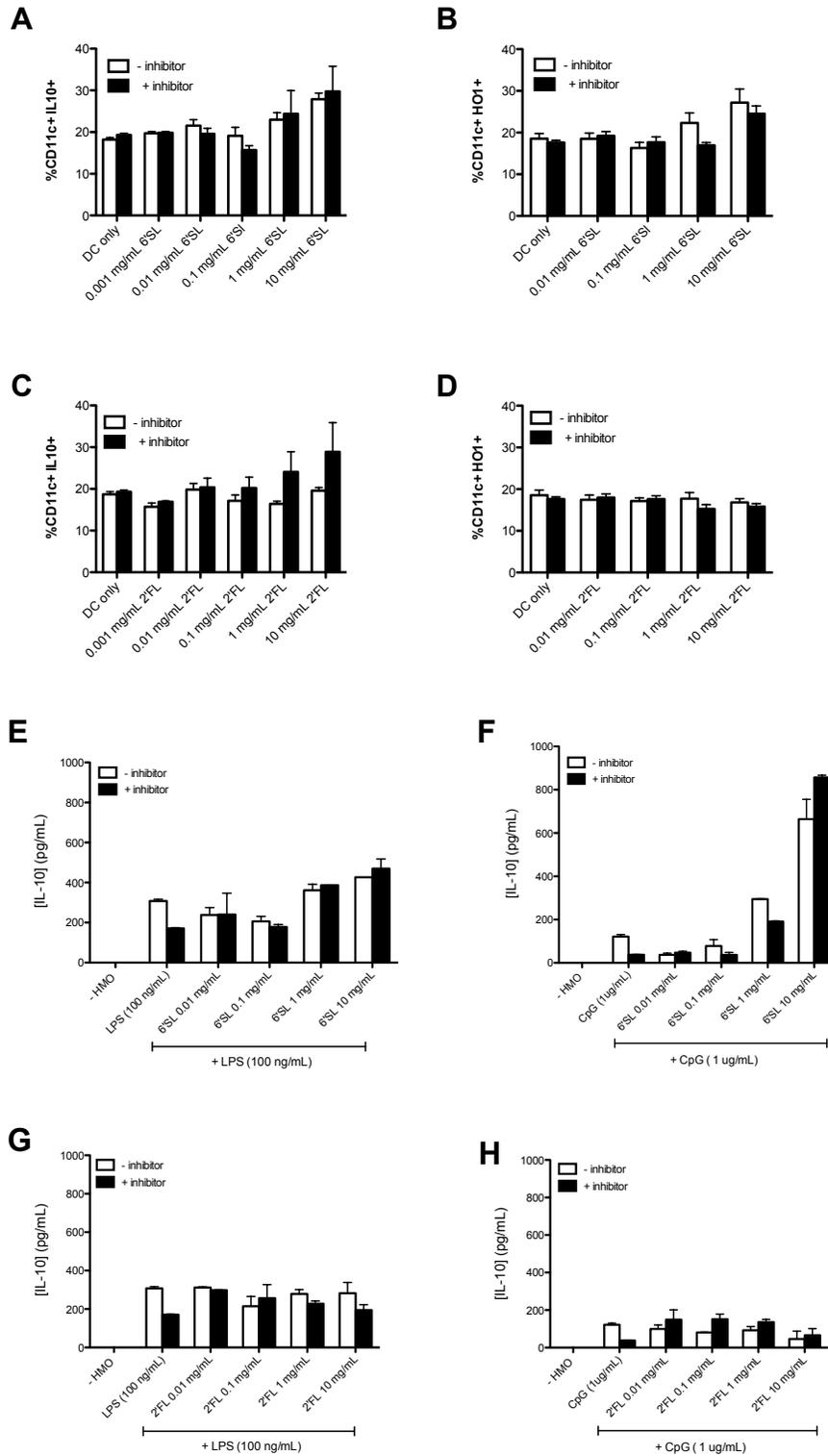
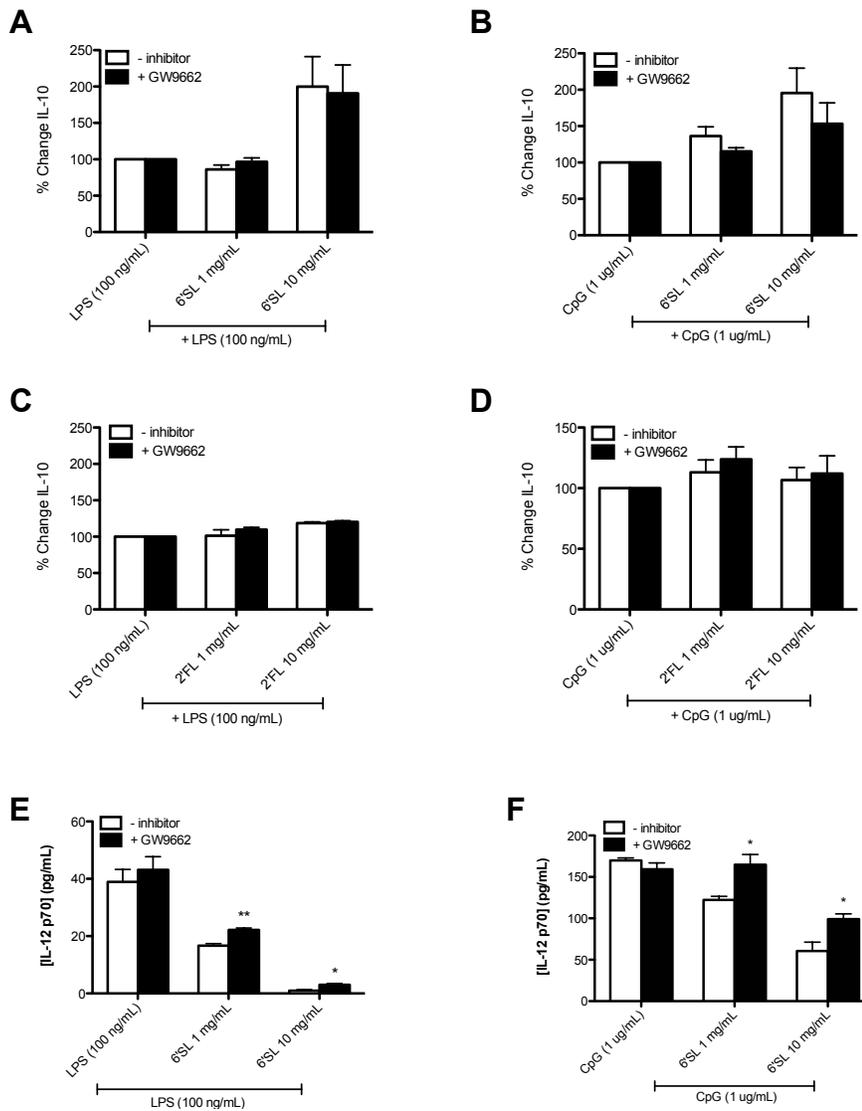


Figure 9: Effect of Sig-F Ab on expression of IL-10 (A, C) and HO-1 (B, D) upon treatment of BMDCs with varying doses of 6'SL (A, B) and 2'FL (C, D). Values expressed as mean \pm SEM with $n \geq 6$. All means compared to untreated BMDC controls. In addition,

effect of Sig-F Ab on production of IL-10 upon TLR-ligand stimulation of BMDCs and treatment with varying doses of 6'SL (E, F) and 2'FL (G, H). Values expressed as mean \pm SEM with $n \geq 3$. All means compared to TLR-ligand stimulated untreated controls. Student's *t* test performed to measure effect of inhibitor on treatment at specific concentrations.



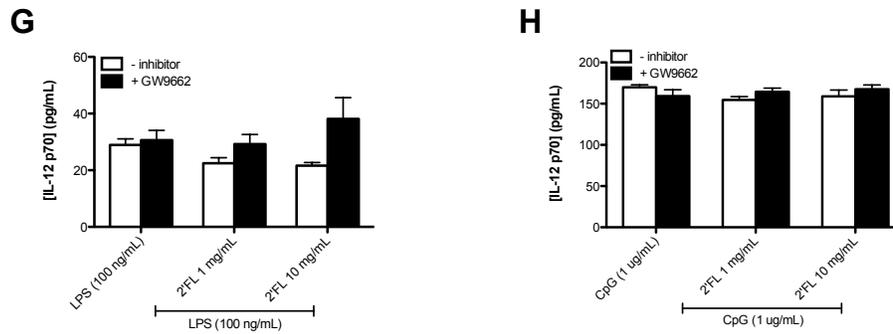


Figure 10: Effect of GW9662 on TLR-ligand-stimulated BMDCs treated with 6'SL and 2'FL on IL-10 (A, B, C, D) and IL-12p70 (E, F, G, H) production. Values expressed as mean absorbance \pm SEM with $n \geq 4$. All means compared to TLR-ligand stimulated untreated controls. Student's *t* test performed to measure effect of GW9662 on treatment at specific concentrations. (**= $p < 0.01$, *= $p < 0.05$).

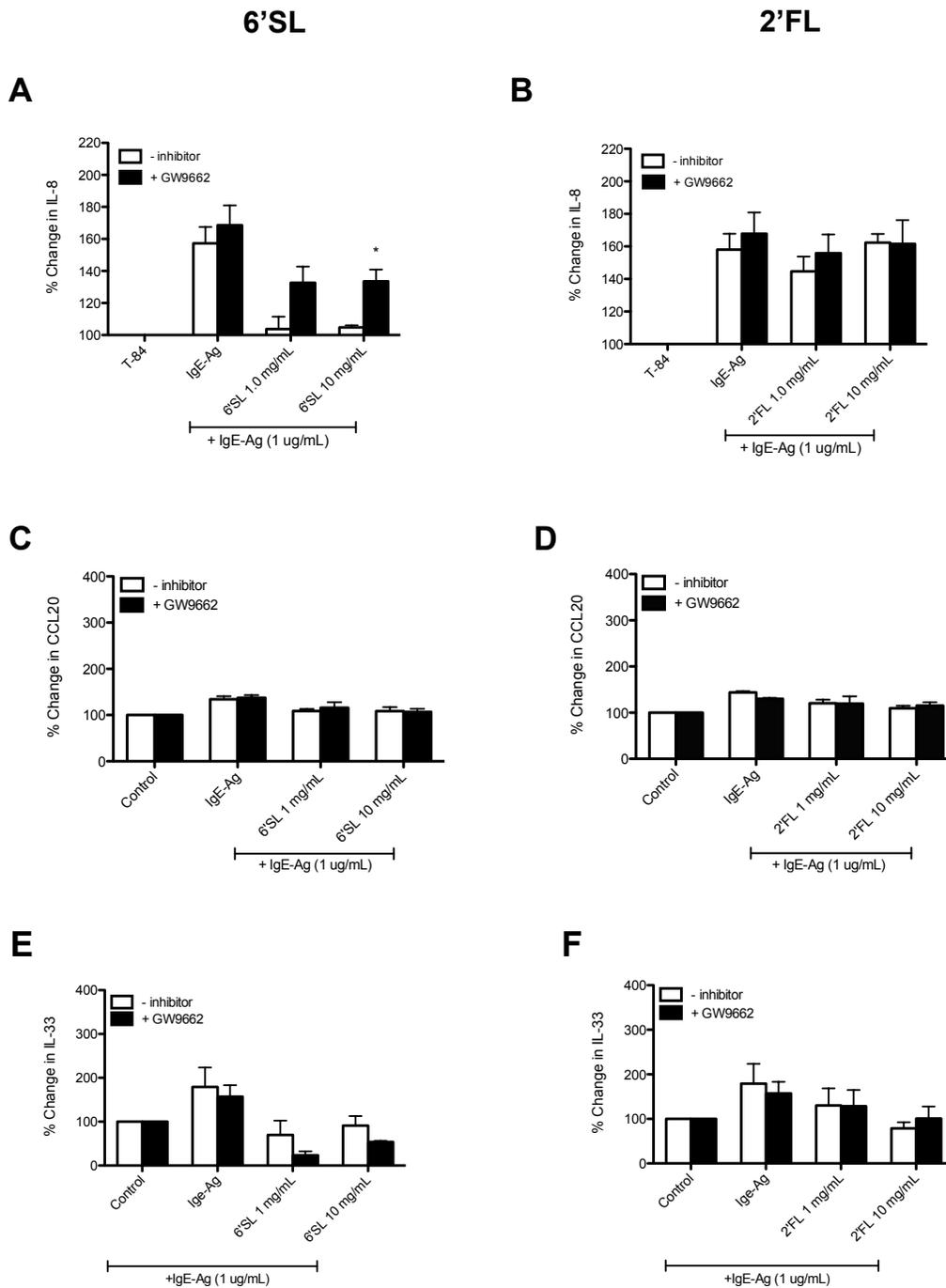


Figure 11: Effect of GW9662 on IgE-Ag stimulated T84 cells treated with 6'SL (A, C, E) or 2'FL (B, D, F) on IL-8 (A, B), CCL20 (C, D), and IL-33 (E, F). Values expressed as mean absorbance \pm SEM with $n \geq 6$. All means compared to TLR-ligand stimulated untreated controls. Student's *t* test performed to measure effect of GW9662 on treatment at specific concentrations. (**= $p < 0.01$, *= $p < 0.05$).

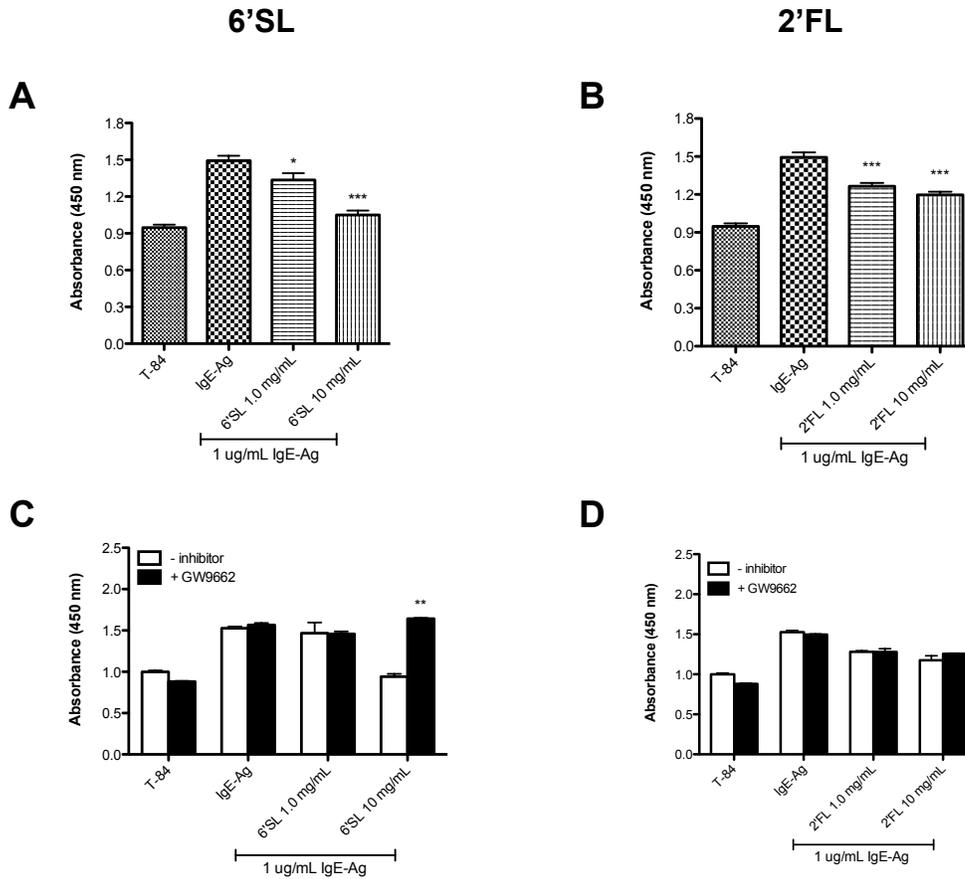


Figure 12: Measurement of NFκB p65 activation in IgE-Ag stimulated T84 cells treated with varying concentrations of 6'SL (A) and 2'FL (B). Values expressed as mean absorbance ± SEM with $n \geq 6$. All means compared to IgE-Ag stimulated untreated controls. In addition, effect of GW9662 on p65 activation in IgE-Ag stimulated T84 cells treated with varying concentrations of 6'SL (C) and 2'FL (D). Values expressed as mean absorbance ± SEM with $n = 2$. All means compared to IgE-Ag stimulated untreated controls. (**= $p < 0.001$, *= $p < 0.01$, **= $p < 0.05$).

DISCUSSION

This study was designed to investigate the direct effects of prebiotic milk oligosaccharides commonly found in human and mouse milk on dendritic cell (DC) and intestinal epithelial cell (IEC) phenotype and function. The present results demonstrate that specific oligosaccharides are capable of having direct immunomodulatory effects on these cells *in vitro*.

Although their role as prebiotic substrates is well established, it has been suggested that MOs can exert immunomodulatory effects even in the absence of commensal microbes (Lindsay et al., 2006; Eiwegger et al., 2010; Zenhom et al., 2011). *In vitro* studies have shown that MOs can act as direct immune modulators by promoting T cell shift to a balanced Th1/Th2-cytokine production (Bode, 2012) and stimulate macrophage release of prostaglandin E₂, IL-10 and TNF- α (Atochina et al., 2005).

Recent studies have shown that a small percentage of MOs are absorbed in the intestinal tract and later excreted in urine (Jantscher-Krenne & Bode, 2012; Eiwegger et al., 2010). In addition, the CD11c+CX₃CR1+ DC subset can extend dendrites to interact with luminal contents of the gut by protruding adjacent epithelial cells (Pabst & Mowat, 2012). Taken together, we hypothesized that in addition to having direct effects on T cells and macrophages, through the process of absorption and direct contact, MOs may interact with DCs to exert immunomodulatory effects. Our results showed that direct treatment of DCs with 1 mg/mL of 6'SL *in vitro* causes a significant increase in IL-10 and HO-1 expression. In addition, 6'SL caused a dramatic increase in CpG- and LPS-induced IL-10 release, in a

dose-dependent manner. In contrast, these effects were not present for any of the remaining oligosaccharides, suggesting that the effect of 6'SL is specific to its structure. Given the role of IL-10 as an anti-inflammatory cytokine and HO-1 as an immunosuppressive enzyme in DCs, our data suggest that 6'SL acts as a direct modulator of DCs, by promoting an anti-inflammatory phenotype and function.

Previously, it has been shown that MOs directly affect EC function by regulating cell cycle changes and possibly contributing to EC renewal in the gut (Kuntz et al., 2008; Kuntz et al., 2009). In addition, Zenhom et al. (2011) demonstrated a reduction in proinflammatory cytokine release by IECs in response to certain oligosaccharides. To extend our understanding of MO-mediated IEC function, we looked at the direct effects of MOs on the T84 cell line. IL-8 is a pro-inflammatory chemokine that regulates the influx of immune cells to mucosal tissues under an allergic response. Given that TNF- α is increased in cell-mediated allergy and is a potent inducer of IL-8 release by IECs (Schuerer-Maly et al., 1994), TNF- α stimulation was used to investigate the effect of different MOs on the T84 cell line. We found that treatment of 6'SL leads to a small but significant reduction in IL-8 release by T84 cells, and LNnT and Lactose also cause slight reductions in IL-8 at low concentrations. The effect of 6'SL persisted in the MODE-K cell line, with TNF- α -induced mKC (the murine homologue for IL-8) release decreasing significantly in the presence of 6'SL at high concentrations. These data suggest that 6'SL may exert an anti-inflammatory effect onto IECs, rendering them less responsive to pro-inflammatory stimulation.

In the gut, resident DCs are neighbours to IECs, the first cells to encounter allergen upon enteral entry into the body. IECs actively influence properties and maturation of bystander

DCs through the production of cytokines. In response to external stimuli, IECs produce TSLP, which stimulates DCs to produce CCL17 and CCL22 (chemokines that attract Th2 cells) (Ruiter & Shreffler, 2012). Ultimately, TSLP-activated DCs prime naïve T cells to produce IL-4, IL-5 and IL-13 (Ruiter & Shreffler, 2012), which are major cytokines in food allergic responses. In addition, stimulated ECs cause DC maturation, involving an upregulation of co-stimulatory receptors such as CD80 and CD86 and increased expression of MHC II molecules. These and other immunological communication mechanisms allow EC-priming of DCs to produce a specific immunological response. With the understanding that MOs directly influence EC function, we therefore looked at the ability of MO-primed ECs to drive differentiation of DC phenotype and function. Our results show that MO-primed ECs do not impact DC maturation; DCs exposed to MO-treated cell-free supernatant had unaltered expression of IL-10, MHC II, CD80 and CD86. However, treatment of DCs with relatively low concentrations of 6'SL-, 2'FL-, and Lactose-primed MODE-K supernatants led to a decreased release of IL-12p70. TSLP and IL-4 produced by IECs promote DCs to produce high levels of IL-12p70 in response to CD40 ligand stimulation (Watanabe et al., 2015). Hence the observed decrease in IL-12p70 by MO-treated cell-free supernatants may involve a reduction in TSLP and/or IL-4 release by IECs. These results demonstrate that changes in DC phenotype are the result of direct contact with MOs, and functional changes are possible through direct as well as MO-primed EC communication.

Several groups have confirmed the expression of CD23 on human intestinal epithelial cells (Li et al., 2007; Kaiserlian et al., 1994; Yang et al., 2000; Kaiserlian et al., 1993) including

the T84 cell line (Li et al., 2006). CD23 functions as a luminal antigen-sampling mechanism, which transports intact IgE-allergen complexes across the IEC barrier. Emerging evidence now exists on the involvement of CD23 in increasing gut permeability and worsening the allergic response by increasing antigen influx when activated by IgE-Ag complexes. In addition to regulating gut permeability through CD23, IECs act as sentinels that transduce signals from the external environment to the mucosal immune system through production of cytokines and chemokines (Li et al., 2007; Eckmann et al., 1994). Tying the function of CD23 and IECs' ability to communicate via secretory messengers, Li et al. (2007) demonstrated that IgE-Ag complexes activate CD23 to trigger IEC release of IL-8 and CCL20 *in vitro*. To study the effects of MOs specifically in relation to allergy, this method of IEC stimulation was adapted from Li et al. (2007). Hence, we investigated the potential ability of MOs 6'SL and 2'FL to impact chemokine and cytokine release in CD23 activated T84 cells. Consistent with Li et al. (2007), our results showed that IgE-Ag stimulated cells release a significantly higher amount of IL-8, CCL20, as well as IL-33. Interestingly, when exposed to 6'SL at high concentrations, the release of these chemokines and cytokine is attenuated, with IL-8 and CCL20 decreasing substantially to almost baseline levels, and IL-33 decreasing, but to a smaller extent. 2'FL, on the other hand, led to a decrease in CCL-20 production only. IL-8 is a chemo-attractant for neutrophils and eosinophils, and is upregulated in the nasal and skin mucosal regions upon allergen encounter (Zweiman et al., 1997; Erin et al., 2005). CCL20 recruits DCs, T cells and B cells bearing the CCL20 receptor CCR6 to the lamina propria under inflammatory conditions. IL-33 plays a crucial role in allergic inflammation; it is produced by IECs in

response to allergen and induces Th2-type inflammatory responses (Kamekura et al., 2012). Hence the inhibition of these chemokines and cytokine by 6'SL shed light on the immunosuppressive function of 6'SL, which renders ECs less prone to allergic stimulation.

Having determined the effect of certain oligosaccharides on DCs and IECs, we next investigated the mechanism of action used by MOs to elicit these responses. A specialized family of lectin receptors found on DCs, ECs and other immune cells are the sialic-acid binding immunoglobulin-type lectin (Siglec) receptors. Siglecs are cell surface transmembrane receptors that regulate function of innate and adaptive immune cells by recognizing soluble sialylated glycans and binding them with specificity (Macauley et al., 2014). One Siglec found on murine DCs, Siglec-F (the isofunctional paralogue of Siglec-8 on human immune cells), can modulate TLR-induced cytokine responses *in vitro*. Since 6'SL is a sialic acid containing unconjugated glycan, we hypothesized that the observed effect of 6'SL and possibly other oligosaccharides may be exerted through Siglec-F. Our results however show that inhibition of Siglec-F does not alter the effect of 6'SL or 2'FL on DCs. Nevertheless, the effect of 6'SL and other sialylated oligosaccharides may be mediated by other Siglecs such as Siglec-E and Siglec-G (mouse orthologues for human Siglecs-9 and -10, respectively), which are also found abundantly on DCs (Macauley et al., 2014). Hence, possible involvement of these receptors on MO-mediated immune responses requires further investigation.

Various studies demonstrate the role of peroxisome proliferator-activated receptor gamma (PPAR γ) in modulating immune responses and inflammation in the gut (Hontecillas & Bassaganya-Riera, 2007; Mohapatra et al., 2010). PPAR γ is highly expressed on immune

cells; it acts as a positive regulator in DC differentiation and is known to play a significant role in down-regulating expression of inflammatory cytokines in ECs (Mohapatra et al., 2010). Interestingly, Zenhom et al. (2011) demonstrated that the sialylated oligosaccharide 3'SL as well as fructooligosaccharides (FOSs) induce a reduction in proinflammatory cytokines via the activation of PPAR γ in IECs, even in the absence of commensal bacteria. We therefore investigated the involvement of PPAR γ in the anti-inflammatory and immunosuppressive effects exerted by specific MOs on DCs and ECs, using GW9662, a potent and specific PPAR γ inhibitor.

As discussed previously, 6'SL had an enhancing effect on IL-10 release and an attenuating effect on IL-12p70 release upon CpG- and LPS- induction of DCs. Interestingly, blocking of PPAR γ with GW9662 resulted in the reversal of these 6'SL-mediated effects, with a decrease in CpG-induced IL-10, and increase in CpG- and LPS-induced IL-12p70, in comparison to unblocked controls. These data suggest that 6'SL exerts its immunomodulatory effects on DCs via this receptor. DCs treated with 6'SL present a tolerogenic phenotype that may be important in driving T cell differentiation, a concept that requires study.

In addition to mediating the effects of 6'SL on DCs, we investigated the involvement of PPAR γ on MO-driven EC function. ECs treated with 6'SL yielded a significant dose-dependent decrease in IgE-Ag stimulated IL-8, and this effect was attenuated when ECs were pre-treated with GW9662. Similar effects were found for IL-33 release, but these results were not statistically significant. Interestingly, the decrease in CCL20 remained unaltered despite addition of the inhibitor, suggesting that a separate mechanism driving

the effect of 6'SL exists. Nonetheless, these data demonstrate that specific prebiotic oligosaccharides such as 6'SL have direct immunosuppressive and anti-inflammatory effects, which are in part mediated by PPAR γ .

NF κ B is a central regulatory transcription factor responsible for immunomodulation in epithelial cells; activation of PPAR γ results in the inhibition of this transcription factor in a PGlyRP3-dependent manner (Hou et al., 2012). A study by Lin et al. (2014) found that sialyllated MOs (pooled and specifically, 3'SL) reduce NF κ B p65 subunit activation *in vitro*, and others have shown prebiotic oligosaccharides inhibit nuclear translocation of NF κ B, resulting in inhibition of proinflammatory cytokines (Lin et al., 2007; Zenhom et al., 2011; Lin et al., 2014). Hence, we next investigated the effects of 6'SL and 2'FL on NF κ B activation. We found that consistent with a reduction in pro-inflammatory cytokines, NF κ B activity is inhibited upon 6'SL treatment, but only at high concentrations. Interestingly, NF κ B activity was reduced in 2'FL treated cells at all concentrations, suggesting that the effect of 2'FL may involve cytokines and/or proteins not considered in this study. Looking at other target genes of NF κ B such as IL-6, IL-13 and IL-17 may shed light onto the effect of 2'FL on NF κ B-mediated immune responses. In addition, we found that inhibition of PPAR γ results in ameliorated activation of p65 upon 6'SL treatment, further supporting our finding that 6'SL exerts its effects through a pathway involving PPAR γ . However, this was true at only high concentration of the MO, which are likely not present at physiological conditions. Alternatively, other regulators of IL-8 such as AP1 may be involved in driving the effect of 6'SL.

Previously, we have shown that oral administration of 6'SL and 2'FL reduce allergic symptoms in a mouse model of OVA-induced allergy (Castillo et al., 2015). In addition, fucosylated oligosaccharides such as 2'FL are able to reduce colon motor contractions *ex vivo* (Bienenstock et al., 2013). Collectively, current and past results suggest that the effects of different MOs are exerted via distinct pathways; while 6'SL may exert direct immunomodulatory effects in the gut, 2'FL may exert its effect by a separate mechanism involving the ENS.

Although present, it is important to note that the large majority of effects exerted by MOs in this study were present only at high concentrations of the sugars (1.0 mg/mL – 10 mg/mL). Previous studies investigating the effects of MOs in cell-based assays regard 15 mg/mL to be an optimal concentration for determining biological effects (Lin et al., 2014). However, while the average concentration of MOs in human milk is 12-14 g/L, no evidence to date exists on the concentration of MOs present in the gut of the breast-fed infant. In fact, indirect evidence suggests an absorption rate of approximately 1%. Hence, it is difficult to predict whether concentrations of MOs yielding direct effects in this study are physiologically relevant in the neonate.

CONCLUSION

In conclusion, this study demonstrates that prebiotic oligosaccharides may have direct anti-inflammatory or immunosuppressive effects on dendritic and epithelial cells by modulating

their phenotype and/or function. Although some effects were measured with 2'fucosylactose and lacto-N-neotetraose treatment, effects were strongest and most consistent for 6'sialyllactose. This alludes to the importance of structure specificity of these diverse group of prebiotic substrates. In addition, our results propose the importance of 6'SL as a dietary nutrient, which may modulate the inflammatory response in IgE-mediated allergy. Lastly, our findings suggest that milk oligosaccharides can exert direct effects on immune cells via the NFκB pathway and more specifically, 6'SL does so in a PPARγ-dependent manner.

To further elucidate the mechanism(s) responsible for the effects observed in this study, other regulatory transcription factors and cytokines involved may be studied. In addition, given the vital role of dendritic cells in establishing oral tolerance and immunity, future studies may focus on investigating the ability of milk oligosaccharide treated-DCs to drive T cell differentiation *in vitro*. Such studies will determine whether MOs' effects are limited to shaping the innate immune responses of the gut, or they play a role in driving adaptive immunity in live animals.

Currently, conflicting evidence exists on the involvement of MOs in conferring protective effects in the neonate, which may be attributed to the differential profile and abundance of MOs produced by individual women. Studies investigating the effects of isolated MOs are therefore crucial, as they clearly demonstrate the potential of these sugars as therapeutic agents for allergic diseases.

SUPPLEMENTARY

<p>%-increase = $\frac{\text{cytokine measurement (pg/mL) upon stimulation and/or treatment}}{\text{cytokine measurement (pg/mL) without stimulation and treatment}} \times 100$</p>
<p>Example:</p> <p><i>Given:</i></p> <p>Baseline IL-8 measurement (without stimulation and treatment) = 1520.820 pg/mL</p> <p>IL-8 measurement with IgE-Ag stimulation = 3591.209 pg/mL</p> <p>IL-8 measurement with IgE-Ag stimulation and 10 mg/mL 6'SL = 1829.043 pg/mL</p> <p>Calculations:</p> <p>A) Baseline measurements set to 100%-increase</p> <p>$\frac{1520.820 \text{ pg/mL}}{1520.820 \text{ pg/mL}} \times 100\% = 100\text{-increase}$</p> <p>B) IgE-Ag stimulated IL-8 release</p> <p>$\frac{3591.209 \text{ pg/mL}}{1520.820 \text{ pg/mL}} \times 100\% = 236.14\text{-increase}$</p> <p>C) IgE-Ag stimulated, 6'SL treated IL-8 release</p> <p>$\frac{1829.043 \text{ pg/mL}}{1520.820 \text{ pg/mL}} \times 100\% = 120.27\text{-increase}$</p>
<p>Sample explanation: Upon IgE-Ag stimulation of T84 cells, a 236.14%-increase in IL-8 was measured; treatment of cells with 6'SL attenuated this effect to 120.27%-increase.</p>
<p><i>Note: All measurements are relative to the baseline release for each cytokine in each experiment.</i></p>

Table S1: Sample calculation for %-increase used in this study.

SACCHARIDE CHARACTERISTICS

	% Purity	Endotoxin EU/mg
Lactose	99 %	0.002
6'Sialyllactose (6'SL)	96.6 %	0.496
2'Fucosyllactose (2'FL)	95.3 %	0.375
3'Sialyllactose (3'SL)	97.1%	0.015
Lacto-N-neotetraose (LNnT)	95.2%	0.340

Table S2: Characteristics of specific saccharides used in study.

DENDRITIC CELLS

	6'SL	2'FL	3'SL	LNnT	Lactose
IL-10 expression	↑	---	---	---	n/a
HO-1 expression	↑	---	---	---	n/a
IL-10 release	↑	n/a	n/a	---	n/a
IL-12p70 release	↓	n/a	n/a	---	n/a
TNF-α release	---	n/a	n/a	---	n/a
<u>Siglec-F Ab Effects</u>					
IL-10 expression	---	↑	n/a	n/a	n/a
HO-1 expression	---	---	n/a	n/a	n/a
IL-10 release	---	---	n/a	n/a	n/a
IL-12p70	---	---	n/a	n/a	n/a
<u>GW9662 Effects</u>					
IL-10 release	---	---	n/a	n/a	n/a
IL-12p70	↑	---	n/a	n/a	n/a

Table S3: Summary of effects found on bone marrow-derived DCs in this study. For full results, including means, SEM and p values, please see RESULTS.

- ↑** = statistically significant increase at some concentrations
- ↓** = statistically significant decrease at some concentrations
- = results not statistically significant at all tested concentrations
- n/a = not applicable (not studied here)

EPITHELIAL CELLS

	6'SL	2'FL	3'SL	LNnT	Lactose
TNF-α Stimulation					
T84 IL-8 release	↓	---	---	↓	↓
MODE-K KC release	↓	---	n/a	n/a	---
IgE-Ag Stimulation					
IL-8	↓	---	n/a	n/a	n/a
CCL20 release	↓	↓	n/a	n/a	n/a
IL-33 release	---	---	n/a	n/a	n/a
NF κ B activity	↓	↓	n/a	n/a	n/a
GW9662 Effects					
IL-8	↑	---	n/a	n/a	n/a
CCL20	---	---	n/a	n/a	n/a
IL-33	---	---	n/a	n/a	n/a
NF κ B activity	↑	---	n/a	n/a	n/a

Table S4: Summary of effects found on ECs in this study. For full results, including means, SEM and p values, please see RESULTS.

↑ = statistically significant increase at some concentrations

↓ = statistically significant decrease at some concentrations

--- = results not statistically significant at all tested concentrations

n/a = not applicable (not studied here)

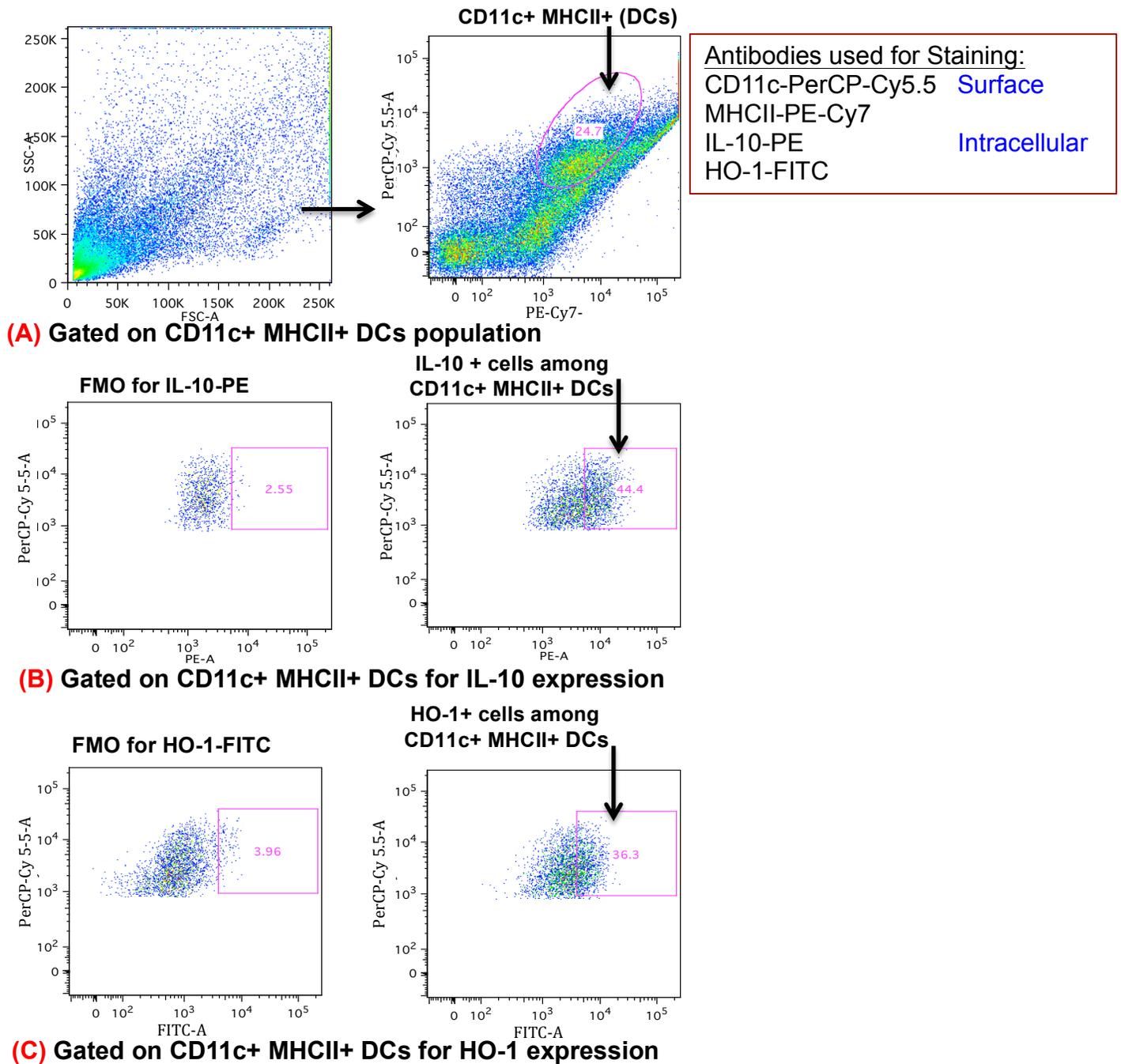


Figure S1: Gating-strategy for (A) CD11c+MHCII+ DCs (B) CD11c+MHCII+ DCs expressing IL-10 and (C) CD11c+MHCII+ DCs expressing HO-1. Strategy and antibodies used remained consistent between experiments, with fluorochrome-conjugations changing between experiments based on availability.

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