A novel method of generating Dendritic cells *in vitro* using the KG-1 cell line and its use as a model for testing effects of Lactic acid bacteria.

By

Parimala Vidya

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ABSTRACT

Dendritic cells (DCs) are prime mediators of innate and adaptive immunity. In humans the DC population comprise only 0.1% of all leukocytes, making their isolation and *ex vivo* manipulation difficult. Since study of DC activity *in vitro* requires large numbers of DCs to be readily available, a cell line model, KG-1, was selected. KG-1 cells are a cytokine-responsive human CD34+ myelomonocytic cell line and can be induced to differentiate to a DC phenotype. A range of differentiation agents and protocols were compared, and differentiation efficiency was determined using both morphological features and cell surface marker expression. Expression of CD83, CD11c, CD123, CD86, HLA-DR and DC-SIGN was assessed by immunofluorescence and flow cytometry. KG-1 cells stimulated with 10 ng/ml PMA and 100 ng/ml Ionomycin were found to be the ideal model for obtaining Dendritic Like Cells (DLCs) *in vitro*. The effect of lactic acid bacteria on KG-1 differentiation was also tested using two immunomodulatory strains, *Lactobacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052. After 5 days of incubation with R0011 the KG-1 cells expressed DC-specific surface markers CD83, CD86, CD11c, CD123, DC-SIGN and HLA-DR. *Lactobacillus rhamnosus* R0011 induced a marked rise in CD83 expression with a mean fluorescence intensity of 115.3 after 5 days, suggesting this strain promoted KG-1 differentiation to DLC. Analysis of cytokine by KG-1 DLC indicated that constitutive production of pro-inflammatory cytokines TNF-α and IL-12 was minimal. However IL-10 and TGF-β were detected after TLR-agonist stimulation of R0011-differentiated KG-1s. This study aimed to develop and assess the KG-1 cell model for screening effects of mediators and microbes on DC.

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LIST OF ABBREVIATIONS

APC	Antigen Presenting Cells
ATCC	American Type Culture Collection
ATRA	all trans Retinoic Acid
CFU	Colony Forming Unit
CD	Cluster of Differentiation
CTL	Cytotoxic T Lymphocytes
DC	Dendritic cell
FBS	Fetal bovine Serum
EDTA	Ethylene diaminetetraacetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
GALT	Gut Associated Lymphoid Tissue
GM-CSF	Granulocyte Macrophage Colony Stimulating Factor
HRP	Horseradish peroxidase
Ig	Immunoglobulin
IL-10	Interleukin-10
IL-12	Interleukin-12
LAB	Lactic acid bacteria
LPS	Lipopolysaccharide
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex

NF-κB	Nuclear factor kappa-light-chain-enhancer of B cells
PBS	Phosphate-Buffer Saline
PAMPs	Pathogen Associated Molecular Patterns
PMA	Phorbol-12-myristate 13-acetate
PP DCs	Peyer's patch dendritic cell
RANK	Receptor Activated Nuclear Factor-κB
RANKL	Receptor Activated Nuclear Factor-κB Ligand
R0011	*Lactobacillus rhamnosus*, strain R0011
R0052	*Lactobacillus helveticus*, strain R0052
IFN-α	Interferon alpha
TNF-α	Tumour Necrosis Factor alpha
TLR	Toll-like receptor
Treg	Regulatory T cell
TRAIL	Tumour necrosis factor- related apoptosis- inducing ligand
TGFβ1	Transforming growth factor β1
TMB	3,3',5,5' – tetramethylbenzidine

INTRODUCTION

The immune response

The Immune system is comprised of various organs, cells and proteins and other mediators that aid the host to fend off infections. Immune responses are mediated through immune cells that can recognize the invading microorganisms and initiate secretion of chemokines and cytokines that further aid in eliminating infectious agents. Infectious agents may be viruses, bacteria, fungi, protozoa or parasitic worms.

The immune response is classified into two types, either innate or adaptive immune responses. The innate immune response is an ancient host immune defence mechanism which is immediate and nonspecific. Innate immunity provides front line defence against pathogens that can cause disease and tissue damage. The innate immune system distinguishes to some extent between self and non-self and responds to both pathogenic and non-pathogenic microorganisms. It also triggers and optimizes the adaptive immune response (Janeway, 2005; Pier, 2004). The innate immune defence system utilizes varied mechanisms which work towards preventing infections. Non-specific components include epithelial surfaces of the body which act as barriers to invasion by microorganisms, antimicrobial peptides, secreted proteins (cytokines, chemokines, plasma proteins and proteins of complement system) and circulating and localized phagocytic cells (macrophages, dendritic cells and neutrophils). Infectious disease often occurs when microorganisms can evade or overwhelm the innate immune response to establish a local infection and then replicate further to allow dissemination in the body. Inflammatory responses are elicited when pathogens spread and are countered

by recruiting effector cells and molecules of the innate immune system from local blood vessels. The rapidly induced innate mechanisms can occur over several days during which adaptive immune system prepares itself to mediate highly specific responses towards pathogens delivered to local lymphoid tissue by Dendritic cells (DCs). The adaptive immune system mechanism clears infectious agents by targeting structures specific to pathogens and has the unique property of developing immunological memory (Janeway, 2005). Innate immunity is rapid, recognizes a broad class of pathogens and can interact with a range of molecular structures. The cells of the innate immune system possess receptors known as pathogen recognition receptors (PRRs) and recognize pathogen-associated molecular patterns (PAMPs) present on microorganisms. Adaptive immunity on the other hand is not inherited in the genome of the organism and requires time for its onset. Key cells of the adaptive immune system are T and B cells which use highly specific receptors. These receptors are encoded in multiple gene segments and undergo a process of gene rearrangement early in B and T cell maturation. The final T-cell receptor (TCR) and B-cell receptor (BCR) are generated through the gene rearrangement process producing lymphocytes with unique and highly specific antigen receptors. Contact of a lymphocyte and TCR or BCR with appropriate antigen leads to lymphocyte activation, provided the appropriate co-stimulatory signals are present. T cells recognize antigens presented by class I and class II MHC molecules, carried on professional APC, which include macrophages and DCs. This mechanism allows discrimination between closely related molecular structures (Janeway, 2005).

Toll-like receptors (TLRs): History and functions.

TLRs were first studied in *Drosophila* in 1988 during embryogenic polarity gene screening (Fernando, 1999). It was also seen that tobacco plants develop resistance against Tobacco Mosaic virus due to the N gene product. The N protein bears homology to the *Drosophila* toll and mammalian IL-1 receptor intracellular domains. This phenomenon was also observed in the *Arabidopsis* RPP5 protein, which protects against the downy mildew pathogen (Meng, 1999). The activation of immune genes in D*rosophila* is dependent upon *Drosophila* Dif and Relish, which are the homologues of mammalian NF-κB. In *Drosophila* the Toll receptors bind to the adaptor tube which is a homolog of mammalian MyD88, a key intermediate in TLR-mediated intracellular signalling. The adaptor tube binds to kinase Pelle, a homologue of IRAK (Interleukin-1 receptor associated kinase) which further leads to transcription of genes that mediate the antimicrobial pathways.

Mechanism of TLR signalling during immune response.

The binding of microbial ligands to their specific TLRs results in homodimerization of the TLRs and recruits MyD88 and MAL to the receptor complex. This in turn recruits IRAK which then binds to Tollip (Toll interacting protein) in the cytoplasm. IRAK activates NF-κB-inducing kinase (NIK) or transforming growth factor-β-activated kinase (TAK-1) through the assistance of co-factor protein TRAF6 (Tumour necrosis factor receptor-associated factor 6). NIK or TAK-1 later phosphorylate IKK (Inhibitor of κB kinase) which in turn phosphorylates the protein inhibitor IκB. Phosphorylation ultimately leads to the degradation of IκB, leading to the release of transcription factor NF-κB which then translocates to the nucleus and causes gene

transcription leading to the production of cytokines (Pier, 2004; Takeda and Akeda, 2004). (Please see the Appendix for a diagram illustrating this pathway).

Types of TLRs

The TLRs constitute 11 different functional receptors that identify molecular patterns present in bacteria, fungi, viruses and parasites (Della-Bella *et al*., 2008). The TLRs bind to PAMPs on the microorganism thus facilitating their recognition by the DC. The various TLRs include TLR2 which recognizes Gram positive and mycobacterial PAMPs as well as bacterial lipopeptide, lipoteichoic acid and peptidoglycan. TLR3 is required for the recognition of double stranded viral RNA, TLR4 for lipopolysaccharide, a chief component of Gram negative outer membranes, and TLR5 recognizes flagellin, a subunit of flagella. TLR7 recognizes imiquimod and single stranded RNA which is a feature of viral infection and TLR9 recognizes bacterial DNA or unmethylated CpG DNA sequence of prokaryotes. Some TLR's work in combination. For example TLR1 together with TLR2 recognizes triacylated bacterial lipopeptide (Furrie *et al*., 2004). Another combination involves TLR2 together with TLR6 and TLR1 recognizing peptidoglycan, yeast cell wall zymosan and phenol soluble modulin (Ida *et al*., 2006; Pier, 2004; Lore *et al*., 2003).

Organs and cells of the immune system

The immune system is comprised of primary lymphoid organs (generative or central lymphoid organs) and secondary lymphoid organs (or peripheral lymphoid organs). The primary lymphoid organs are bone marrow and thymus, and the secondary

lymphoid organs are the spleen, lymph nodes, and mucosa associated lymphoid tissue (MALT). MALT includes GALT: the mucosal barrier composed of the epithelial lining of the gastrointestinal tract as well as organized lymphoid tissue of the gastrointestinal tract such as Peyer's patches (PPs) and dispersed lymphoid cells within the epithelium and lamina propria (Piers, 2004). Cells involved in immune mechanisms are from precursors in the bone marrow. During hematopoesis, bone marrow derived stem cells differentiate into either mature immune cells or precursor cells which further differentiate into mature cells in other organs of the body. Stem cells give rise to both lymphoid and myeloid progenitor cells. The lymphoid stem cells give rise to T-cells, B-cells and natural killer cells. The myeloid stem cells give rise to neutrophils, eosinophils, basophils, mast cells and also monocytes. Monocytes further give rise to dendritic cells and macrophages (Janeway, 2005).

Dendritic cells

Dendritic cells (DC) are now known to be the major antigen presenting cells (APC) of the immune system. DC arises from DC precursors from hematopoietic progenitor cells (HPC) in the bone marrow (Paul, 2003; Stephens *et al*, 2008; St Louis *et al*., 1999). The DC precursors are monocytes and plasmacytoid cells which are also known as pre-DC and pre-DC2 respectively (Paul, 2003). DCs are present in blood and secondary lymphoid organ and are also found localized in skin (Langerhan cells), lungs, intestine and GALT (Gut associated lymphoid tissue). They were identified for the first time in peripheral lymphoid organs of mice by Steinmen and colleagues in 1972 (Steinman and Cohn, 1973). DCs belong to the innate immune system and act as front

line sentinels of host defence against microbial invasion. They initiate and control the adaptive immune response by stimulating T and B cells of the immune system and presenting antigens to T cells (Ida *et al*., 2006). T cell receptors (TCRs) recognize antigens presented by Major Histocompatibility Complex (MHC) proteins on DCs. Presentation of antigens by class I MHC stimulates cytotoxic T lymphocyte (CTL) activity, which causes the destruction of virus infected cells. Presentation of antigen by MHC class II molecule stimulates helper T cell (Th) activity which results in immune regulatory and activating effects (Satthaporn and Eremin, 2001).

DC may be classified as either immature DC (iDC) or mature DC. iDCs are found scattered in nonlymphoid organs throughout the body and are unable to stimulate T cells. iDCs can take up the antigens at the peripheral sites and then migrate to the T cell areas of the secondary lymphoid organs where they undergo maturation, process the phagocytised antigen and present it using MHC class I and MHC class II proteins and so stimulate naive $CD4^+$ T cells and $CD8^+$ cytotoxic T cells (Satthaporn and Eremin, 2001; Sallusto and Lanzavecchia, 1994).

A novel cell line model used to generate DCs *in vitro*

DCs are found as a minute population in blood and this makes large scale studies laborious as they are difficult to isolate from peripheral blood. Study of DC from other locations in humans such as the GALT, can be even more challenging due to difficulty in obtaining appropriate tissue. Adding a further difficulty to this, DCs can be easily damaged in the isolation process. Therefore a new cell line model using KG-1

cells would be useful to circumvent this problem. KG-1 cells are a cytokine-responsive human $CD34^+$ myelomonocytic cell line derived from bone marrow aspirate of a patient with erythroleukemia which later evolved into acute myelogenous leukemia (Koeffler, H.P and Goldie, D.W, 1978; St.Louis *et al*., 1999). On stimulation with certain agents KG-1 cells have been reported to differentiate into DLCs (Dendritic like cells) which are characterized by production of "dendrites" or "neurites" and also by expression of DC specific markers on their cell surface (St Louis *et al.*, 1999; Teobald *et al*., 2008; Berges *et al*., 2005; Hulette *et al*., 2001; Bharadwaj *et al*., 2005).

Dendritic cell surface markers and DC lineage

Cluster of differentiation or CD numbers are used to designate the presence of specific molecules on the surface of cells within the immune system. These cell surface markers are often identified by specific antibodies and help to identify the cell types, their differentiation stage and even the activity of the cell. The study of the cell surface markers present on DCs provides a basis for comparison with the markers that are produced on differentiated KG-1s. The major cell surface markers found on DCs are CD83, CD11c, CD123, CD86, HLA-DR, DC-SIGN (Dendritic cell Specific Intercellular adhesion molecule 3 (ICAM-3)-Grabbing Non integrin and CD80 (St. Louis *et al.*,1999; Della Bella *et al*., 2008).

Although the precise function of CD83 is unknown it is thought to be a mature DC specific marker that plays a role in antigen presentation and or lymphocyte activation. Structurally it is a membrane of the immunoglobulin super family and is made up of a

single chain transmembrane glycoprotein (43KD) (St. Louis *et al*., 1999; Della Bella *et al*., 2008; Lechmann. *et al*., 2001). CD11c is found on myeloid DCs (mDCs) as well as Natural Killer cells (NK cells), B and T cell subsets and is involved in adhesion and cytotoxic T lymphocyte (CTL) associated killing. It is a 145-150KD type I transmembrane glycoprotein. The ligands for CD11c are CD54, fibrinogen, iC3b, ICAM-1 and ICAM-4 (St.Louis *et al*., 1999; Kurts *et al*., 2008).

CD123 is a plasmacytoid DC (pDC)-specific marker and is the Interleukin-3 receptor. The protein IL-3 receptor is a dimer made up of an α sub unit and a signal-transducing β subunit which together bind to IL-3 with high affinity. Structurally CD123 is made up of only the 70 KD transmembrane α chain of the IL-3 receptor (St. Louis *et al*., 1999; Ida *et al*., 2005; Della Bella *et al.*, 2008). CD209, also known as DC-SIGN, are molecules expressed on the surface of mDCs. CD209 binds to ICAM-3, ICAM-2 and Butyrophilin (BTN2A1) and mediates dendritic cell migration and T cell proliferation. CD209 is a receptor for HIV-1, West Nile virus, hepatitis C, and certain bacteria and parasites. It plays a role in capture and internalization of these pathogens. CD209 is a 44 KD type II transmembrane glycoprotein and a member of the C-type lectin family (St. Louis *et al*., 1999; van Kooyk, 2003).

HLA-DR (MHC class II) is a marker of professional APCs and its expression increases with DC activation. HLA-DR is required for peptide presentation to $CD4^+$ T cells. It is a heterodimeric cell surface glycoprotein made up of 36KD α (heavy chain) and a 27KD β (light) chain (St. Louis *et al*., 1999). CD80 (B7-I) and CD86 (B7-2) are

co-stimulatory molecules expressed by APCs. They play a critical role in T-cell activation by binding to CD28 on the T cell surface. CD86 is involved in immunoglobulin class switching and triggering NK cell mediated cytotoxicity (St. Louis *et al*., 1999; Ackerman and Cresswell, 2003).

The DC lineage

Dendritic cells and monocytes are derived from a common precursor cell referred to as the macrophage and dendritic cell precursor or MDP (Laffont and Powrie, 2009). MDPs differentiate into common DC precursors (CDPs), which further differentiate into DC subsets. DCs can also be derived from monocytes under inflammatory conditions and in certain tissue locations, such as intestinal tissues. Human blood DCs can be grouped into either mDC or pDC types. The mDC are HLA-DR+ (MHC class II), $CD11c^{high}$ and $CD123^{low}$ (Ida *et al*., 2006; Masten *et al*., 2006). They are found as immature cells in skin and mucus membranes. On exposure to a "danger signal" such as an invading pathogen, they mature and migrate to the local draining lymph node. Within the lymph node they process and present antigen to T cells and express co-stimulatory molecules CD80 and CD86 to activate T cells specific for the antigen presented. mDCs produce the cytokines IL-12 and TNF-α (Ida *et al*., 2006). pDC are important in rapid responses to viral infections and are characterized as $HLA\text{-}DR^{+}$, $CD11c^{low}$ and $CD123^{high}$. They produce IFN-α and TNF-α in large amounts (Ida *et al*., 2006), and also produce a variety of chemokines. mDCs and pDCs differ in their response to TLR agonists. mDC respond to theTLR4 agonist Lipopolysaccharide (LPS)

and TLR3 agonist Poly (I:C) while pDCs respond to the TLR7 agonist imiquimod and the TLR9 agonist CpG oligonucleotide (Della-Bella *et al*., 2008).

Table 1: Cell Surface markers present on mDCs and pDCs.

DC lineage	Cell surface markers	Cytokines produced	Response to TLR-agonist
Myeloid DC (mDC)	HLA-DR+ (MHC class II) CD11c high, CD 123 low	IL-12 and TNF-α	LPS (TLR4) and poly (I:C) (TLR3)
Plasmacytoid DC (pDC)	HLA-DR+, CD11c low and CD123 high	IFN-α and TNF-α	TLR7 and CpG oligonucleotide (TLR9)

Roles of DC Cytokines

DCs produce cytokines upon stimulation with TLR-agonists and other stimuli. The chief cytokines produced are IL-12, TNF-α, IL-10, TGF-β and IFN-α. Determination of the cytokine profile produced by DC helps in determining the DC subset or lineage.

IL-12 is a proinflammatory cytokine that plays a role in both innate and adaptive immunity (Watford *et al*., 2003). It is naturally produced by epithelial cells, dendritic cells, macrophages and B cells during infection. IL-12 is a multimer linked by disulphide bonds, forming a p70 heterodimer which consists of p35 and p40 subunits. p40 is a dominant effector molecule produced by epithelial cells. IL-12 binds to IL-12 receptors found on T cells and NK cells. IL-12 plays a critical role in the Th1 response

leading to Th1 cell differentiation from Th0 cells and promotion of Th1-associated activities, including macrophage activation (Paul, 2003; Ashman *et al.*, 2011).

TNF-α was identified in 1975 and has an important role in immune system development, inflammation and apoptosis. It is produced by DC, mast cells, monocytes, macrophages, T and B lymphocytes, NK cells, neutrophils, endothelial cells, smooth and cardiac muscle cells, fibroblasts and osteoclasts (Bradley, 2008). TNF-α is also involved in cell differentiation and cell proliferation. TNF-α triggers signal transduction by binding to receptors TNFR1 and TNFR2, resulting in active NF-κB entering the nucleus of the cell, initiating transcription of several genes associated with inflammation (Bradley, 2008; Paul, 2003). DCs and T cells express TNF family members RANK (Receptor Activator of Nuclear Factor-κB) and RANKL (ReceptorActivator of Nuclear factor-κB Ligand) respectively. RANK is a receptor of RANKL.DCs, after antigen presentation to and activation of naive T cells, are eliminated by apoptosis through TNF family molecules TRAIL(Tumour necrosis factor-related apoptosis- inducing ligand), FasL and TNF-α produced by activated T cells. This is done to prevent excessive T cell activation by mature DCs. Failure of DC elimination is observed in patients with autoimmune lymphoproliferativesyndrome (ALPS) which is an inherited autoimmune disease of lymphocyte homeostasis (Leibbrandt, 2010).

IFN-α is involved in innate immunity and is chiefly produced by pDC during viral infections. IFN-α is synthesised when intracellular MyD88 is activated following ligand binding to TLR 7, 8 or 9. Activated MyD88 in turn induces antiviral responses

by forming a complex with transcription factor IRF7. The death domain of MyD88 interacts with the inhibitor domain of IRF7, resulting in the activation of IFN-α dependent promoters. IRF7 also binds TRAF6 leading to INF-α synthesis (Sato *et al*., 2004). IFN-α is then released to induce protective anti-viral defences in neighbouring cells, such as induction of RNA activated protein kinase. As IFN-α is produced only by pDCs and not mDCs, its identification either in the culture supernatant or within the cell helps in classifying the *in vitro* derived DLCs as belonging to the pDC lineage.

IL-10 is an 18.4KDa regulatory cytokine that down regulates the synthesis of pro-inflammatory cytokines including IL-2, IL-3, TNF-α, IFN-γ and GM-CSF. It is secreted by DCs, $CD4^+CD25^+Foxp3^+$ Treg cells in the gut mucosa, B cells, monocytes and keratinocytes (Siddiqui and Powrie, 2008; Zeuthen *et al.*, 2006). IL-10 plays a major role in inhibiting Th1 function and favours Th2 development (Paul, 2003).

TGF-β is a key regulatory molecule having effects on a variety of cells which include monocytes, T_{reg} cells, B cells, NK cells, macrophages, epithelial cells. It is an anti-inflammatory cytokine endogenously secreted by GALT DCs which drive Treg cell differentiation from naive T cells in the GALT (Siddiqui and Powrie, 2008). TGF-β is also involved in B cell class switching to IgA. TGF-β has three isoforms: TGF-β1, TGF-β2 and TGF-β3 with TGF-β1 being the most prominent among them. TGF-β is secreted in latent form and needs to be liberated from the latency associated protein, LAP and latency TGF-β binding protein, LTBP, to release biologically active TGF-β (Li *et al.*, 2006).

Other DC subsets

Although conventionally DCs were classified based on their origin as either being myeloid or lymphoid, DCs are also classified based on the tissue where they reside steadily. Using location as reference point, DCs have been classified as either interstitial or tissue-derived DC and blood or lymphoid-derived DC (Adorini *et al.*, 2009). The tissue-derived DCs are skin DC, thymus DC, lung DC, hepatic DC, spleen DC, Peyer's patches DC (PP DCs), Mesenteric Lymph node DC (MLN DCs) and DCs of the intestinal tract. In the intestine two subtypes of DCs were identified: $CD103^+$ CX_3CR1^- DC, which arise from pre-dendritic cells found in bone marrow and require growth factor flt3 for their development, and the monocyte-derived $CD103^+$ CX_3CR1^+ DC which depend on growth factor M-CSF (macrophage colony stimulating factor) for their development (Laffont and Powrie, 2009). In addition there are many more DC subsets that have been identified. For example cDC are postulated to originate directly from lymphoid progenitors or upon viral induced activation of immature pDC. This cDC subtype was found to be similar to the conventional DC originally identified by Steinman and Cohn. cDC are powerful stimulators of T cells and are of two subtypes: $CD8^-$ ($CD11b^+$, myeloid) and $CD8^+$ ($CD103^+$ in tissues) (Reizis, 2011).

Another subtype of DC identified were tolerogenic DC (tDC) which originate from immature DC (iDC). tDc can be either derived naturally from hematopoietic precursors or may be induced from other DCs having received instructive signals that lead them towards their tolerogenic phenotype. tDCs are induced during disease which may be due to pathogens or due to tumour formation. Additionally it was also seen that

T_{reg} cells induced tDC by a mechanism termed as "infectious tolerance". tDC can be produced by experimental manipulation using biomolecules like TGF-β, Hepatocyte Growth Factor (HGF) and vitamin D3 metabolites, by using immunosuppressive drugs prednisolone or dexamethasone or by genetic manipulation (Maldonado and von Andrian, 2010).

GALT and the Dendritic cell

The intestinal tract of mammals is constantly exposed to the external environment and can therefore be easily colonized and invaded by viruses, bacteria, fungi and parasites. Immature DCs are localized in the mucosa of the small intestinal tract where they reside in the PPs, lamina propria and the draining MLNs making up the GALT (Smith and Nagler-Anderson, 2005). PPs are overlaid with special cells known as M (microfold) cells that can sample antigens and microorganisms in the gut lumen. These M cells deliver antigen by transcytosis to the subepithelial dome that contains antigen presenting cells including DC. Intestinal DC include $CD103^+$ $CX3CR1^-$ DC, which are derived from pre-dendritic cells in response to the growth factor Flt3, and $CD103^-$ $CX3CR1^+$ DC, which develop from monocytes in response to M-CSF (Laffont and Powrie, 2009). CX3CR1 is a chemokine receptor, and $CX3CR1^+$DCs in the lamina propria of the small intestine extend their dendrites between epithelial tight junctions to directly sample the luminal contents (Artis, 2008; Mohaamadzadeh *et al., 2*008) facilitating their role as APCs that act as principle stimulators of naive T-helper cells, and potentially playing a role in local tissue inflammatory responses. In contrast, $CD103^+CX3CR1^-$ DC are thought to regulate the generation of T_{reg} cells and

immunoglobulin A (IgA)-producing B cells through the production of the cytokines TGF-β and IL-10 which strongly contribute to immune homeostasis (Zeuthen *et al.*, 2005; Laffront and Powrie, 2009). IgA production by the immune cells in the gut is a result of microbial colonization in the intestine**.** The human intestine can secrete 3 grams of secretory IgA (SIgA) in the intestinal lumen each day. The major sites for IgA generation are the PPs which develop during embryogenesis and the isolated lymphoid follicles (ILFs) that develop following bacterial colonization early after birth (Suzuki and Fagarasan, 2008). SIgA acts as a barrier for the invasion of both intestinal microbiota and pathogens. PPs are the major sites for IgA production and contain germinal centers (GC) which are special microenvironments allowing interactions between the B cells, DC and follicular T helper cells. Interaction between these cells activates B cell proliferation and expression of activation-induced cytidine deaminase (AID) which is a critical molecule required for class switch recombination (CSR) from IgM to IgA. PP DCs are $CD11b^+$ $CD11c^+$ $CCR6^+$ whereas those within the T cell rich region and the MLNs are $CD11c^+$ $CD8\alpha^+$ $CCR7^+$. PP DCs are important in controlling the mucosa-tropism of lymphocytes by induction of homing molecules α4β7 integrin and CCR9, a chemokine receptor, on antigen primed B and T lymphocytes, through production of retinoic acid from vitamin A (Takahashi *et al.*, 2009).

Studies have shown that IgA CSR is regulated by inducible nitric oxide synthase (iNOS) which is expressed by PP DCs. iNOS production is induced in PP DCs by TLRs recognizing commensal bacteria. iNOS controls T-cell dependent IgA CSR by TGF-β whereas T-cell- independent IgA CSR is regulated by APRIL (A Proliferating

Inducing Ligand) and BAFF (B-cell-Activating Factor), which can be produced by DCs as well as by IECs (Mohamadzadeh *et al*, 2008). In summary, intestinal DCs are now believed to play key roles not only in antigen presentation to T cells, but also in directing development of regulatory T cells (Treg) and in supporting IgA class switching in the MALT.

Role of DCs in intestinal homeostasis

PP DCs produce large amounts of IL-10, an immunomodulatory cytokine that plays a role in preventing autoimmunity. Lamina propria DCs take up antigen (which may include commensal bacteria and apoptotic intestinal epithelial cells in addition to antigen from infectious agents) to Mesenteric lymph nodes (MLNs). DCs that have migrated to MLNs or those located in the PPs interact with B and T cells and initiate anti-inflammatory activity in the intestine due to IL-10 production. Intestinal DCs promote the development of forkhead box P3 $(FOXP3)^{+}$ regulatory T cells (T_{reg} cells) from naive T cells thereby diverting T cells that may strongly react to the antigen to a T cell that is tolerogenic in its nature. The induction of FOXP3 expression by naive T cells is mediated by TGF-β produced by intestinal DCs. FOXP3 production is also dependent upon retinoic acid synthesis by DC. It has been noted that IL-10, TGF-β and retinoic acid are involved in preventing autoimmune diseases (Coombes and Powrie, 2008).

Gastrointestinal microbiota, and their interaction with immune system

The mammalian intestine is a home to a vast and diverse commensal microbiota which in return promote angiogenesis, development of intestinal epithelium and

protection against tissue injury. They also aid in digestion, absorption and storage of nutrients, especially the plant-derived nutrients that would be otherwise inaccessible to the host. In addition the gut microbiota prevents colonization by pathogens by competing with them for nutrients and an environmental niche in the intestine. They also secrete antimicrobial peptides that protect the host against pathogens. "Dysbiosis" is defined as a change in the composition of the commensal microbiota in the intestine which may lead to Irritable Bowel Syndrome (IBD) and necrotizing enterocolitis (Artis, 2008). It is known that the immune cells of the gut are influenced by the stimuli coming from the commensal microbiota. Intestinal homeostasis is a mechanism by which the immune system discriminates between pathogens and the intestinal microbiota. Pathogens in the intestinal mucosa elicit an immune response, while the commensal microbiota tends to promote IgA production and regulatory activity. Any imbalance in this mucosal immune system may lead to problems such as food allergies and Crohn's disease which are characterized by inappropriate immune response to food antigens and intestinal bacteria respectively (Zeuthen *et al.*, 2005; Vanderpool *et al*., 2008).

The Hygiene hypothesis suggests that if there is an altered exposure to bacteria during establishment of the intestinal microbiota during infancy there is development of an altered immune response. In a closely related concept "Old friends" are harmless microorganisms that are present throughout mammalian evolution. Frequent contact with "old friends" is hypothesized to lessen incidence of allergies (Neish, 2008). Probiotics may act as "old friends" through their effects on the immune system.

Recent evidence suggests that probiotics of the *Lactobacillus* group regulate DC expression and cytokine production. DCs are the chief stimulators of naive T- helper cells and are involved in the maintenance of the delicate balance between Th1and Th2 cells. The development of Th1 and Th2 response is influenced by DC production of cytokines or chemokines. It has been reported that probiotic stimulation of DCs induced production of cytokines IL-10, IL-12 and TGF-β, resulting in generation of T_{reg} cells and IgA producing B cells (Zeuthen *et al.,* 2006; Neish, 2009; Walker, 2008). IL-10 is also produced by Treg, and suppresses IL-12 activity, preventing an inflammatory cascade of Th1 cytokines and cellular migration. IL-10 also prevents the activation of antigen presenting cells, inhibits DC maturation, T-cell proliferation, and induces a state of antigen-specific tolerance and T_{reg} cell induction (Drakes, *et al.,* 2003). Different species of *Lactobacilli* vary in their ability to induce cytokine production by DCs. Cytokine production patterns by DC also depend upon the concentration of the *Lactobacilli* species used. For example treatment with low concentrations of *Lactobacilli casei* induced the production of higher levels of IL-6, IL-12 and TNF-α but no IL-10 production by DC. *Lactobacilli reuteri* on the other hand down- regulated IL-12 and TNF-α production but induced the production of IL-10, suggesting that *L. reuteri* would have anti-inflammatory effects (Christensen *et al*., 2002).

Potential health benefits of probiotics

Probiotic is a term derived from the Greek word meaning “supporting or favouring life” and has been defined by World Health Organization (WHO) as live microorganisms which when administered in adequate amounts confer a health benefit on

the host." (Guarner & Schaafsma, 1998). Probiotics have been studied intensely in recent years as they may play an important role in influencing both humoral and cell-mediated immunity. The commonly used probiotics are *Lactobacillus, Bifidobacterium* and *Saccharomyces boulardii* in food. Species of lactic acid bacteria (LAB) that are chiefly associated with probiotic activity include *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus fermentum, Lactobacillus johnsonii, Lactobacillus paracasei, Lactobacillus plantarum, Lactobacillus reuteri, Lactobacillus rhamnosus, Lactobacillus salivarius, Lactobacillus lactis, Bifidobacterium lactis* and *Bifidobacterium longum* (Sanders, 1999).

The immunomodulatory effects associated with probiotics include strengthening of non-specific defence against infection and tumours, adjuvant effects in antigen specific immune responses and increase in secretory IgA production (Sanders, 2010). Most probiotics are Gram positive lactic acid bacteria and their cell walls are composed of a thick peptidoglycan layer interspersed with teichoic acids and polysaccharide. Exceptions include the *E. coli* Nissle strain, which are Gram negative bacteria, and *Saccharomyces boulardii,* a yeast. Upon entering the intestine, probiotics interact with intestinal epithelial cells (IECs) and DCs. Probiotics encounter DCs in the intestinal tract in two ways: Lamina propria DCs continuously sample the environment by passing their dendrites between IECs, and secondly, DC can interact with the bacteria which have reached the GALT through M cells. DCs recognize probiotics by the MAMPs (microorganism associated molecular pattern) found on the bacterial cell surface which interact with PRRs (pathogen recognition receptors), including TLRs expressed on DCs. In the case of certain Lactobacillus species, TLR2 is engaged (Lebeer *et al.*, 2010; Hart *et*

al., 2004). The various MAMPs found on probiotics include flagella, fimbriae (both in case of *E.coli Nissle* 1971 *and Lactobacillus rhamnosus* GG), secreted proteins (*Lactobacillus rhamnosus* GG p40 and p75 and *Lactobacillus johnsonii* EFTu and GroEL), glycan ligands (*Lactobacillus acidophilus* SlpA), capsular polysaccharide (*Lactobacillus casei Shirota)*, Lipoteichoic acid (*Lactobacillus plantarum*), Lipopolysaccharide, lipid A (*E.coli Nissle* 1917 hexa acyl lipid A) and peptidoglycan (*Lactobacillus plantarum* DAP-PG) (Lebeer *et al*., 2010). This diverse array of bacterial components serving as MAMPs suggest that there are multiple ways in which probiotics can interact with DCs and influence the immune response.

Probiotics are also reported to aid in physiological functions such as lactose digestion and prevention of colonization by pathogenic bacteria by varied mechanisms. Some of the mechanisms include secreting bacteriocins, adhesins, lactic acid, QS (Quorum Sensing) signals, short chain fatty acids (butyric acid, acetic acid and propionic acid) and decrease pH. They induce increased mucin production in the intestine thereby interfering with pathogen attachment to intestinal epithelial cells. They also enhance tight junction functioning and prevent apoptosis (Lebeer *et al.*, 2010). It has also been suggested that probiotics may exert anti-colon cancer effects by binding to mutagens or by de-activating carcinogens, through inhibition of carcinogen producing enzymes (beta-glucuronidase and nitroreductase) produced by colonic microbes (Sanders, 1999). Certain probiotics produce colonization resistance inhibitors such as hydrogen peroxide and biosurfactants thereby preventing colonization by urogenital pathogens. Probiotics have also been reported to be involved in reducing blood lipids by assimilation of cholesterol

and increased excretion of bile salts due to deconjugation mediated by a hydrolase enzyme. Certain strains may are also be involved in anti-hypertensive effect mediated by the enzyme peptidases which act on milk proteins, yielding tripeptides which in turn inhibit the angiotensin 1 convertingenzyme (ACE). Studies have demonstrated that *Lactobacillus helveticus* is capable of releasing anti-hypertensive peptides from the milk protein casein such as ACE inhibitory tripeptides Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) (Narva *et al.,* 2004). Furthermore the cell wall components of certain probiotics have been reported to inhibit activity of the angiotensin converting enzyme (ACE), suggesting multiple mechanisms for ACE inhibition by probiotics in foods (Lye *et al*., 2009). Probiotics may also influence effects of dietary components such as soy. Studies have shown that probiotic bacteria can hydrolyze isoflavone glucosides into bioactive aglycone form which are absorbed faster and in greater amounts than in natural glucosides in soy milk (Lye *et al*., 2009**).** Phytoestrogens are isoflavones which are structurally similar to estrogens and can bind to estrogen receptors they are sometimes used by women for hormone replacement (Sanders, 1999).

Differentiation of KG-1 cells into DLCs

KG-1 cells can be differentiated into DLCs using phorbol myristate acetate (PMA) or a combination of PMA + Ionomycin or PMA + TNF-α (St.Louis *et al*., 1999; Teobald *et al.*, 2008; Berges *et al*., 2005). Other agents that can be used to induce differentiation are GM-CSF (Granulocyte macrophage colony stimulating factor) and TNF-α. KG-1 cells grow in Iscove's medium and appear as round non-adherent cells. After stimulation with PMA they appear as large, loosely adherent cells with irregular

shapes, prominent dendritic processes and hair-like cytoplasmic projections. This morphological characteristic is very much similar to *in vivo* DC and helps in their identification. Differentiation can be further confirmed by assessing expression of DC-specific cell surface molecules (St. Louis *et al.*, 1999; Della Bella *et al*., 2008).

Mechanism of action of the differentiation agents

KG-1 cells can be differentiated into DLCs by the combination of PMA and Ionomycin (St. Louis *et al*., 1999; Ackerman and Cresswell, 2008; Teobald *et al*., 2008). PMA acts as an analogue of diacyl glycerol and activates the enzyme Protein Kinase C. This in turn regulates different signal transduction pathways and other cellular metabolic activities (Goel *et al*., 2007). Protein Kinase C activation causes morphological changes in KG-1 cells by inducing surface molecule expression and results in allostimulatory capacity for T-cells (St.Louis et al., 1999; Hulette *et al*., 2001; Berges *et al*, 2005). PMA stimulation of KG-1 cells involves activation of the NF-κB transcription family through up regulation of Re1B. Nuclear localization of Re1B results in increased antigen presentation cell activity in both KG-1 cells and human DCs thus implying that PMA adopts a similar pathway in both of these cells (St.Louis *et al*., 1999; Berges *et al*., 2005). Ionomycin is a calcium ionophore, and a polyether antibiotic produced by *Streptomyces congloblastus* ATCC 31005. Ionomycin is involved in the Ca^{++}/calmodulin signalling pathway and also in induction of hydrolysis of phosphoinositides, and can act in synergy with PMA in inducing cell activation (Chatila *et al*., 1989).

Retinoic acid or vitamin A is essential for normal immune function and is involved in both innate and adaptive immunity. It is required to maintain balance of Th 1 and Th2 responses **(**Massacand *et al.*,2008**).** Without vitamin A the responses are skewed towards the Th1 response and antigen presenting cells have been shown to be the target of retinoic acid in the restoration of Th2 response (Hengesbach and Hoag, 2004). Retinoic acid receptors (RARs) are the main targets for regulating myelopoiesis and are required for granulocyte development. Retinoic acid binds to RARs present on DNA which in turn bind to Retinoid X Receptor elements (RXRE) found in promoter regions, leading to either induction or repression of transcription of genes responsible for differentiation of various immune cells (Coombes and Powrie, 2008; Duester, 2008). When treated with retinyl acetate and 13-*cis* retinoic acid, mice show enlargement of lymphoid tissues due to an increase in numbers of macrophages and DCs. Studies have indicated that retinoic acid also promotes mDC differentiation and their migration to the small intestine (Hengesbach and Hoag, 2004).

Lactobacillus species as a KG-1 differentiation agent

Although *Lactobacillus* strains were never tested for their differentiation capacity on KG-1s, it is known that they possess immunomodulatory and immunostimulatory capacity based on work done using peripheral blood DCs (Hart *et al.*, 2003; Christensen *et al*., 2002). It was noted that the bacterial capacity to stimulate production of IL-10, IL-12, TNF-α and the amount of cell surface markers by human monocyte-derived DC not only varied among the different bacterial genera but also varied among the various species of Lactobacillus tested (Zeuthen *et al*., 2006). It should

also be noted that there are relatively few studies addressing effects of lactobacilli on DC, in spite of the increasing evidence for the impact of the gut microbiota on intestinal DC. The KG-1 model provides a potential system to examine effects of lactobacilli, and by extension gut microbes, on DC differentiation.

OBJECTIVES AND STUDY DESIGN

In humans the DC population composes only 0.1% of all leukocytes thus making their isolation and *ex vivo* manipulation difficult. Since many studies of DCs require large numbers of readily available DCs, a cell line model using KG-1 was selected. The overall objective of this study was to examine the utility of an *in vitro* Dendritic cell (DC) system derived from the KG-1 cell line and to utilize this model for testing effects of selected lactic acid bacteria on dendritic cells.

Specific objectives were as follows:

1. Determine the optimal stimuli and conditions necessary to differentiate KG-1 cells into DLCs using various combinations of differentiation agents

2. To determine the cell surface markers expressed by KG-1 DLCs in comparison with the cell surface markers expressed by undifferentiated KG-1's.

3. Determine effects of selected probiotics (*Lactobacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052) on KG-1 differentiation to the DC phenotype.

4. Determine and measure the types of cytokines produced by undifferentiated KG-1s and KG-1 DLCs to further determine the DC lineage.

5. Determine and measure the types of cytokines produced by DLCs obtained by PMA + Ionomycin differentiation after TLR-agonist stimulation, using TLR

agonists best suited for mDC and pDC activation (Poly (I:C) (TLR3 agonist), Lipopolysaccharide (TLR4 agonist), Flagellin (TLR5 agonist), Imiquimod (TLR7 agonist) and unmethylated CpG DNA (TLR9 agonist).

6. To determine the effects of probiotics on KG-1 DLC DC responses to TLR-agonists by measuring effects on cytokine production.

MATERIALS AND METHODS

Cell culturing

KG-1 cells obtained from the American Type Culture Collection (ATCC) were maintained in Iscove's Modified Dulbecco's Medium (IMDM) (Invitrogen Inc.) supplemented with 20% Fetal Bovine Serum (FBS, Sigma-Aldrich Canada), and 0.05 mg/ml Gentamicin. Cells were grown in 75 cm^2 tissue culture flasks (Cellstar, Greiner Bio-one Mississauga, Ontario). Cultures were maintained in a humidified incubator at 37°C in 5% CO_2 (Thermo Corporation, Toronto). Sub-culturing of the cells was performed every 2-3 days.

Preparation of Lactobacillus strains

Lactobacillus rhamnosus (strain R0011) and *Lactobacillus helveticus* (strain R0052) were obtained from the Institut Rosell (Lallemand Inc; Montreal, Quebec) as industrially packed lyophilized cultures, at defined CFU/g. Bacteria were reconstituted by resuspending an aliquot of lyophilate in 20 ml of Phosphate buffer (PBS) and centrifuging at 3000 xg for 20 minutes to remove extraneous materials. After centrifugation the supernatant was removed carefully and the pellet resuspended in PBS and centrifuged again two times adopting the same procedure. Finally the pellet obtained was suspended in 5ml PBS and the number of colony forming units/ml determined by serial dilution and plating on de Man, Rogosa and Sharpe (MRS) agar. The concentrations of *Lactobacillus* used for differentiation were 2×10^8 CFU/ml and 1×10^8 CFU/ml.

Differentiation of KG-1s to Dendritic Like Cells (DLCs*) in vitro*

Several differentiation agents and their combinations were used to induce KG-1 cells to differentiate into DLCs, including Phorbol myristate acetate (PMA), Ionomycin, Tumour Necrosis Factor α (TNF-α), all trans Retinoic Acid (atRA). KG-1cells were cultured with differentiation agents at an initial concentration of 5 x 10^4cells/ml (determined using Guava Viacount) unless otherwise specified. All tests of differentiation agents were compared with controls which contained un-stimulated KG-1s cultured under the same conditions and concentration, and all tests were performed in 6 well tissue culture plates (BD Falcon). To determine the efficacy of PMA as a differentiation agent, PMA (Sigma-Aldrich, Catalogue #1585) was tested for effects on KG-1 differentiation over a range of concentrations (10 ng/ml, 15 ng/ml, 20 ng/ml, 30 ng/ml PMA) over a period of 7 days. After incubation, KG-1 cells were observed under an inverted light microscope for production of "neurite" or "dendritic" processes.

The differentiated cells were identified as DLCs using direct immunofluorescence and flow cytometry (using a Guava Personal Cell Analysis or Guava PCA) to measure cell surface marker expression. In addition, KG-1 cells (at a concentration of 5 x10^4 cells/ml) were stimulated with the combination of PMA at 10 ng/ml + Ionomycin at 100 ng/ml (Sigma-Aldrich, Canada) for 3 or 5 days. Additional testing of PMA and Ionomycin at these concentrations with KG-1 cells at 8 x10^4 cells/ml for 5 days was also carried out, again observing morphological changes and cell surface marker expression. The effect of PMA and Ionomycin was also tested at a higher concentration (20 ng/ml PMA+ 200 ng/ml Ionomycin) for 7 days. The combination of 10

ng/ml PMA + 10 ng/ml TNF-α (Sigma-Aldrich) was also tested, observing KG-1 differentiation over 7 days. all trans Retinoic acid (atRA) (Sigma Aldrich; Catalogue #R2625) was tested at a concentration of 1μM (Darmanin *et al*., 2007), observing effects on KG-1 differentiation over 5 days. All preparation of atRA was performed in subdued light as atRA is highly light sensitive. A stock solution of atRA at 0.001M stores for two weeks without deterioration in absolute ethanol at -70ºC. Stock solutions in absolute ethanol were dispensed in dark colour vials (suitable for storing light sensitive materials), flushed with nitrogen gas for 10 minutes and stored at -70C. Samples for assay were withdrawn using a needle and syringe.

In addition, *Lactobacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052 (at concentrations of 2 x 10^8 CFU/ml and 1 x10^8 CFU/ml) were also tested for their KG-1 differentiation capacity. *Lactobacillus rhamnosus* R0011 at a concentration of 1 x10^8 CFU/ml was selected as the optimal strain and concentration, based on initial testing of effects on KG-1 viability.

Cell viability measurement

Cell viability was determined using the Guava Viacount assay, performed using a Millipore® Guava Personal Cell Analysis (PCA; Millipore, Billerica, MA, catalogue #0500-1090). The Viacount Reagent (Millipore, Catalogue #4000-0041) contains two proprietary DNA binding dyes, and differentially stains viable and non-viable cells based on their differential permability to a membrane-impermeant DNA binding dye. (Only non-viable cells are permeable to this dye.) A membrane-permeant dye in the Viacount

Reagent mixture stains all nucleated cells, whether alive or dead. The Guava PCA counts the stained nucleated events and then uses the forward light scatter (FSC) properties to distinguish free nuclei and cellular debris from cells to obtain an accurate viable count. Cytosoft™ Software was used for data acquisition and analysis by enabling the EasyFit™ feature. The EasyFit performs analysis in three dimensions and allows better discrimination of live cells, dead cells and debris when they overlap with each other. Cells were diluted 1/20 in Viacount Reagent and incubated for 5 minutes before analyzing the sample. The Guava PCA was calibrated before reading samples using the Guava Check software and Guava Check kit (Millipore, Catalogue # 4500-0020). Data was collected (1000-2000 events per sample) using the Guava ViaCount software and saved to an FCS 2.0 file and an Excel spread sheet file.

Immunofluorescence and flow cytometric analysis

Flow cytometry uses the hydrodynamic principle of passing fluorochrome-labelled cells through a laser beam to excite the fluorochrome, resulting in emission of photons. The scattered photons are collected by optical detectors and converted into electrical pulses. The most common optical detector is a photo multiplier tube (PMT) which collects electrical impulses and amplifies them. The amplified signals are then processed by an Analog to Digital Converter (ADC) into graphical signals that appear as histograms. One-parameter histograms were generated in this research with the x- axis depicting the parameter measured (Fluorescence Intensity) and the y-axis depicting the cell count.

Cell surface marker analysis was initially conducted on two kinds of samples. The first sample was drawn from the top of the well and was labelled as “non adherent” cells and the second type of sample was withdrawn from the bottom part of the well and labelled as “adherent” cells. The cells at the bottom of the well were harvested by detaching them with a solution of 1mM EDTA (Sigma Aldrich) + PBS for 10 minutes at 37°C in 5% CO_2. The detached cells were spun at 300xg for 10 minutes and the pellet was suspended in 1ml of cell staining buffer (2% FBS in PBS). The cells were counted by Guava Viacount and were later stained with PE-labelled anti-human antibodies or their isotype controls by incubating them in the dark for 30 minutes on ice. After incubation, the cells were spun at 300xg for 5 minutes and washed twice with 1ml of cell staining buffer. The final pellet was re-suspended in 0.5 ml cell staining buffer and placed on ice in the dark until analysis (3 to 5 minutes). The processed samples were analyzed by flow cytometry using the “Guava Express” program.

Monoclonal antibodies used for direct immunofluorescence were PE-labelled anti-Human CD83 (BioLegend, SanDiego, CA, Catalogue #305307), PE -labelled anti-Human CD11c (BioLegend, Catalogue #301605), PE-labelled anti-Human CD123 (BioLegend, Catalogue #306005), PE-labelled anti-Human CD86 (BioLegend, Catalogue #305406), PE labelled anti-Human HLA-DR (BioLegend, Catalogue #307605) or PE-labelled anti-Human CD80 (BioLegend, Catalogue #305307) and PE-labelled anti-Human DC-SIGN (BioLegend, Catalogue #343003). PE-labelled mouse IgG1, κ (BioLegend, Catalogue #400114), PE-labelled mouse IgG2b, κ (BioLegend, Catalogue #400314) and PE-labelled mouse IgG2a, κ (BioLegend, Catalogue #400211) were used

as isotype control antibodies, matched to the appropriate test antibodies. The Guava Express Assay identifies specific cell populations based on their expression of cell surface molecules. The software uses two fluorescence parameters in combination with forward angle light scatter (FSC) to identify cells with specific phenotypic markers. Fluorophore-conjugated antibodies are able to bind specific surface molecules on the cells, labelling them, and the Guava PCA can detect the intensity of fluorescence signals on individual cells as well as determining the number of cells labelled. The fluorophore used throughout for direct immunofluorescence was phycoerythrin (PE). Data including mean fluorescence intensity (MFI), percent coefficient of variance (CV%), and the resulting histograms generated were exported to FCS 3.0 files and saved in Excel spreadsheet files. Detailed analysis including use of the Overton subtraction for determination of percentage of cells staining positive and the MFI of selected cell populations was conducted using FCS Express version 3.0 software.

Table 2: Anti-Human antibodies required for identifying dendritic cells differentiated from KG-1 cells.

Cell surface markers	Markers Role	Antibodies specific for the cell surface marker	Corresponding isotype control antibody
CD83	Specific dendritic cell marker	PE anti-human CD83	Mouse IgG1, κ
CD80	Co-stimulatory molecule; ligand for CD28	PE anti-human CD80	Mouse IgG1, κ
CD86	Co-stimulatory molecule; ligand for CD28	PE anti-human CD86	Mouse IgG2b, κ
CD123	pDC (plasmacytoid DC marker)	PE anti-human CD123	Mouse IgG1, κ
CD11c	mDC marker (myeloid DC marker)	PE anti-human CD11c	Mouse IgG1, κ
CD209 (DC-SIGN) Dendritic Cell Specific Intercellular adhesion molecule 3 (ICAM-3)-Grabbing Nonintegrin	Myeloid dendritic cells	PE anti-human CD209 (DC-SIGN) Antibody	Mouse IgG2b, κ
HLA-DR (MHC class II)	Markers of APCs; increases with DC activation	PE-labelled anti HLA-DR (anti class II MHC)	Mouse IgG2a, κ

Determination of cytokine production profiles and kinetics

KG-1 DLCs obtained after differentiating KG-1 cells with the combination of PMA (10 ng/ml) and Ionomycin (100 ng/ml) were collected from the bottom of 6 well culture plates after treatment with 1mM EDTA+ PBS solution for 10 minutes at 37°C in 5% CO_2. The adherent cells from replicate wells were pooled, cells were washed by centrifugation, and viability and cell concentration measured using the Guava Viacount assay. The viability of KG-1 DLCs was typically 90%. Differentiated KG-1 DLCs were then cultured in 96 well tissue culture plates (Cellstar, Greiner Bio-One) to determine cytokine production profiles.

To determine cytokine production profiles, KG-1 DLCs were tested at concentrations of 1 $x10^6$ cells/ml and 2 $x10^6$ cells/ml, using a panel of TLR agonists. LPS (Sigma Aldrich, Catalogue #L2654), a TLR4 agonist, was tested at 0.1 µg/ml, 1 µg/ml and 10 µg/ml concentrations to determine the optimal dose for cytokine induction. The TLR3 agonist Poly (I:C) (Sigma Aldrich, Catalogue # P1530) was tested at concentrations of 100 µg/ml, 50 µg/ml, 25 µg/ml and 5 µg/ml. The TLR5 agonist Flagellin (Invivogen) was tested at concentrations of 10 ng/ml, 1 ng/ml and 0.1 ng/ml. The TLR7 agonist Imiquimod (Sigma Aldrich, Catalogue #I5159) was tested at concentrations 10 µg/ml, 5 µg/ml and 2.5 µg/ml and the TLR9 agonist unmethylated CpG DNA (Imgenex Crop.) was tested at concentrations of 20 µg/ml, 10 µg/ml and 5 µg/ml**.** KG-1 DLC were cultured with TLR agonists in a final volume of 200µL per well, in triplicates, and negative control cultures containing KG-1 DLCs (cultured without TLR agonists) were included in all tests. Cell culture supernatants were withdrawn after 4

hours and 16 hours by first centrifuging the 96 well plates and then transferring the supernatant to a fresh 96 well tissue culture plate. The plates were immediately frozen at -80°C until analysis for production of cytokines IL-12, TNF-α and IL-10 and TGF-β1 by ELISA. The plates used for TGF-β sample collection were treated with a silicon solution (Sigmacote, Sigma Chemical Cot) to prevent TGF-β attachment to the plastic surface. The same procedures were also used to determine cytokine production profiles of KG-1 DLCs obtained by differentiation with *Lactobacillus rhamnosus* R0011, and to determine the effects of R0011-induced differentiation on the KG-1 DLC response to TLR agonists.

Cytokine measurement by Enzyme Linked Immunosorbent Assay (ELISA)

All buffers and reagents were prepared and used in accordance with the manufacturers' protocols for IL-12 (p70) (BioLegend Inc, San Diego, California, Catalogue # 430201), TNF-α (BioLegend Inc, San Diego, California, Catalogue # 431701), IL-10 ELISA kits (Biosource International Inc, Camarillo, California, Catalogue # CHC1323) and TGF-β1 (R&D systems Inc, Minneapolis, USA, Catalogue # DY240). Polystyrene high binding, Microlon 600 ELISA plates (Greiner Bio-one, Catalogue # 655061) was used for all ELISA assays. Table 1 and 2 compares buffer compositions and protocols for each ELISA kit. A standard curve was conducted for each ELISA to allow accurate determination of the concentration of each cytokine in the samples tested. For measurement of IL-12(p70) and TNF-α, plates were coated with either anti-IL-12 capture antibodies or anti-TNFα capture antibodies as per manufacturer's protocol and plates incubated overnight at 4ºC. A blocking step was carried out using 1% BSA in PBS (Assay diluent) and incubating plates for 1 hour with

shaking at room temperature to block non-specific binding. Biotinylated anti-IL-12 detection antibody and the biotinylated anti-TNFα detection antibody were prepared according to manufacturers's protocol. Avidin-conjugated horseradish peroxidase (HRP) was used at a1:1000 dilution in assay buffer, and 1.8 N H_2SO_4 was used as a stop solution for all ELISAs.

The IL-10 ELISA was performed using anti-human IL-10 capture antibodies at a concentration of 1 µg/ml in coating Buffer, and anti-human IL-10 detection antibody at a concentration of 0.16 µg/ml in assay buffer. HRP-conjugated streptavidin was used at a 1/1250 dilution, and 1.8 N H_2SO_4 was used as a stop solution.

Human recombinant IL-12(p70), TNF-α, IL-10 and TGF-β1 were used as standards in the appropriate ELISAs and the plates were read at 450nm wavelength (reference wavelength of 650nm) using a plate reader (Bio-Tek Instrumentation, Nepean, Ontario). Data was collected with KC4 program for windows (Bio-Tek) and compiled using Microsoft® Excel® (Microsoft Corp., Redmond, Washington, U.S.A).

Statistical Analysis

Data are presented as mean ± Standard Error of the Mean (where n permits) and analyzed using a one-way analysis of variance (ANOVA). In case of ELISAs, each sample was tested in triplicate in each ELISA and assigned an average value. Therefore, a value represented as n = 1 is equivalent to a triplicate average for any single sample. For flow cytometry, 1000 to 2000 events were collected for each sample tested.

Table 3: Composition of assay solutions for TNF-α, IL-12(p70), IL-10 and TGF-β1 ELISA kits.

	TNF-α	**IL-12(p70)**	**IL-10**	**TGF-β1**
Solutions	Mass per Litre	Mass per litre	Mass per litre	Molar
Coating Buffer	8.4 g $NaHCO_3$, 3.56g Na_2CO_3, pH 9.5	8.4 g $NaHCO_3$, 3.56g Na_2CO_3, pH 9.5	8.0 g NaCl, 1.13g Na_2HPO_4, 0.2g KH_2PO_4 0.2g KCl, pH to 7.4	137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, 0.2 µm filtered
Assay Buffer	8.0g NaCL, 1.16g Na_2HPO_4, 0.2g KH_2PO_4, 0.2g KCL, 10% FBS, pH 7.4	8.0g NaCL, 1.16g Na_2HPO_4, 0.2g KH_2PO_4, 0.2g KCL, 10% FBS, pH 7.4	8.0 g NaCl, 1.13 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl, 5.0 g BSA, pH to 7.4	137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 1.5 mM KH_2PO_4 1.4% BSA, 0.05% Tween-20, pH 7.4
Wash Buffer	8.0g Nacl, 1.16g Na_2HPO_4, 0.2g KH_2PO_4, 0.2g KCL, 0.05% Tween-20, pH 7.4	8.0g Nacl, 1.16g Na_2HPO_4, 0.2g KH_2PO_4, 0.2g KCL, 0.05% Tween-20, pH 7.4	0.2g KH_2PO_4, 1.9g $K_2HPO_4.3H_2O$, 0.4g EDTA, 0.5ml Tween-20, pH 7.4	137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.05% Tween-20, pH 7.4
Substrate solution	TMB	TMB	1:1 H_2O_2:TMB	1:1 H_2O_2:TMB
Stop solution	1.8M H_2SO_4	2 N H_2SO_4	1.8M H_2SO_4 (Excess)	2N H_2SO_4 (Excess)

RESULTS

Effects of PMA on KG-1 differentiation

KG-1 cells treated with PMA were compared with controls containing unstimulated KG-1 cells incubated under identical condition for 7 days (Figure 1). KG-1 cells at a concentration of $5x10^4$ cells/ml treated with 10 ng/ml PMA for 7 days showed poor differentiation, as indicated by production of only 0-1 cells producing "neurite-like processes" per randomly selected field of vision as observed by microscopy. A representative field of view is shown (Figure 2). Other PMA concentrations (15 ng/ml and 20 ng/ml) tested on KG-1s for 7 days produced only 0-2 cells differentiated per randomly selected field of vision (Figure 5 and 6) and PMA at 30 ng/ml did not induce any improvement in "neurite- like process" production.

KG-1 cells treated with PMA at 10 ng/mL for 7 days showed lower expression of CD83 than did control cells (CD83 MFI = 43.9; control MFI = 80.5). After stimulation of KG-1 with 20 ng/ml PMA, CD83 expression was also lower (MFI = 47.4) compared to the control (MFI = 59.19) (Figure 10). This indicated that PMA at concentrations of 10 ng/ml and 20 ng/ml caused minimal KG-1 differentiation. Moreover, both the adherent and non-adherent cell populations from KG-1 cultures treated with 10 ng/ml PMA expressed low CD83 levels (MFI = 79.3 and 74.3 respectively). There was not much difference between the control MFI value and the test MFI of adherent and non-adherent cells for CD83 expression (Figure14).

Effects of the combination of PMA and TNF-α on KG-1 differentiation

KG-1s at a concentration of $5x10^4$ cells/ml treated with 10 ng/ml PMA+ 10 ng/ml TNF-α for 7 days showed 12-14 cells per randomly selected field of vision producing "neurite- like" processes, but there was also extensive cell death after 7 days of incubation. PMA and TNFα-treated KG-1 cell cultures were compared with control cultures containing unstimulated KG-1 cells (Figure 10 and 9). Analysis of cell surface molecule expression by KG-1's cultured at an initial concentration of $8x10^4$ cells/ml and differentiated by 10 ng/ml PMA+ 10 ng/ml TNF-α for 5 days showed lower MFI values for CD83 and CD86 expression than did non-differentiated KG-1 controls. This change in CD83 and CD86 expression was observed on both adherent and non-adherent cells indicating that at this concentration, treatment with PMA and TNF-α led to a decrease in CD83 and CD86 expression (Figure 17).

Effect of the combination of PMA and Ionomycin on KG-1 differentiation

KG-1 cells at a concentration of $5x10^4$ cells/ml treated with the combination of 10 ng/ml PMA + 100 ng/ml Ionomycin showed small "neurite-like" processes on 3-5 differentiated cells per randomly selected field of vision after 3 days of incubation (Figure 11). On extension of the incubation time for 5 days there was not much loss of cell viability. Again, controls containing unstimulated KG-1's were used for comparison throughout (Figure 12). Initial analysis of the whole KG-1 cell population indicated that this combination of differentiation agents induced an increase in CD83 and CD86 expression relative to untreated controls, and that expression of these cell surface molecules increased with time. PMA + Ionomycin-treated KG-1 cells had an MFI value

of 44.6 for CD83 expression after 3 days of incubation, which rose to 95.8 after 5 days of incubation. CD86 expression also increased, from an MFI of 58.9 on day 3 to an MFI of 106.4 on day 5 (Figure 15). Further analysis comparing cell surface molecule expression by the adherent versus non-adherent differentiated KG-1 cells revealed little difference between them, as MFI values for CD83 and CD86 were similar for both groups of cells, whether obtained from the top portion of the well, or from the bottom after removal with EDTA (Figure 16). When the KG-1 cell concentration was raised to $8x10^4$ cells/ml, there was an increase in CD83 expression by PMA + Ionomycin-treated KG-1 cells (MFI = 124.9) compared to control cells (MFI = 89.9). CD11c expression was also elevated on treated KG-1 cells (MFI = 426.2) compared to control cell CD11c expression (MFI = 155.2). CD123 showed a marked rise in expression while CD86 expression was decreased by this differentiation protocol. DC-SIGN and HLA-DR expression did not vary much between differentiated and control cells (Figure 18). (One way ANOVA; p = 0.04.)

KG-1's at a concentration of $5x10^4$ cells/ml treated with the combination of 20 ng/ml PMA and 200 ng/ml Ionomycin for 7 days showed extensive cell death (by microscopy), therefore this differentiation protocol was not further characterized.

Effects of all trans Retinoic Acid (atRA) as a KG-1 differentiation agent

Cell surface molecules expressed by KG-1cells 8 $x10^4$ cells/ml stimulated with atRA showed some increase in CD83 expression (MFI = 54.5) compared to control KG1 cells (MFI = 47.9) after 1 day of incubation (Figure 19). 3 days after atRA stimulation,

CD83 expression had increased to an MFI of 69.1, compared to the control cell MFI of 67.5, indicating that effects of atRA on CD83 expression were minimal. Expression of CD11c showed a statistically significant rise in response to atRA treatment, increasing from an MFI of 132.3 on control cells to an MFI of 231.2 on atRA-treated cells (One way ANOVA; $p < 0.0001$). CD123 expression was also significantly elevated, from an MFI of 79.7 on the control cells to an MFI of 146.5 on the atRA-treated KG-1 cells (Figure 20) (One way ANOVA; $p < 0.0001$). However, the cell yield was low and the viability of the atRA-treated KG-1 cells was 89.7% compared to the control sample viability of 92.7%.

Effects of *Lactobacillus rhamnosus* R0011 on KG-1 differentiation

Cell surface molecule expression was measured on adherent KG-1's after 5 days of treatment with *L. rhamnosus* R0011**.** CD83 expression was higher on strain R0011-treated cells than on control KG-1 cells, showing that KG-1s had differentiated into DLC's. Expression of CD123 was higher on R0011-treated cells than on control cells. However, expression of CD11c, CD86 and HLA-DR did not vary much on R0011-treated KG-1 cells relative to control cells. DC-SIGN expression was low on both control and R0011-treated KG-1 cells (One way ANOVA $p = 0.0001$.) (Figure 21).

Cytokine production profile of KG-1s and KG-1 DLCs

Undifferentiated KG-1 cells and PMA + Ionomycin-derived DLCs produced minimal amounts of cytokines IL-12 and TNF-α even when stimulated with TLR-agonists, whether measured at 6 or 24 hours after stimulation (Table 3). Although the standard curves for these ELISAs indicated they were performing optimally, levels of

these cytokines in the KG-1 media samples were often below the reliable detection limit. Cytokine production by KG-1 cells differentiated with *Lactobacillus rhamnosus* R0011 and treated with TLR-agonists was also minimal after 4 and 16 hrs (Table 4). Stimulation of R0011-differentiated KG-1s with either flagellin (at 10 ng/ml) or Poly I:C at 50 µg/ml) (Table 5) induced IL-10 production at 4 hours, with lower levels evident at 16 hours after TLR agonist addition. PMA+ Ionomycin-derived KG-1 DLC's produced almost no TNF-α and IL-12 when stimulated with TLR-agonists. PMA + Ionomycin-derived KG-1 DLCs showed a decrease in IL-12 and TNF-α production after 16 hours. Preliminary results indicated that stimulation with flagellin induced TGF-β1 production by both PMA + Ionomycin-differentiated KG-1s and strain R0011-differentiated KG-1 DLC (data not shown).

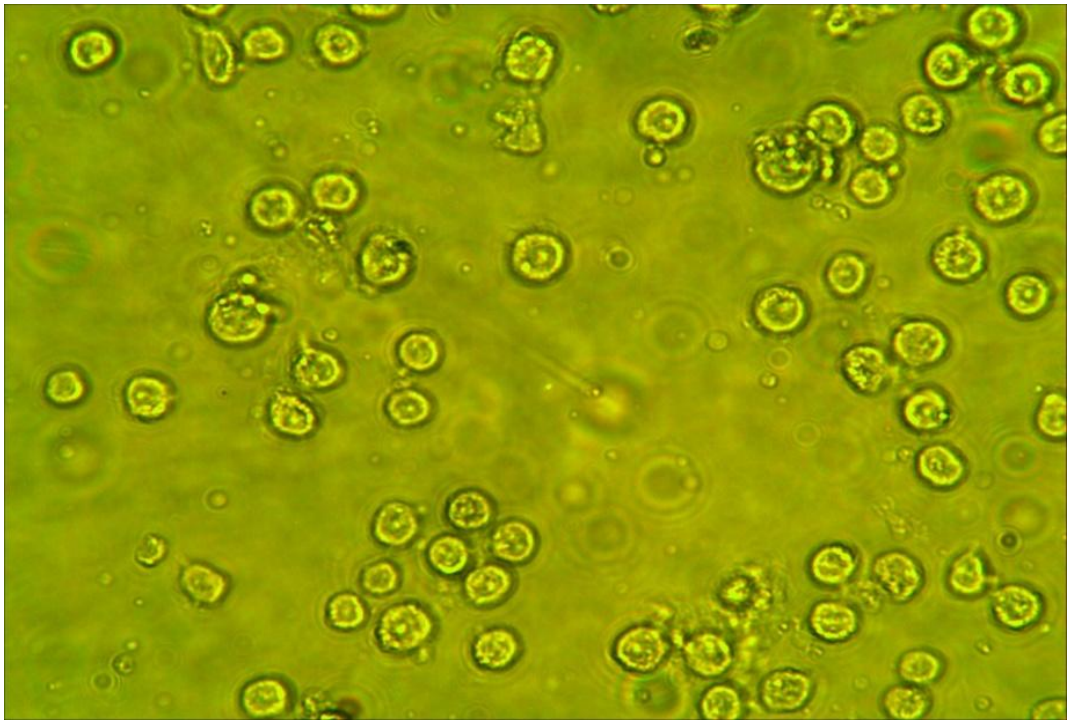

Figure 1. Control KG-1 cells after 7 days of incubation. Results obtained by light microscopic examination (40X magnification) of control samples containing KG-1 cells at a concentration of 5 x 10^4 cells/ml.

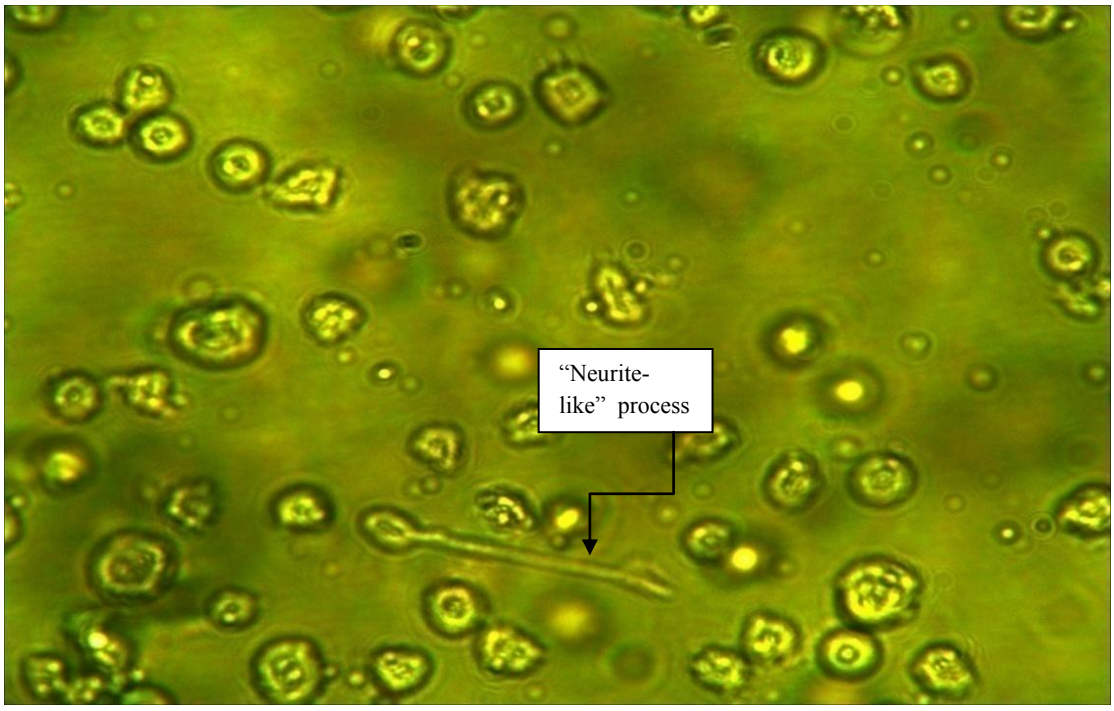

Figure 2. PMA-treated KG-1 cells after 7 days of incubation. KG-1 cells were seeded at a concentration of $5x10^4$ cells/ml and 10 ng/ml PMA. Production of distinct "neurite- like" processes was evident after 7 days by light microscopy at 40X magnification.

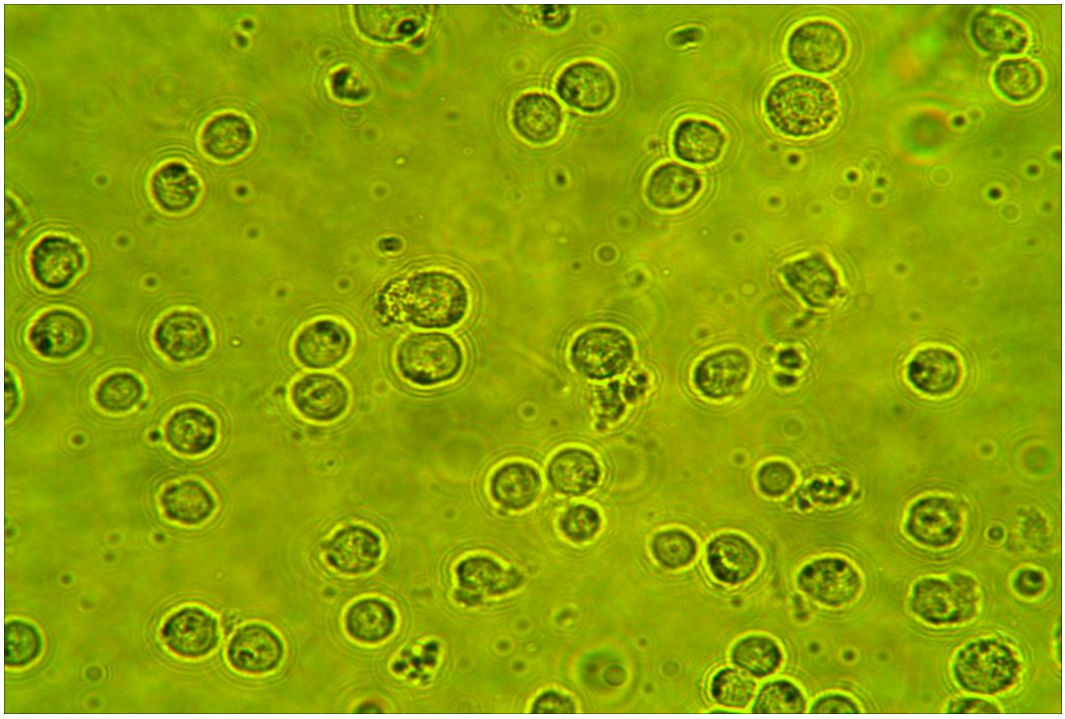

Figure 3. Control KG-1 cells. KG-1 cells seeded at a concentration of 5 x10^4 cells/ml after 7 days incubation (40X magnification).

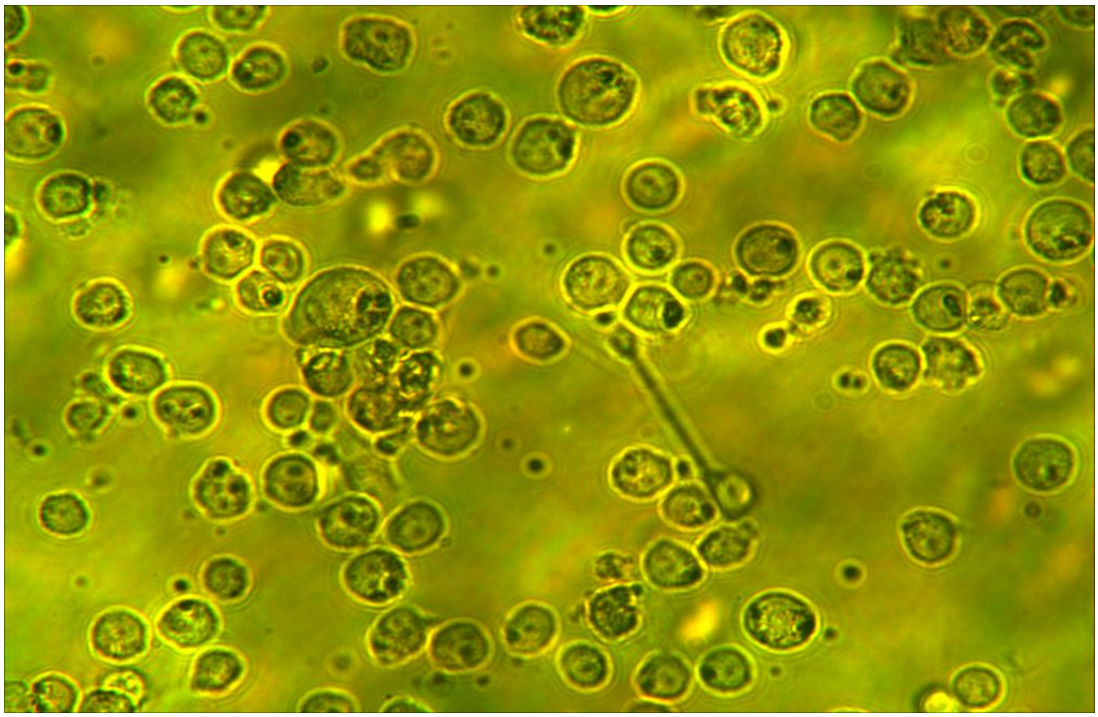

Figure 4. PMA-treated KG-1 cells after 7 days of incubation. KG-1 cells were seeded at an initial concentration of 5 x10^4 cells/ml with PMA at 10 ng/ml. Neurite-like extensions typical of Dendritic cell morphology were evident by light microscopy under 40X magnification.

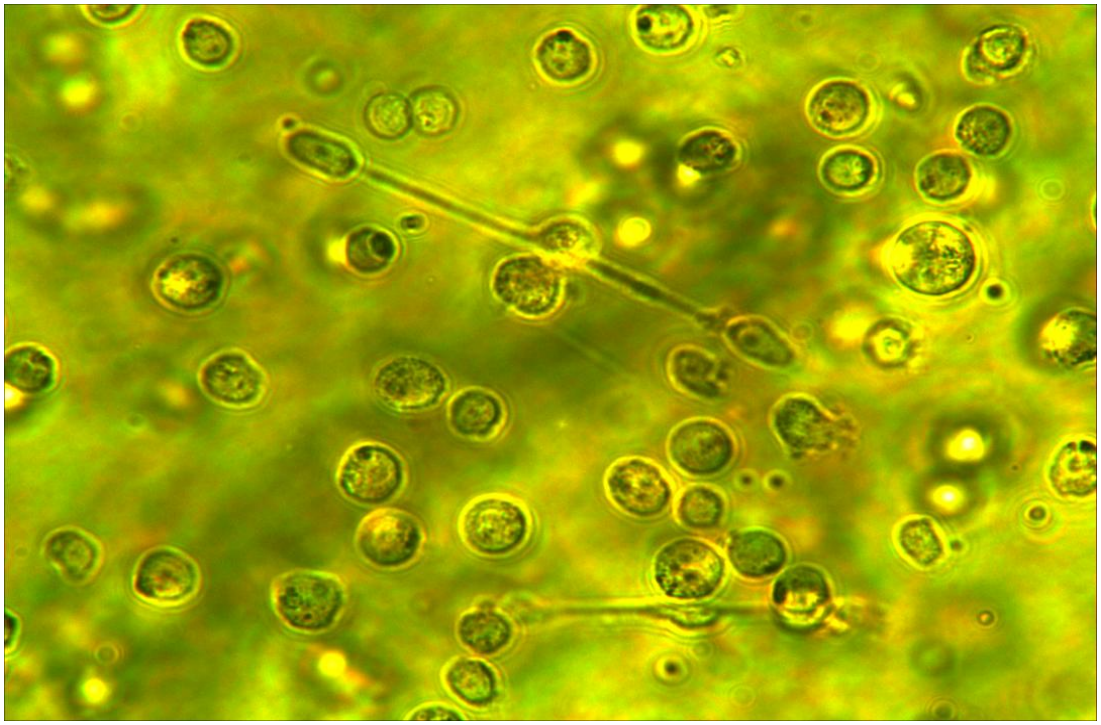

Figure 5. PMA-treated KG-1 cells seeded at a concentration of 5 x10^4 cells/ml and incubated with 15 ng/ml PMA. Production of "neurite-like" processes was observed by light microscopy under 40X magnification after incubation for 7 days.

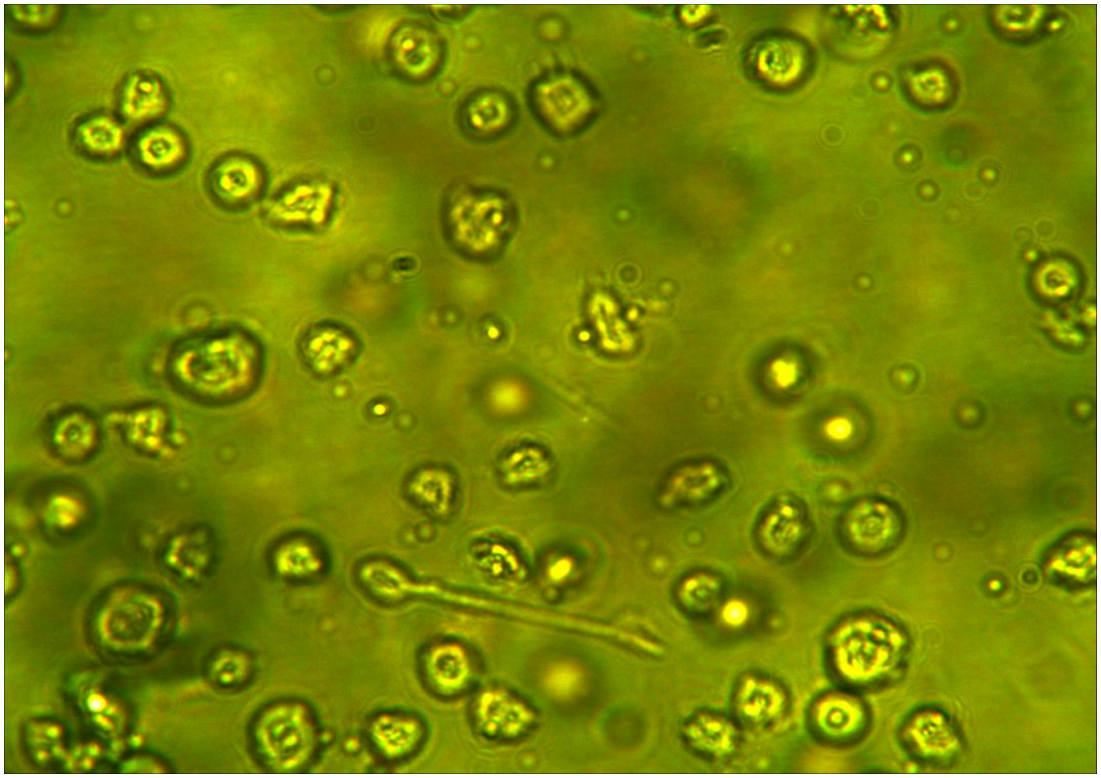

Figure 6. PMA-treated KG-1 cells seeded at a concentration of 5 x 10^4 cells/ml and incubated with 20 ng/ml PMA. Production of "neurite-like" processes was observed by light microscopy (40X magnification) after incubation for 7 days.

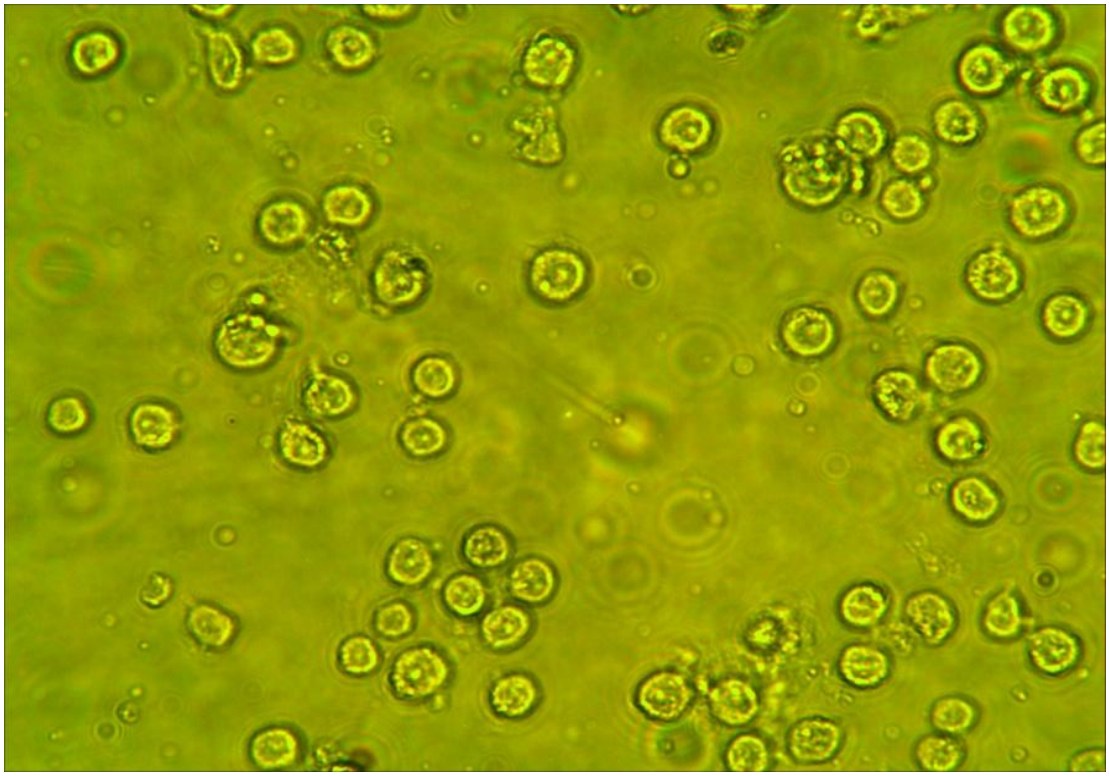

Figure 7: Control KG-1 cells. KG-1 cells at an initial concentration of 5 x10^4 cells/ml for 3 days used for comparison with PMA + TNF-α treated KG-1 cells (40X magnification).

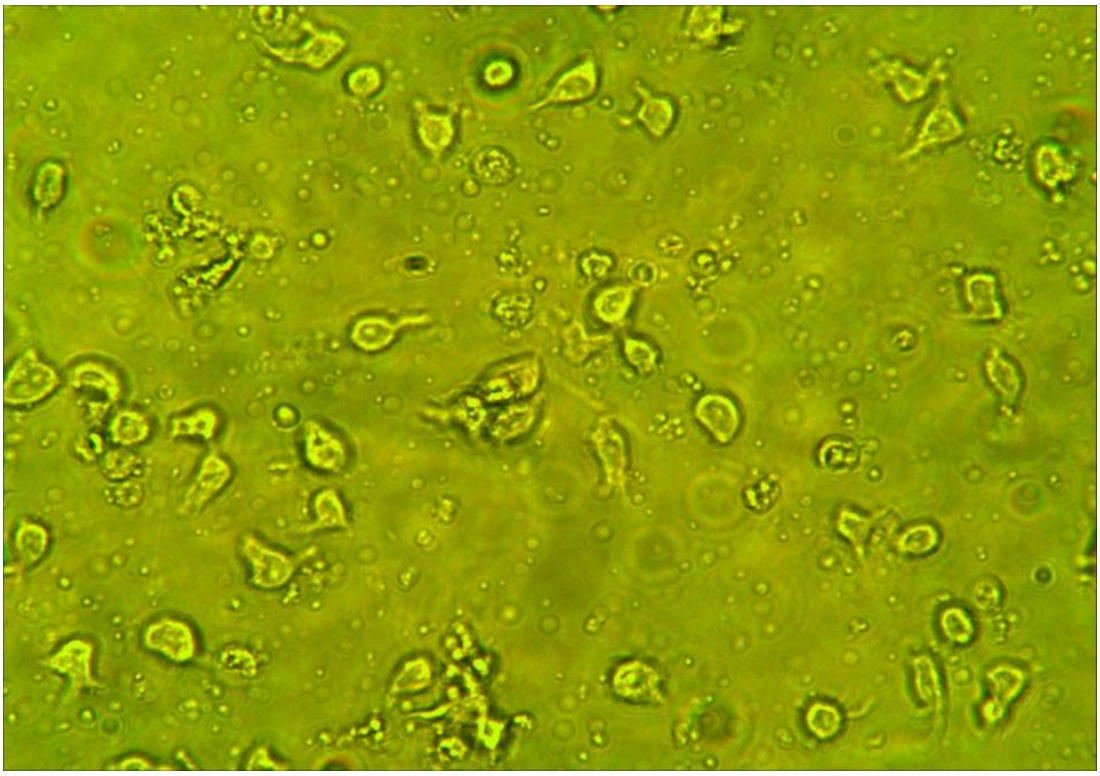

Figure 8: PMA + TNF-α treated cells after 3 days of incubation. KG-1 cells seeded at a concentration of 5 $x10^4$ cells/ml and treated with 10 ng/ml PMA + 10 ng/ml TNF-α showing extensive cell death as observed by light microscopy under 40X magnification.

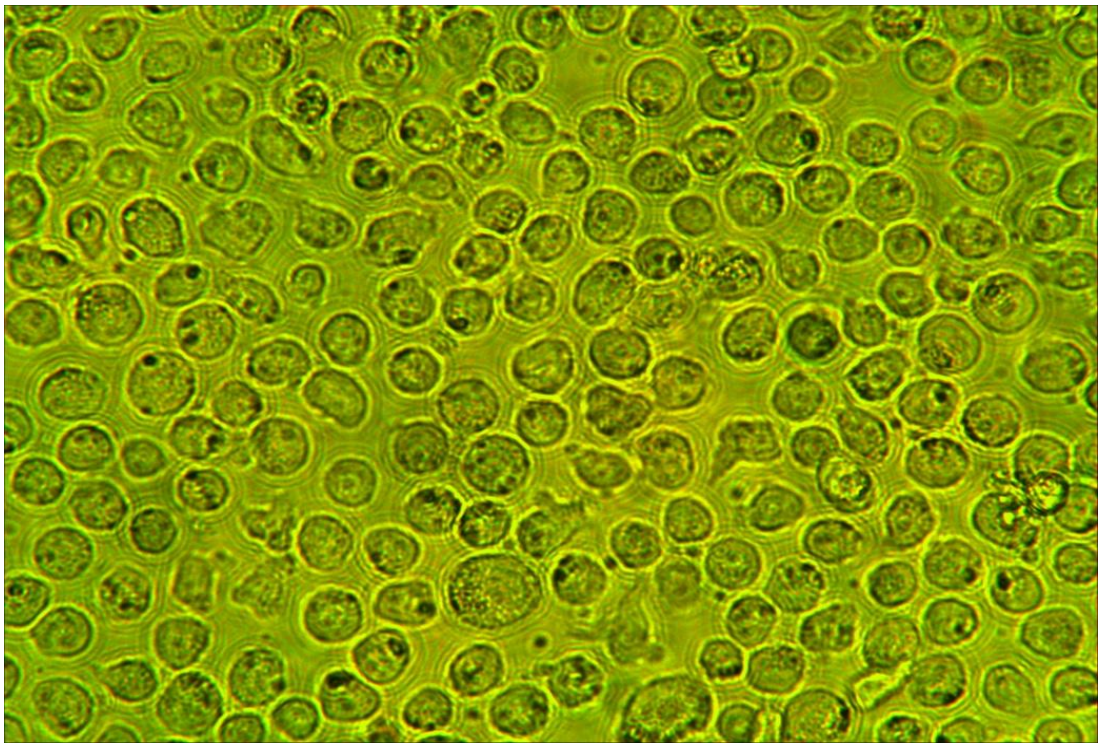

Figure 9: Control KG-1 cells after 7 days incubation. KG-1 cells at a concentration of 5 x10^4 cells/ml used for comparison with PMA + TNF-α treated KG-1s (40X magnification).

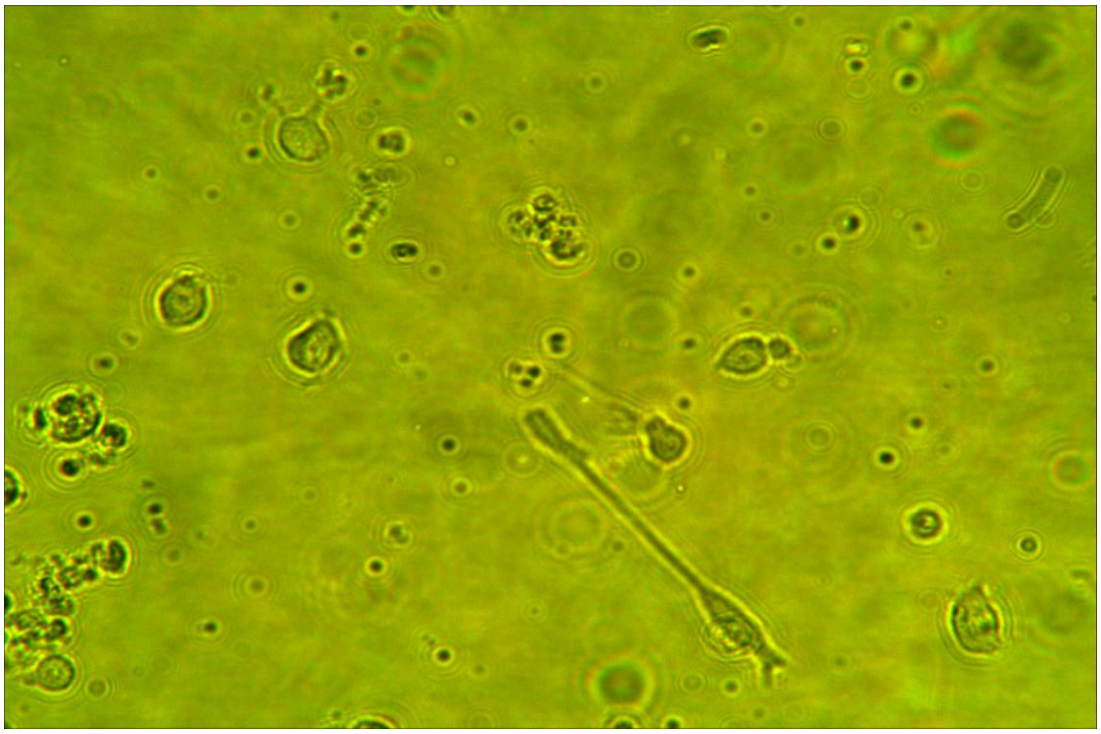

Figure 10: KG-1 cells treated with PMA and TNF-α after 7 days incubation. Neurite-like processes were observed by light microscopy under 40X magnification. KG-1 cells were seeded at a concentration of at 5 x10^4 cells/ml with PMA at 10 ng/ml and TNF-α at 10 ng/ml.

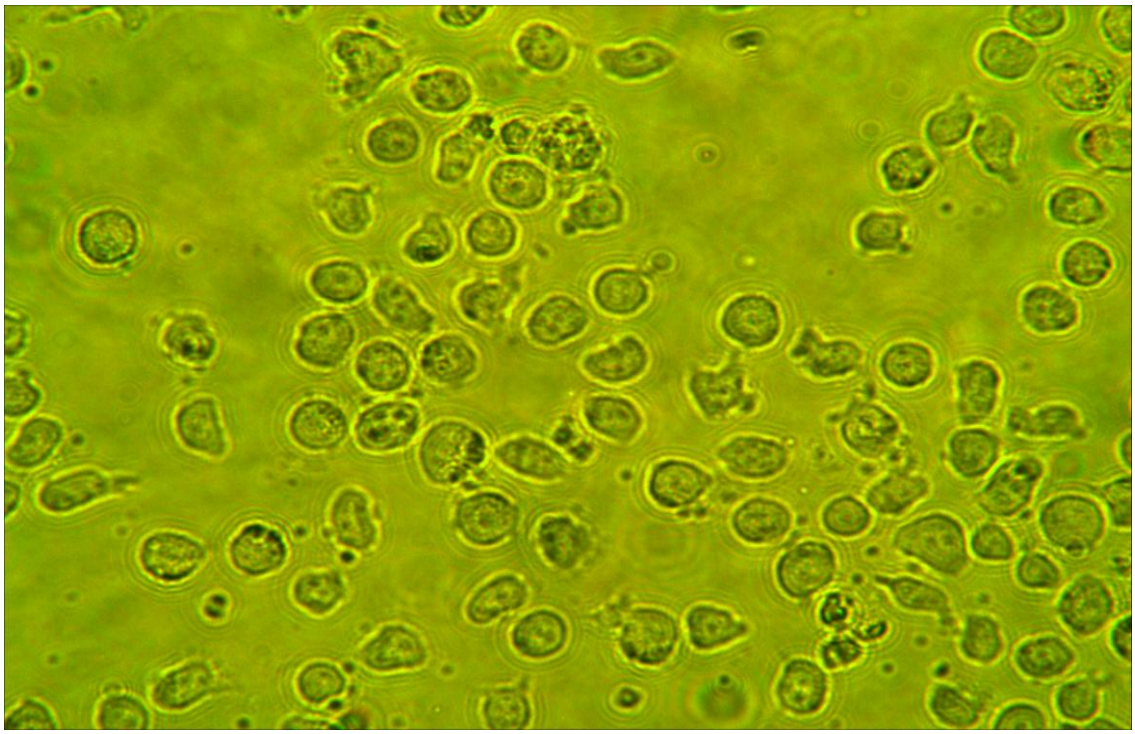

Figure 11: KG-1 control cells seeded at an initial concentration of 5 x10^4 cells/ml for comparison with PMA + Ionomycin-treated KG-1 cells. Light microscopy under 40X magnification illustrates appearance of control KG-1 cells after 3 days of incubation.

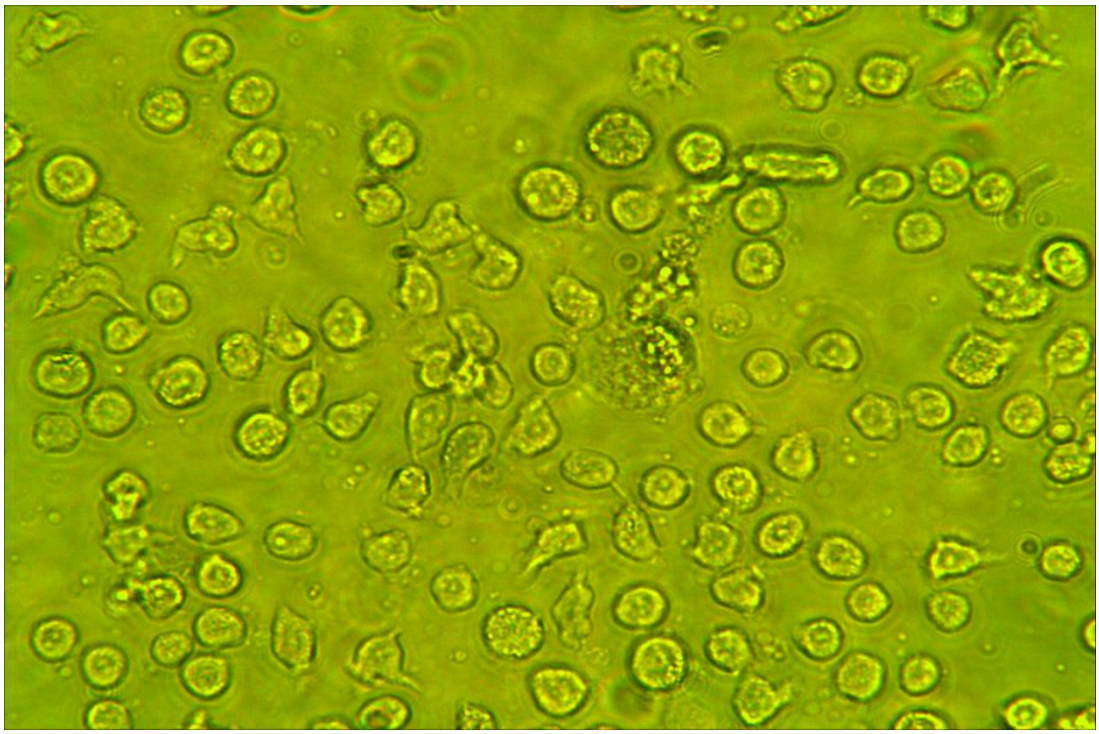

Figure 12. KG-1 cells treated with PMA + Ionomycin after 3 days incubation. KG-1 cells seeded at a concentration of 5 x10^4 cells/ml and treated with 10 ng/ml PMA +10 ng/ml Ionomycin for 3 days. Production of "neurite- like" processes observed at 40X magnification under light microscopy.

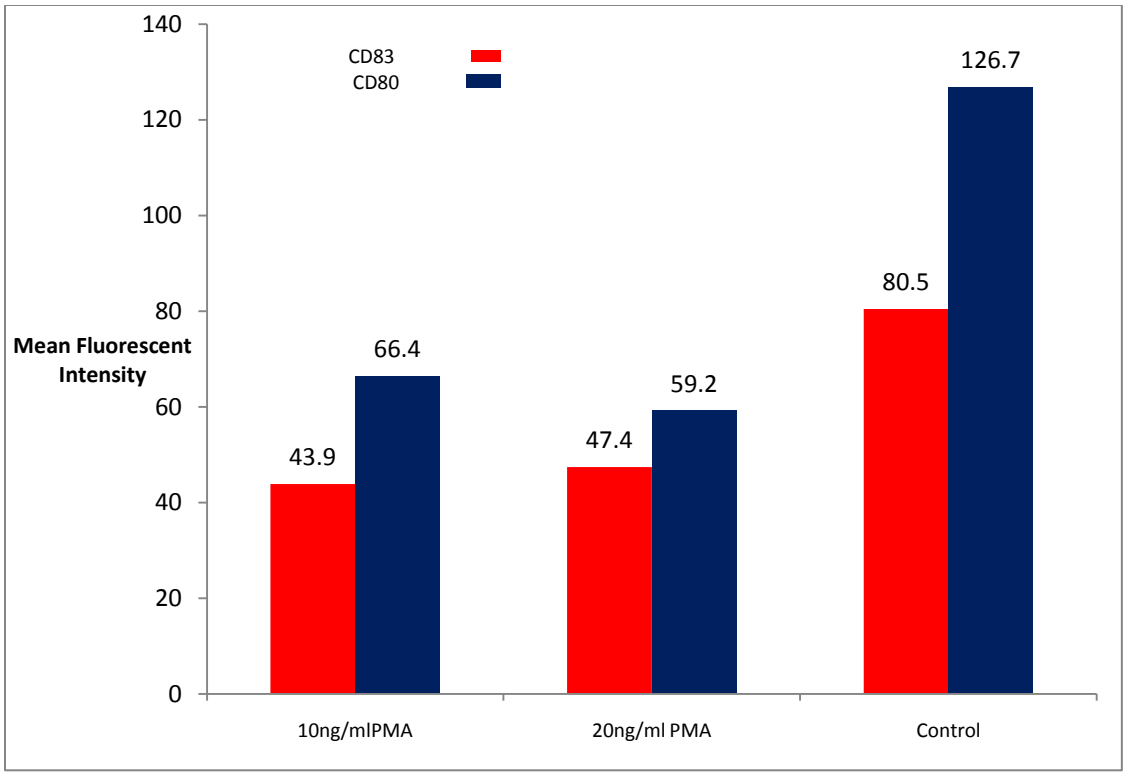

Figure 13: CD83 and CD80 expression by KG-1 cells (cultured at 5 x10^4 cells/ml) stimulated with 10 ng/ml PMA or 20 ng/ml PMA after 7 days incubation, compared to controls incubated under identical conditions without PMA. Results are shown as Mean Fluorescence Intensity (MFI) and illustrate representative cell surface molecule expression. Cell surface marker expression was determined by Guava PCA after staining cells with PE-labelled anti-human CD83 or CD80 antibodies, and were compared to isotype matched controls (n = 1). Mean MFIs are shown above bars for ease of comparison.

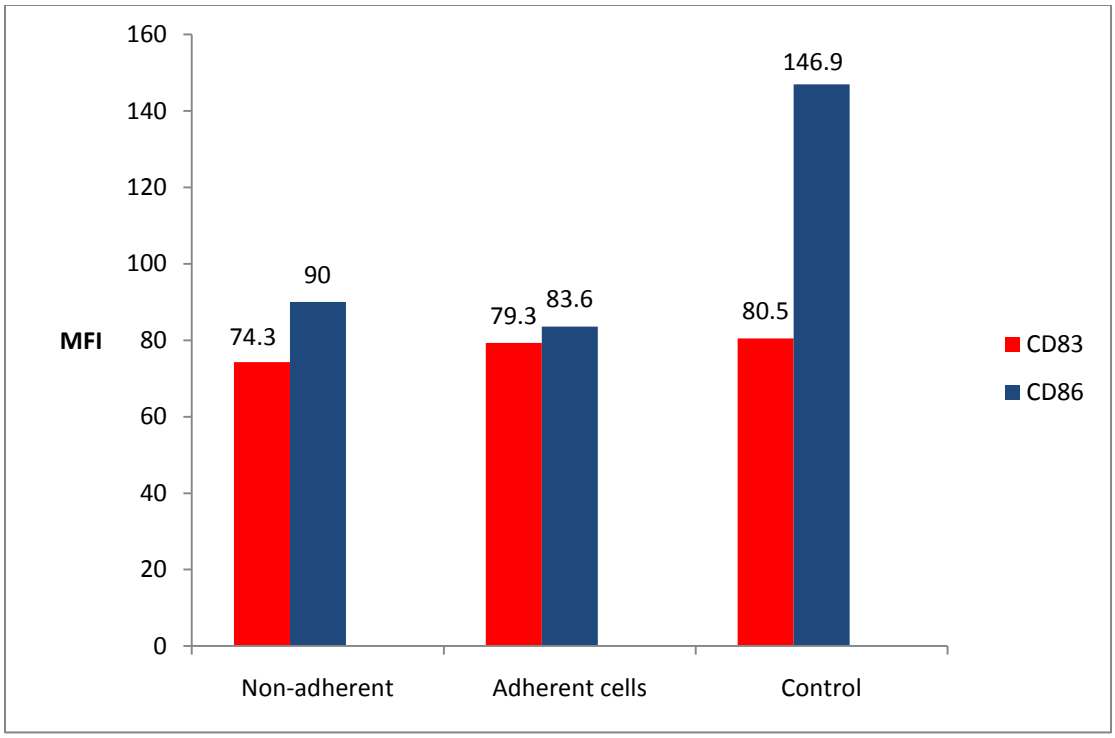

Figure 14: CD83 and CD86 expression on non-adherent and adherent KG-1 cells stimulated with PMA (10 ng/ml) for 7 days, compared to non-stimulated control KG-1 cells. MFI is indicated above each bar for ease of comparison. Adherent cells were obtained from the bottom portion of the tissue culture well and the non-adherent cells were obtained from top portion of the culture well (n = 1). Mean MFIs are shown above bars for ease of comparison.

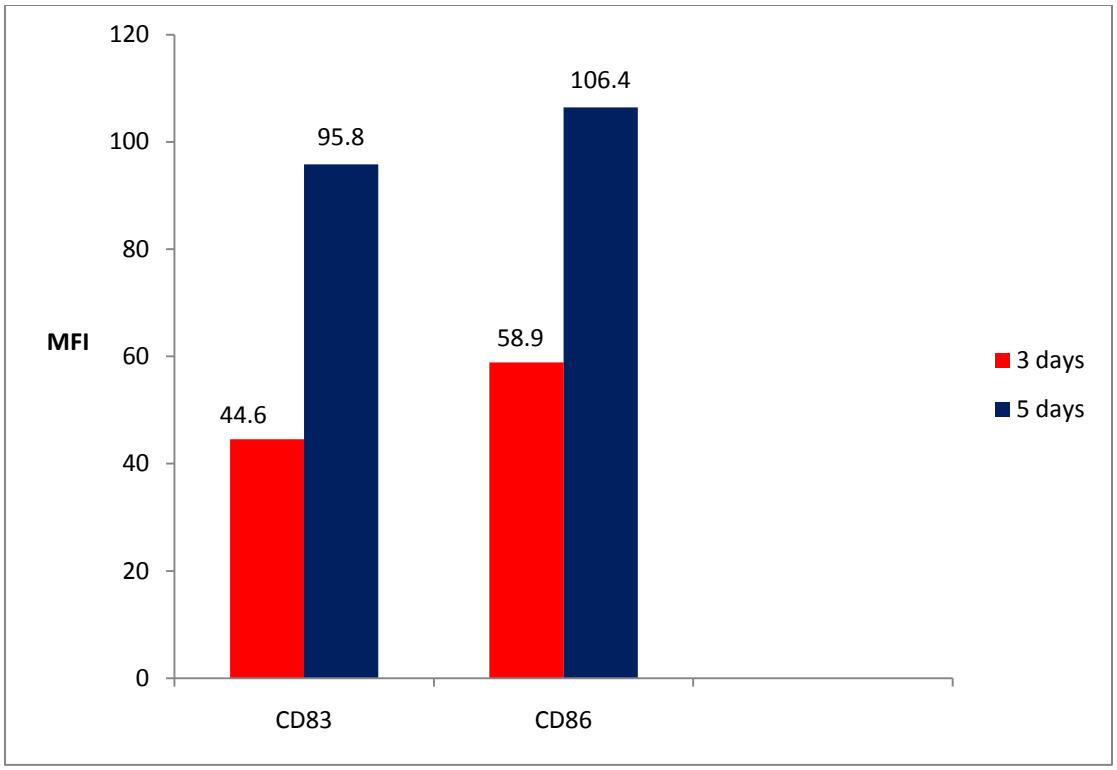

Figure 15: CD83 and CD86 expression by KG-1's differentiated with PMA+ Ionomycin. KG-1 cells seeded at $5x10^4$ cells/ml concentration were differentiated using 10 ng/ml PMA+ 100 ng/ml Ionomycin, and cell surface molecule expression was measured at 3 and 5 days by direct immunofluorescence (n = 1). MFI is indicated above each bar for ease of comparison**.**

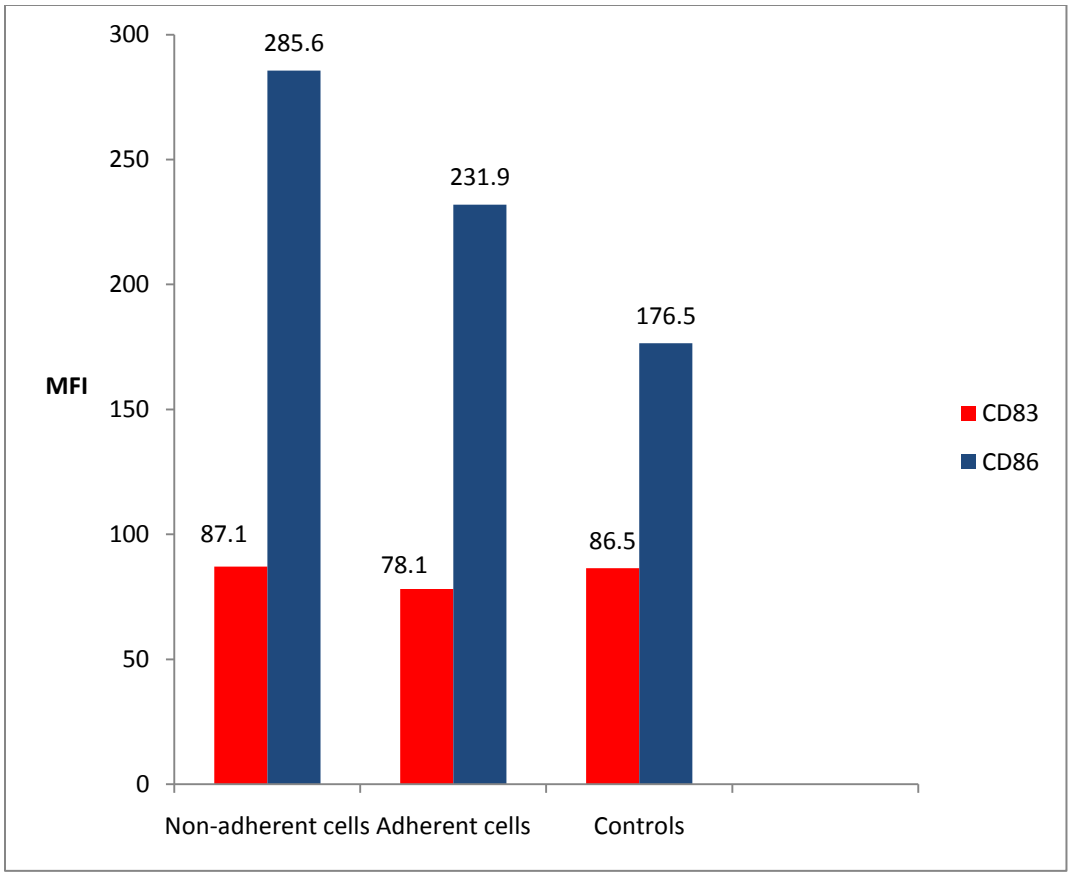

Figure 16: CD83 and CD86 expression on adherent and non-adherent KG-1's differentiated by 10 ng/ml PMA+ 100 ng/ml Ionomycin for 5 days. KG-1s were seeded at an initial concentration of $5x10^4$ cells/ml and treated cells were compared with control KG-1 cells incubated under identical conditions (n = 1). Mean MFIs are shown above bars for ease of comparison.

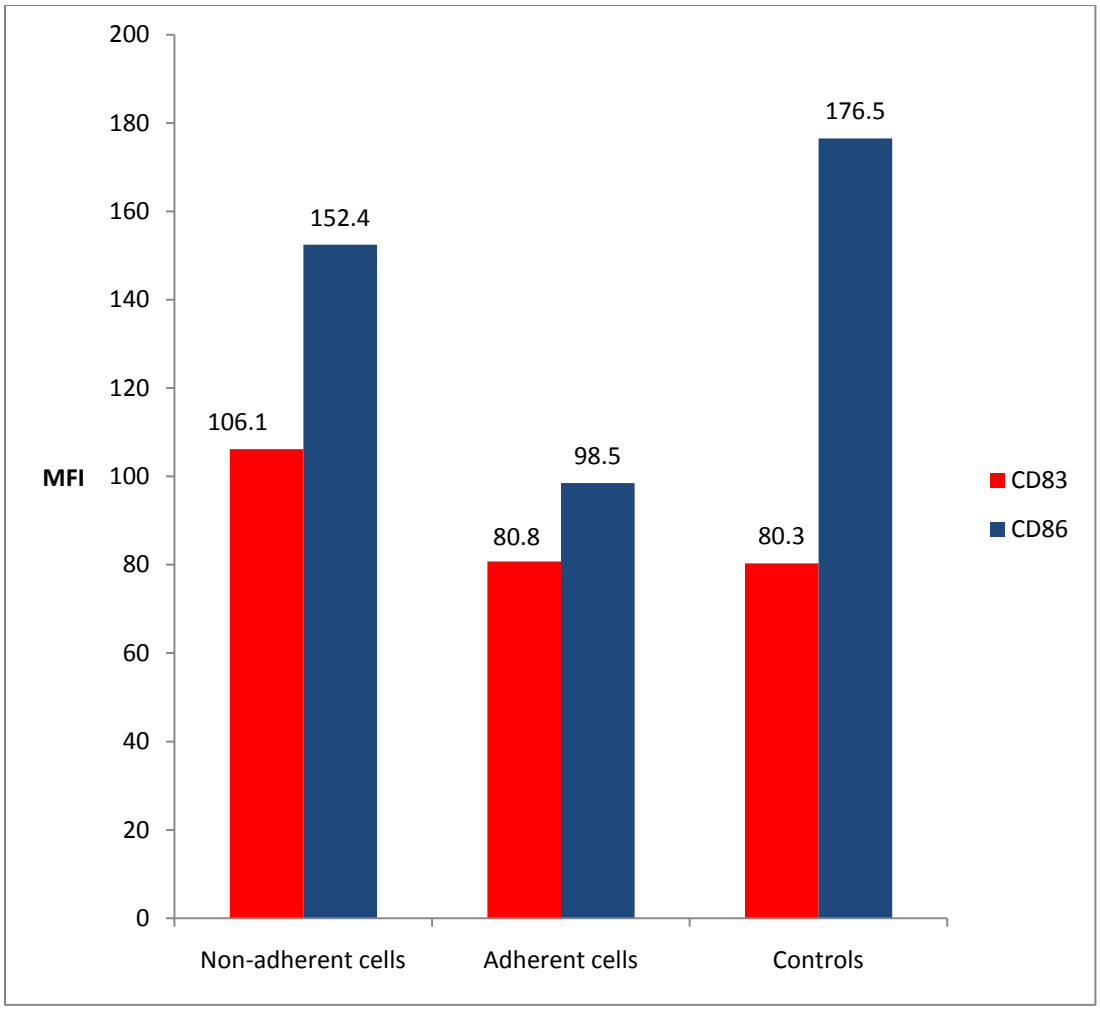

Figure 17: CD83 and CD86 expression on adherent and non-adherent KG-1 cells differentiated with PMA + TNF-α. KG-1's seeded at a concentration of $8x10^4$ cells/ml were differentiated using 10 ng/ml PMA+ 10 ng/ml TNF-α for 5 days (n = 1). Mean MFIs are shown above bars for ease of comparison.

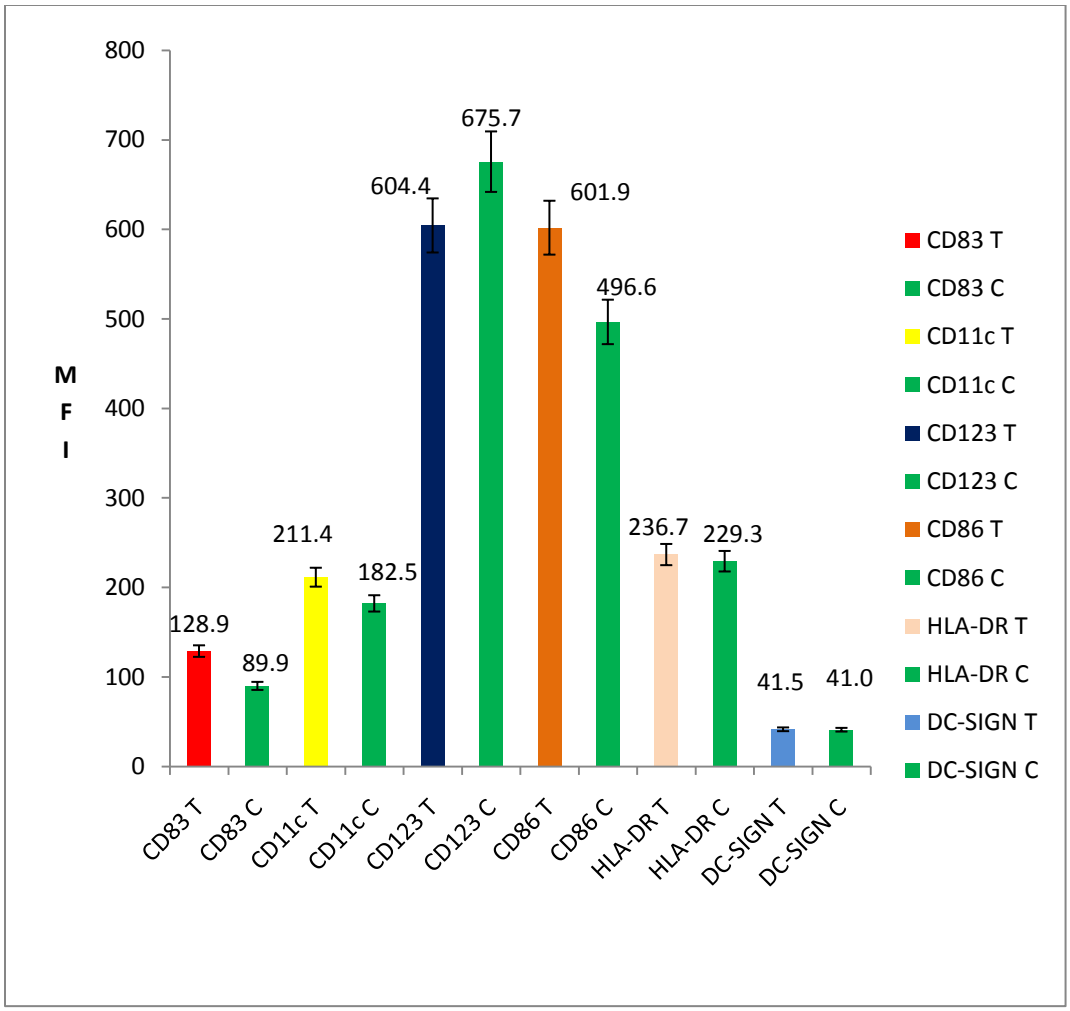

Figure 18: Cell surface molecule expression on PMA+ Ionomycin treated KG-1 cells. KG-1 cells seeded at $8x10^4$ cells/ml and differentiated by 10 ng/ml PMA+ 10 ng/ml Ionomycin for 5 days (n = 3). MFIs of treated cells (denoted as T) are compared with the control MFIs (denoted as C). Mean MFIs are shown above bars for ease of comparison. One way ANOVA; p = 0.04.

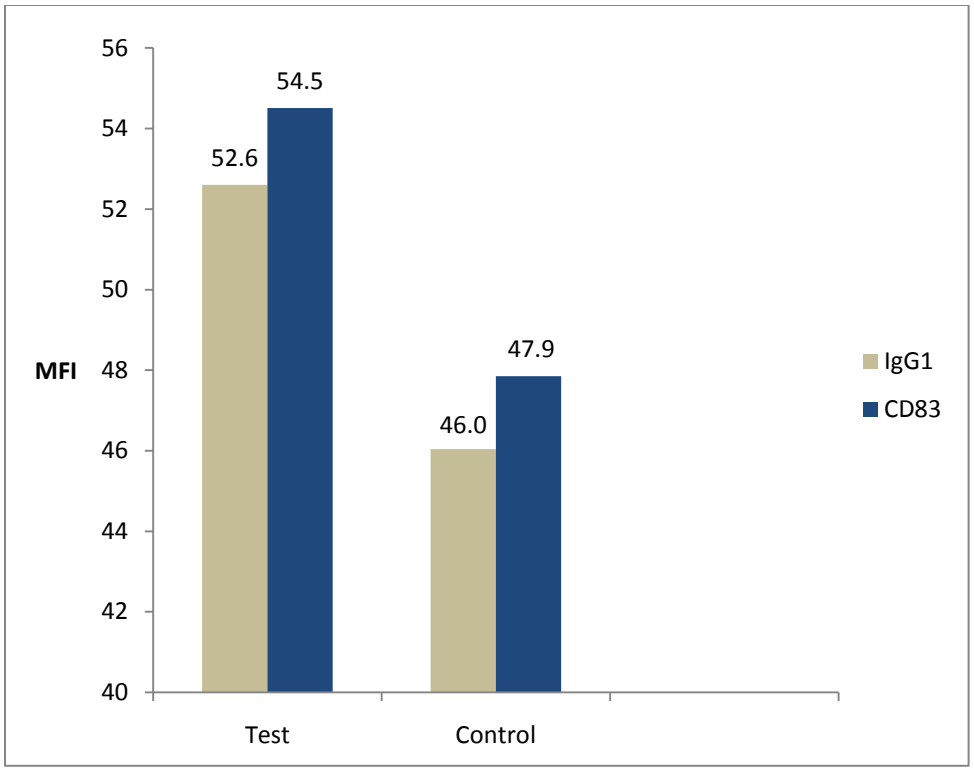

Figure 19: CD83 expression by KG-1s following treatment with atRA. KG-1 cells were cultured at an initial concentration of 8 x 10^4 cells/ml with atRA (1µM/ml) and control cells were cultured at the same concentration without atRA. CD83 expression was measured after 1 day of incubation and is expressed as MFI, shown relative to isotype control (IgG1) (n = 1). Mean MFIs are shown above bars for ease of comparison.

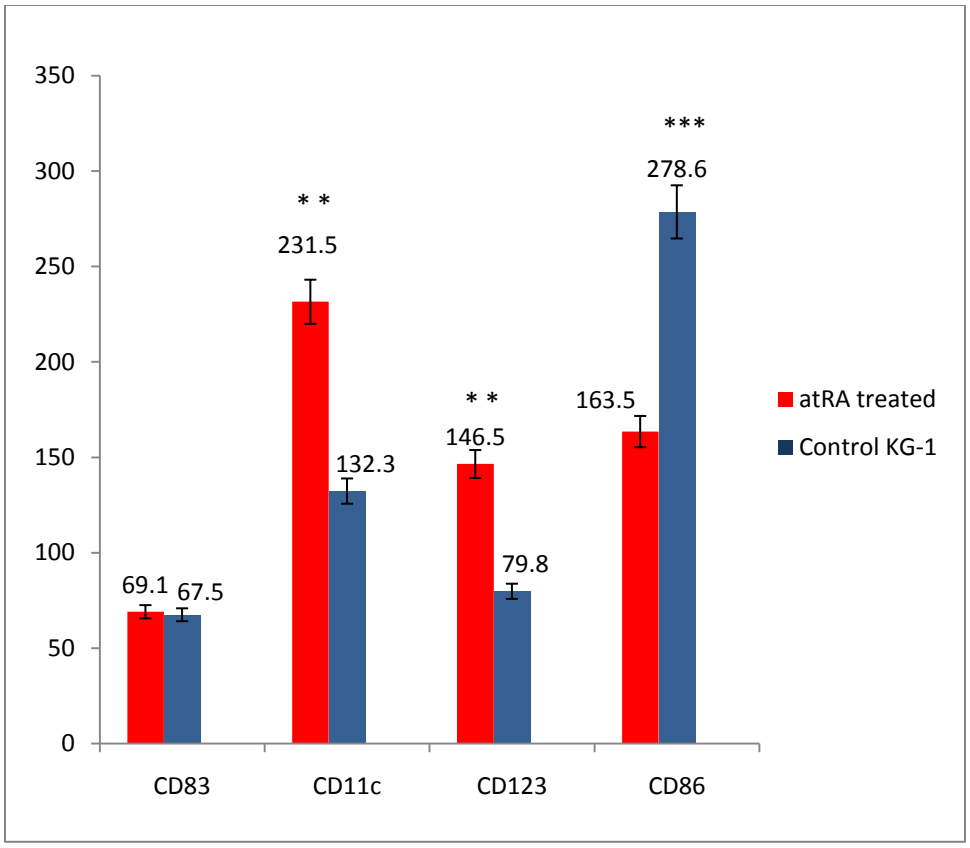

Figure 20: Expression of CD83, CD11c, CD123 and CD86 by KG-1 cells treated with atRA. KG-1 cells were seeded at 8 x 10^4 cells/ml and treated with atRA (1µM/ml) and cell surface molecule expression was measured after 3 days incubation. Control samples contained KG-1s at the same concentration. (n = 3). Mean MFI is shown above each bar. One way ANOVA; $p < 0.0001$. Two asterisks denote significant difference from control where as 3 asterisks indicate significant difference from test.

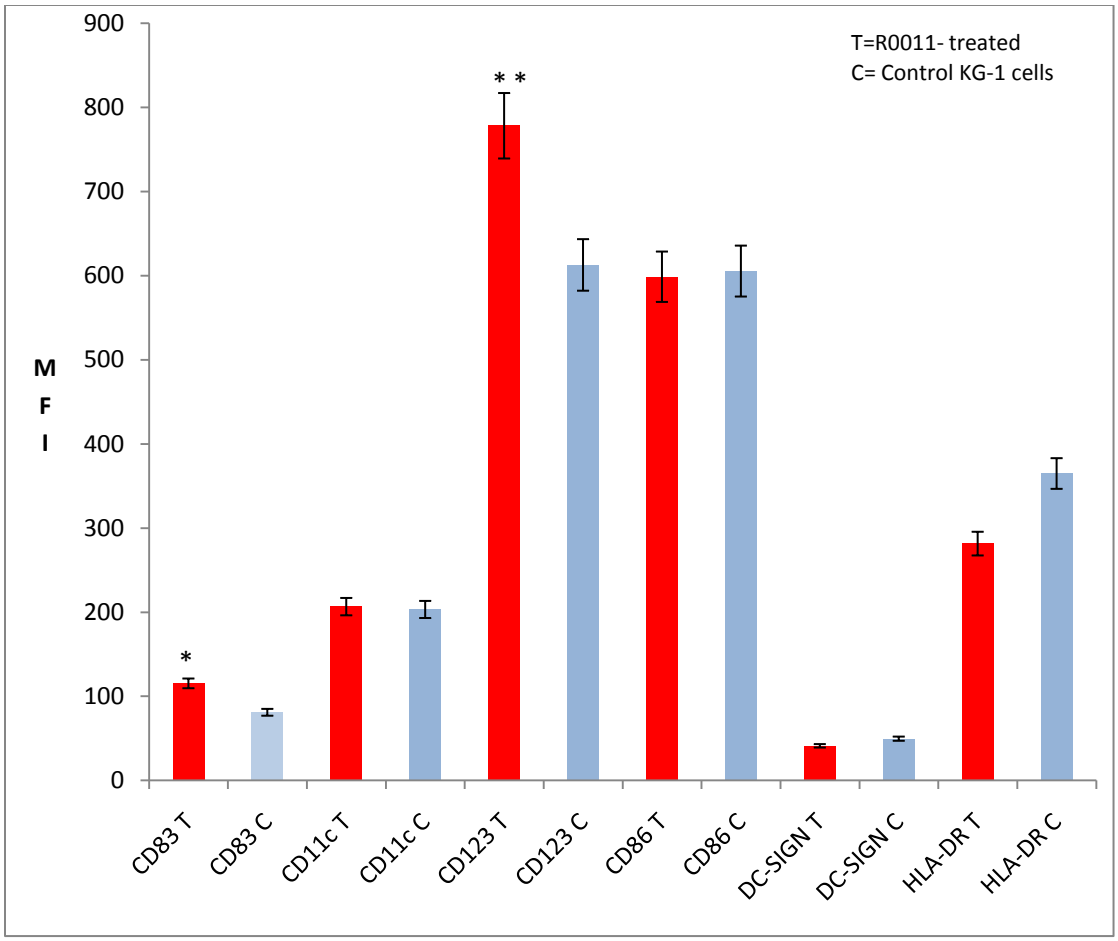

Figure 21. Expression of CD83, CD11c, CD123, CD86, DC-SIGN, HLA-DR on KG-1cells treated with *Lactobacillus rhamnosus* R0011 compared to untreated control KG-1 cells. KG-1 cells seeded at a concentration of 5 $x10^4$ cells/ml were stimulated with *Lactobacillus rhamnosus* R0011 at a concentration of 1 $x10^4$ cells/ml for 5 days. Results are shown as mean MFI of R0011-treated (red bars) and control (blue bars) cells (n = 3). One way ANOVA p =0.0001. Asterisks denote significant difference from control.

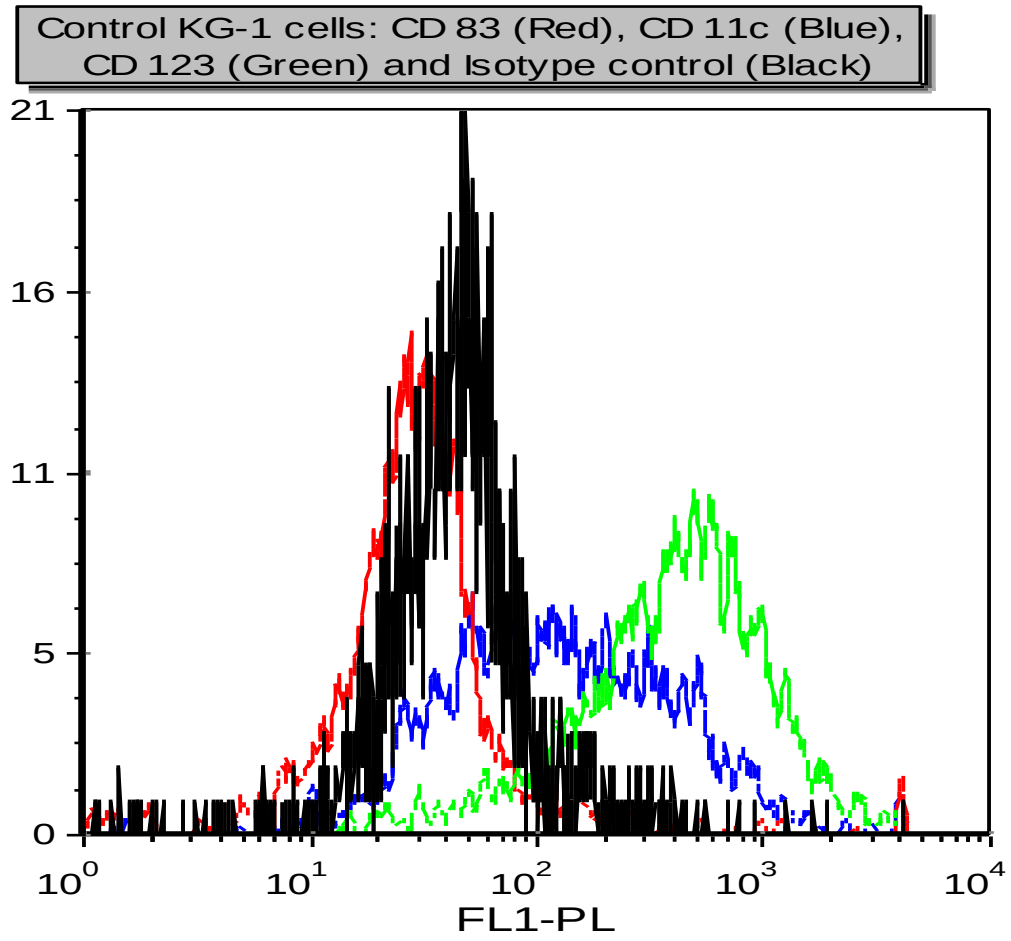

Figure 22: Cell surface expression of CD83 on KG-1 control cells. The histogram shows the level of fluorescence for CD83 expression in red, CD11c expression in blue, CD123 expression in green and the isotype control (IgG1) in black. The histogram was generated by FCS Express Version 3 software from analysis of data obtained by direct immunofluorescence and flow cytometry using a Guava PCA. The Overton subtraction values were 1.5% positive for CD83 expression, 48.4% positive for CD11c expression and 80.2% positive for CD123 expression relative to isotype control.

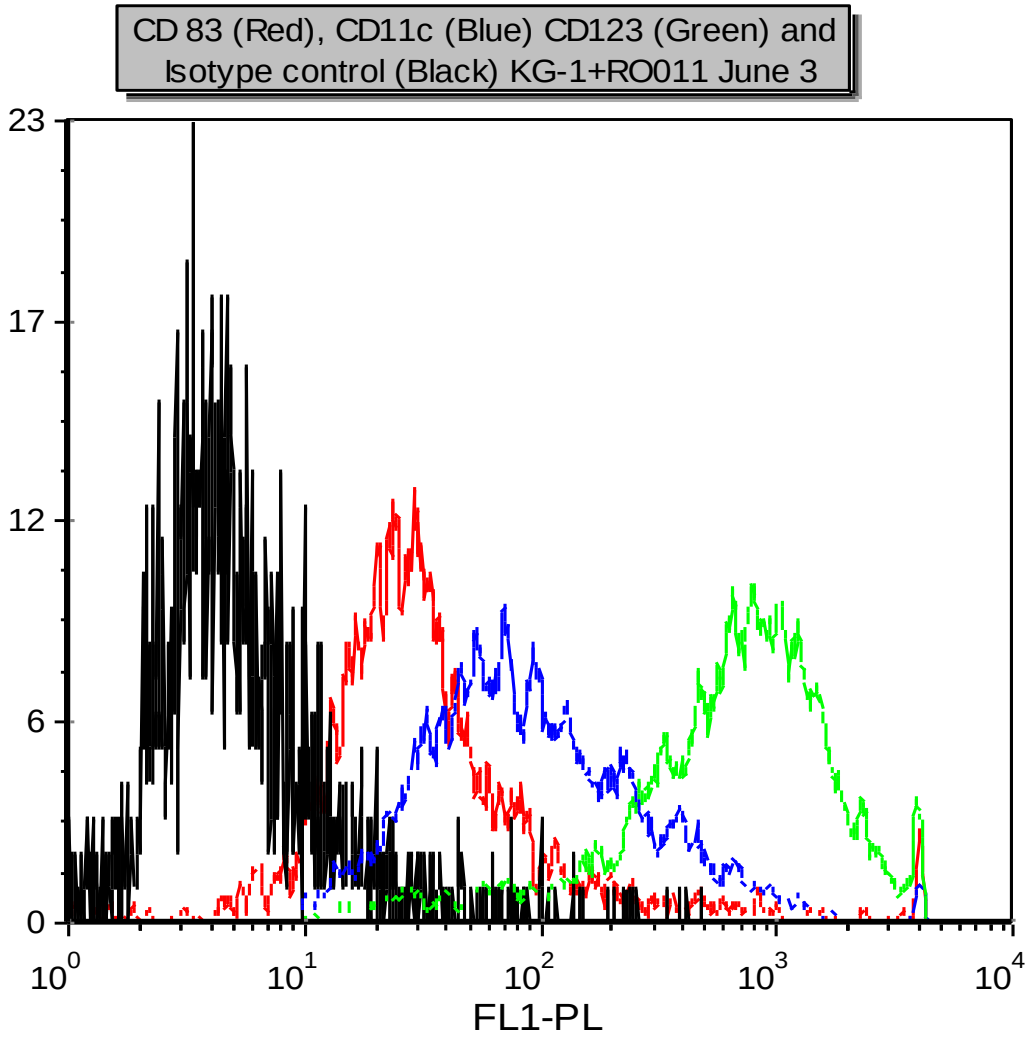

Figure 23: Cell surface marker expression of CD83, CD11c and CD123 on KG-1 cells differentiated with *Lactobacillus rhamnosus* R0011. KG-1s at $5x10^4$ cells/ml were treated with R0011 at $1x10^8$ CFU/ml for 5 days. The histogram shows CD83 expression in red, CD11c in blue, CD123 in green and the isotype control IgG1κ in black. Overton subtraction indicated 76% positive for CD83 expression, 87.4% positive for CD11c expression and 81.8% positive for CD123 expression.

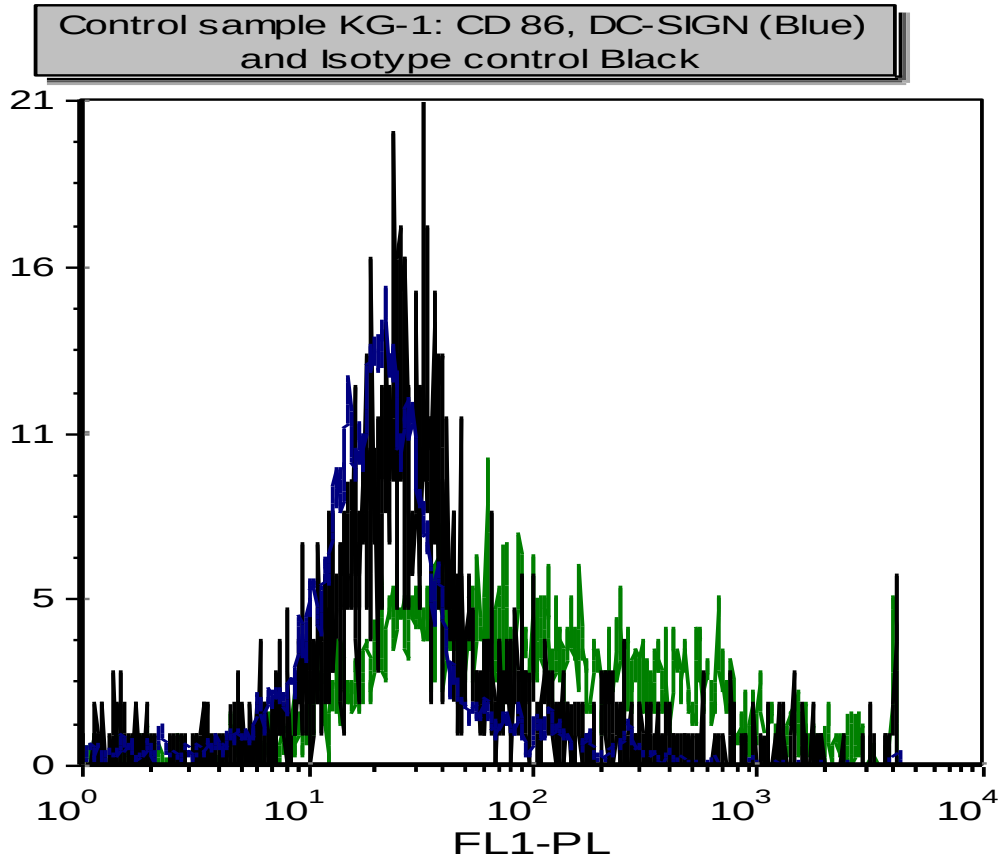

Figure 24: Cell surface marker CD86 and DC-SIGN expression on control KG-1 cells. The histogram shows CD86 expression in green, DC-SIGN in navy blue and Isotype control IgG2b, κ in black. Overton subtraction indicated 38.2% positive for CD86 expression 0.6% positive for DC-SIGN expression.

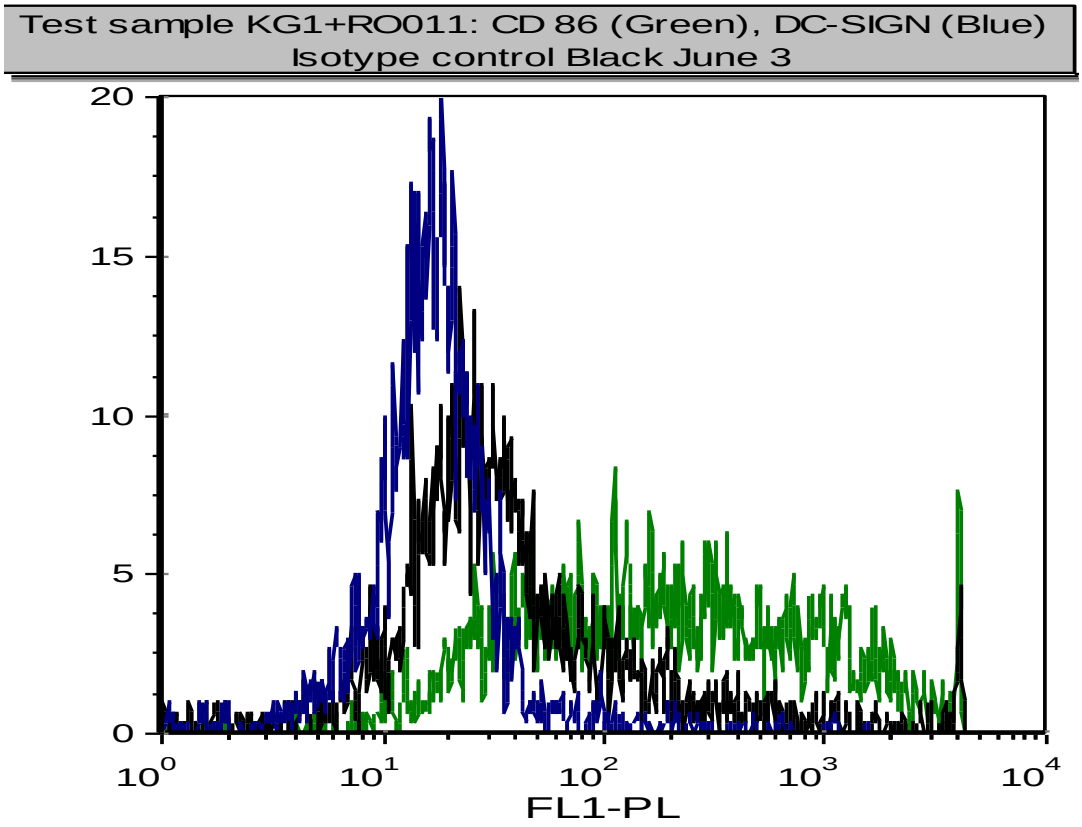

Figure 25: Cell surface marker CD86 and DC-SIGN expression on KG-1 cells treated with R0011. The histogram shows CD86 marker in green, and DC-SIGN in navy blue and Isotype control IgG2b, κ in black. Overton subtraction indicated 47.1% positive for CD86 expression and less than 1% positive for DC-SIGN expression (0.8%).

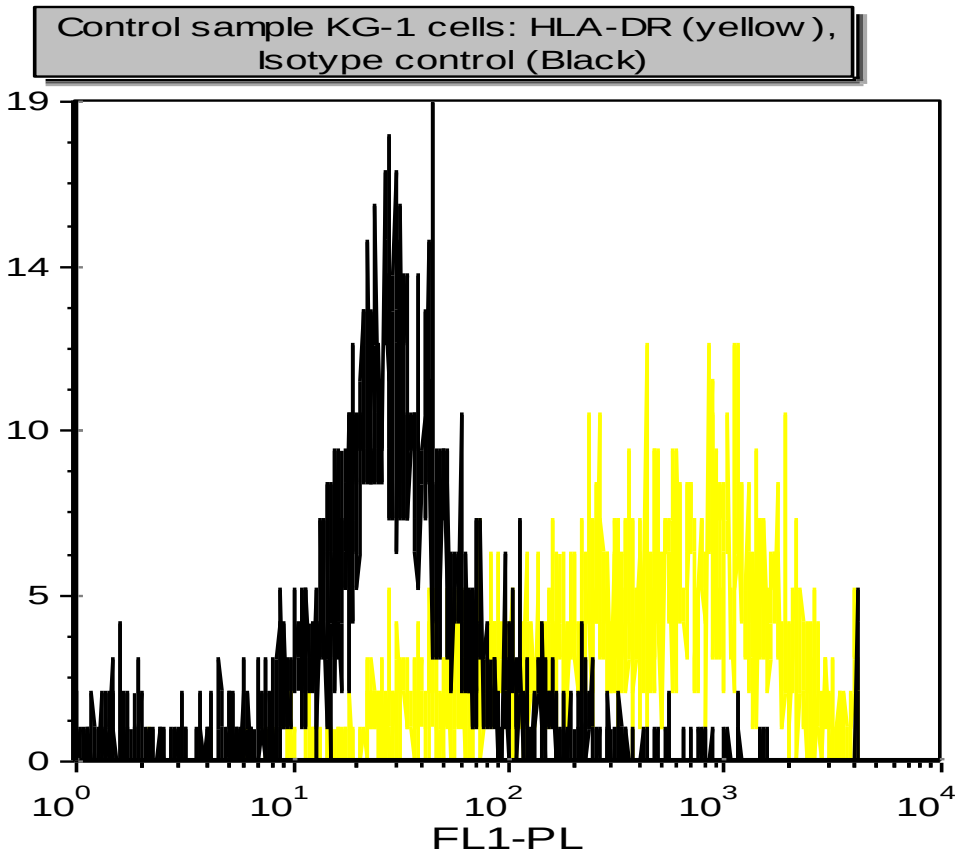

Figure 26: Cell surface marker HLA-DR on control KG-1 cells. HLA-DR expression is shown in yellow and Isotype control IgG2a in black. Overton subtraction indicated 73.1% positive for HLA-DR expression relative to isotype control.

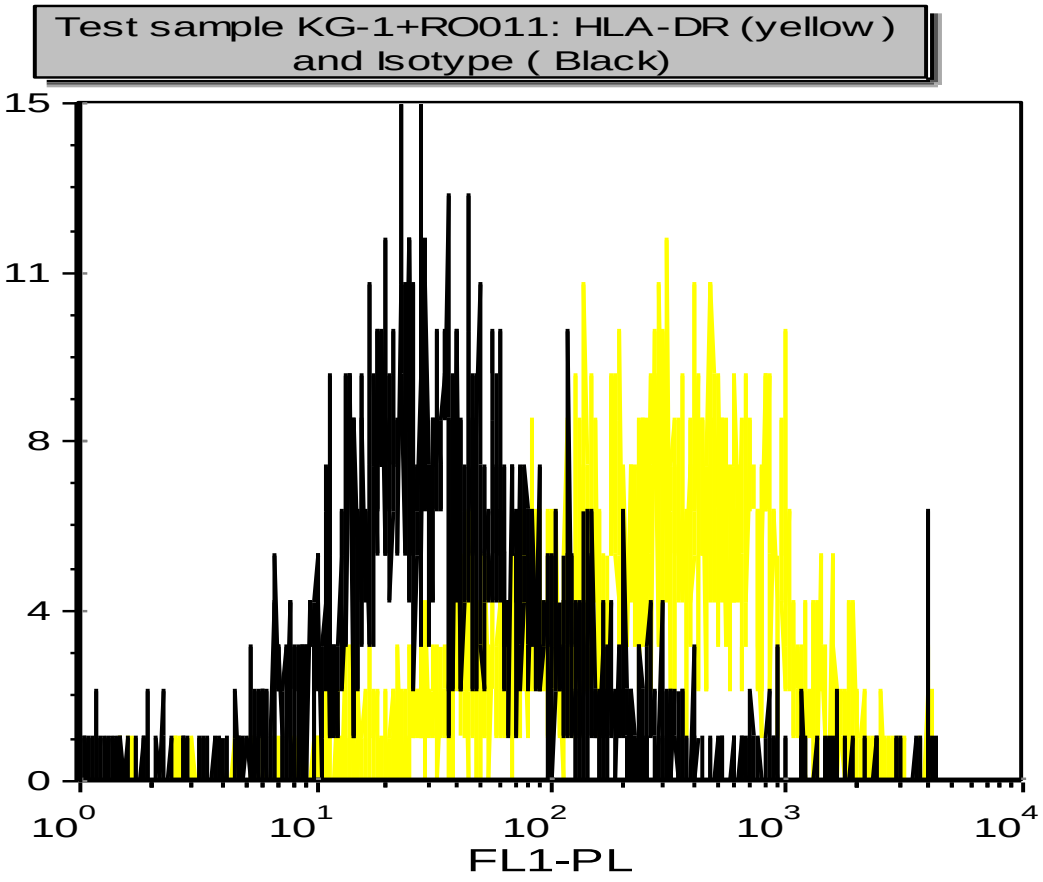

Figure 27: Cell surface marker HLA-DR expression by KG-1s differentiated with *Lactobacillus rhamnosus* R0011. HLA-DR in yellow and Isotype control IgG2a in black. Overton subtraction indicated 59.5% positive for HLA-DR expression.

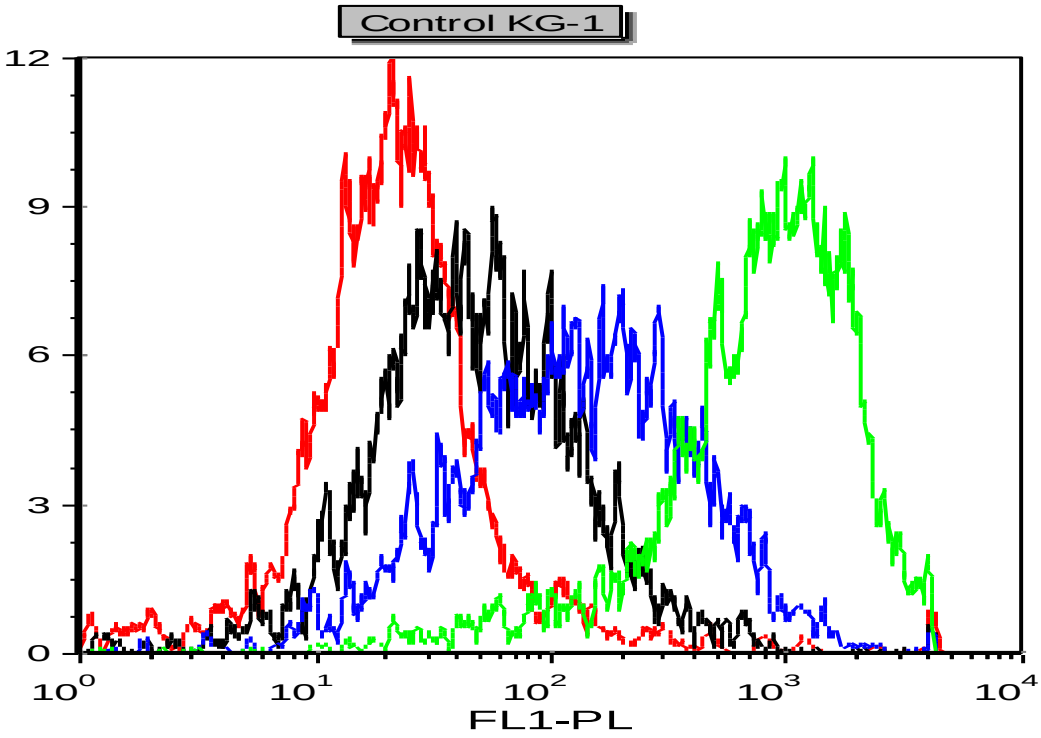

Figure 28: Cell surface marker expression of CD83, CD11c and CD123 on control KG-1 cells. KG-1s at $8x10^4$ cells/ml were incubated for 5 days. The histogram shows CD83 marker expression in red, CD11c in blue, CD123 in green and Isotype control IgG1, κ in black. Overton subtraction indicated 0.1% positive for CD83, 36.8% positive for CD11c and 82.2% positive for CD123.

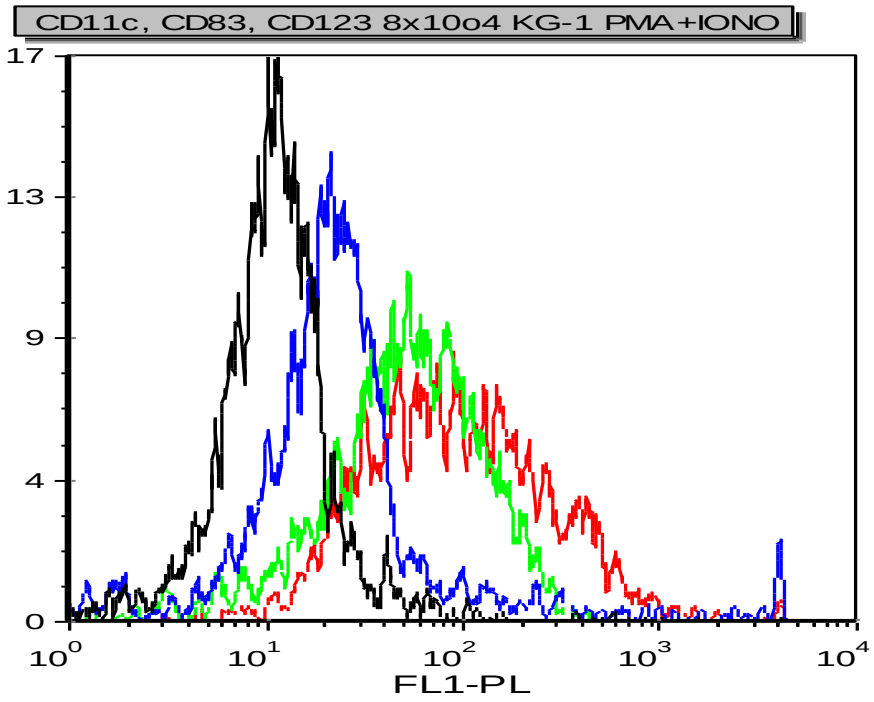

Figure 29: Cell surface marker expression of CD83, CD11c and CD123 on KG-1 cells differentiated with PMA+ Ionomycin. KG-1s at $8x10^4$ cells/ml were treated with 10 ng/ml PMA and 10 ng/ml Ionomycin for 5 days. The histogram shows CD83 expression in red, CD11c in blue, CD123 in green and Isotype control IgG1, κ in black. Overton subtraction indicated 47.7% positive for CD83, 83.5% positive for CD11c and 72.9% positive for CD123 expression.

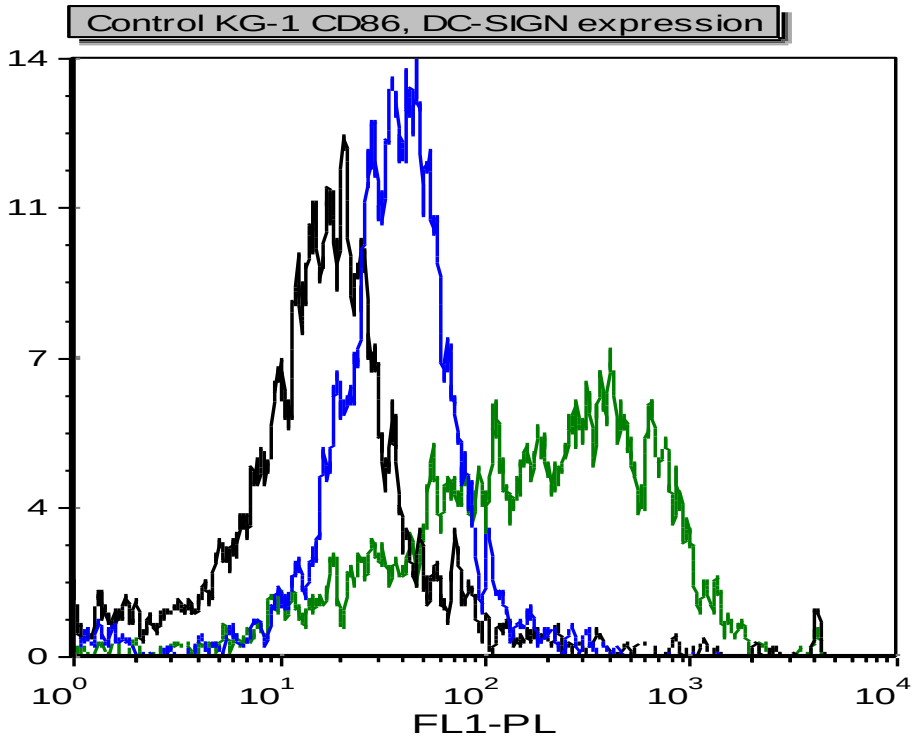

Figure 30: Cell surface markers (CD86 and DC-SIGN) expressed by control KG-1s. KG-1 cells were seeded at $8x10^4$ cells/ml concentration and incubated for 5 days. CD86 in green, DC-SIGN in blue and the Isotype marker IgG2b, κ in black. Overton subtraction indicated 68.8% positive for CD86 expression and 2.2% positive for DC-SIGN expression.

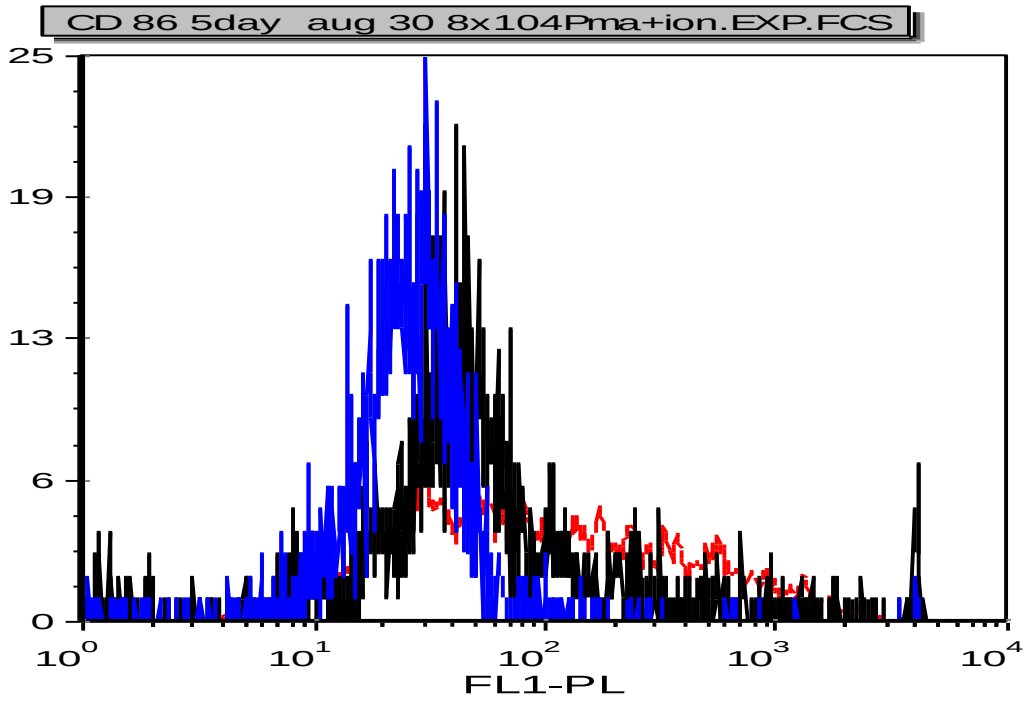

Figure 31: Cell surface markers expressed by PMA+ Ionomycin-treated KG-1. KG-1 cells were seeded at $8x10^4$ cells/ml concentration and incubated for 5 days. CD86 in red, DC-SIGN in blue and the Isotype marker IgG2b, k in black. Overton subtraction indicated 25.1% positive for CD86 expression and 21.9% positive for DC-SIGN expression**.**

Table 4. TNF-α production after 6 hours and 24 hours by unstimulated KG-1 and PMA + Ionomycin-treated KG-1 DLC

	TNF-α in pg/ml (6 hrs)		TNF-α in pg/ml (24 hrs)	
TLR agonist	KG-1s	DLCs	KG-1s	DLCs
LPS (µg/ml)				
0.1	none[1]	none	none	none
5	none	none	none	none
10	none	none	none	none
Flagellin (ng/ml)				
0.1	none	none	none	none
1.0	none	none	none	none
10.0	none	none	none	none
Imiquimod (µg/ml)				
2.5	none	none	none	none
5.0	none	none	none	none
10.0	none	none	none	none
Poly (I:C) µg/ml				
5	none	none	none	none
25	none	none	none	none
100	62.1±0.01	none	none	none
No TLR-agonists[2]	54.5± 11.5	none	213.4±13.4	none

None[1] indicates no TNF-α production detected by ELISA. Control cells were cultured without TLR-agonists. Results are shown as a mean of 3 replicates ± S.E. (n = 3). No TLR-agonists[2] indicate control KG-1 cells not treated with any TLR-agonists.

Table 5: TNF-α and IL-12 production by KG-1 cells differentiated with *Lactobacillus rhamnosus* R0011.

TLR-agonists	cytokine production at 4 hrs		cytokine production at 16 hrs	
LPS in µg/ml	TNF-α	IL-12	TNF-α	IL-12
0.1	none[1]	none	none	none
5	none	none	none	none
10	none	none	none	none
Flagellin in ng/ml				
0.1	none	none	none	none
1.0	none	none	none	none
10.0	none	none	none	none
Imiquimod in µg/ml				
2.5	none	none	none	none
5.0	none	none	none	none
10.0	none	none	none	none
Poly (I:C) in µg/ml				
5	none	none	none	none
25	none	none	none	none
100	none	none	none	none
KG-1s + L.r[3]				
(No TLR-agonists)	none	none	none	none

None[1] indicates no TNF-α or IL-12. L.r[3] indicates *Lactobacillus rhamnosus* R0011. Results are a mean of 3 replicates ± S.E. (n=3). KG-1s + L.r[3] indicates no TLR-agonists were added and this combination served as a negative control.

Table 6: IL-10 production by KG-1 cells differentiated with *Lactobacillus rhamnosus* R0011.

TLR-agonist	levels (pg/ml) after 4 hrs	levels (pg/ml) after 16 hrs
LPS (µg/ml)		
0.1	297.7± 0.6	86.7± 0.02
5	N[2]	70.2 ± 0.01
10	39.9± 0.002	51.6± 0.008
Flagellin (ng/ml)		
0.1	none[1]	253.0 ± 0.06
1.0	198.7± 0.04	none
10.0	2192.0 ± 0.35	143.0± 0.04
Imiquimod (µg/ml)		
2.5	none	none
5.0	none	none
10.0	none	none
Poly (I:C) µg/ml		
5	none	none
25	28.9 ± 0.001	none
100	1557.8 ± 0.2	none
KG-1s + L.r[3]	1249.9± 0.08	170.8±0.05

None[1] indicates no IL-10 production detected by ELISA. L.r indicates *Lactobacillus rhamnosus* R0011. N[2] indicates a negligible amount of IL-10 was detected. Results are shown as the mean of 3 replicates ± S.E. (n = 3). KG-1s + L.r[3] indicates no TLR-agonists were added and this combination served as a negative control.

Table 7. IL-12 production by PMA+ Ionomycin-differentiated DLCs after TLR-agonist stimulation.

TLR-agonist	levels (pg/ml) at 4 hrs	levels (pg/ml) at 16 hrs
LPS (µg/ml)		
0.1	88.9± 0.1	270 ± 0.17
5	81.7± 0.09	47.4 ± 0.14
10	78.1± 0.1	none
Flagellin (ng/ml)		
0.1	none[1]	none
1.0	none	none
10.0	54.5± 0.07	353.0 ± 0.1
Imiquimod (µg/ml)		
2.5	none	none
5.0	73.5 ± 0.08	none
10.0	70.9 ± 0.04	none
Poly (I:C) (µg/ml)		
5	51 ± 0.002	none
25	116 ± 0.1	none
50	59.8 ± 0.02	241.2 ± 0.2
KG-1s (No TLR-agonists)	138.4± 0.006	

None[1] indicates no IL-12 production detected by ELISA. Results are shown as a mean of 3 replicates ± S.E. (n = 3).

Table 8: Comparison of effects of differentiation agents on KG-1 DLC cell surface marker expression.

Cell surface markers	atRA		PMA+Ionomycin		R0011	
	% positive	MFI	% positive	MFI	% positive	MFI
CD83	N	69.1	47.7	128.9	76	115.4
CD123	64.8	231.2	72.8	604.4	81.8	778.2
CD11c	44.5	146.5	82.5	211.4	87.4	206.6
CD86	25.6	163.5	25.1	601.9	47.1	598.7
HLA-DR	n/a	n/a	32.3	236.7	59.5	281.5
DC-SIGN	n/a	n/a	21.5	41.5	0.6	41.2

MFI indicates mean fluorescence intensity. N indicates negligible and n/a applies to those tests not performed. Percent positive was determined using the Overton subtraction, and is expressed relative to isotype controls.

DISCUSSION

DC has gained much research attention in recent years with respect to their role as prime mediators of innate and adaptive immunity. For example, there is currently a great deal of speculation as to the potential for recruiting mucosal DCs to elicit the desired levels of SIgA secretion in the intestine by using oral vaccines which include genetically modified probiotics (Takahashi, 2009). There is also currently much debate regarding the ability of the gut microbiota to influence DC activity and phenotype (Rook and Brunet, 2004). Such current questions prompted our study to develop a system to differentiate DCs *in vitro* and use this system to test effects of lactic acid bacteria. Differentiation studies on KG-1 cells were conducted for the first time by St. Louis *et al.*, 1999. Differentiation agents studied included PMA, PMA + Ionomycin, PMA + TNF-α and GM-CSF with IL-4 or TNF-α, with treatment periods of 3 to 7 days. After incubation with the differentiation agents, KG-1 cells were analyzed for cell surface marker expression and emphasis was placed on expression of CD83, MHC Class II and co-stimulatory molecule CD86 and CD80 expression. In addition the presence of Re1B transcription factor mRNA along with DC-CK1 chemokine mRNA was analysed by RT-PCR to confirm that the KG-1 cells had differentiated into KG-1 DLCs. The PMA + Ionomycin differentiation protocol was adopted in studies of KG-1s conducted by Ackerman and Cresswell to determine the regulation of MHC I transport (Ackerman and Cresswell, 2003). Both of these studies were limited to determining the expression of cell surface markers and no further study to elucidate the DLC phenotypes was undertaken by these investigators. Furthermore no analyses of cytokine production profiles produced by KG-1 cells were determined. More recent work by other groups (Teobald *et al.,* 2008 and Berges *et al.,* 2005) focused on determining whether the KG-1 DLCs were in a mature or

immature state by examining cell surface marker expression, Re1B transcription factor upregulation and production of proteasome subunits. Thus to date, little research has been done to determine the kinds of cytokines produced by KG-1 cells.

The approaches used in the current study were based chiefly on the differentiation protocols published by St.Louis *et al* (St. Louis *et al.*, 1999) and Ackerman and Cresswell (Ackerman and Cresswell, 2003). KG-1 cultures were observed by light microscopy for the production of "neurite-like" processes, cell adherence and cell death during the testing of each differentiation agent. In the very early phase, cultures initiated with 5 x10^4 KG-1 cells/ml and treated with 10 ng/ml PMA revealed very few cells producing "neurite-like" processes by light microscopy (0-1 differentiated cells per randomly selected field of vision). These results indicated that 10 ng/ml PMA was not potent enough to effectively generate DLCs and indicated the need to test higher concentrations of PMA (15 ng/ml, 20 ng/ml and 30 ng/ml PMA). However, these higher concentrations of PMA did not induce better differentiation compared to10 ng/ml PMA and therefore use of PMA alone as a differentiation agent was abandoned. In the second phase of the differentiation studies other combinations of differentiation agents together with 10ng/ml PMA were tested. The combinations tested were 10 ng/ml PMA + 100 ng/ml Ionomycin, 20 ng/ml PMA + 200 ng/ml Ionomycin and 10 ng/ml PMA + 10 ng/ml TNF-α. The combination of 10 ng/ml PMA + 100 ng/ml Ionomycin produced small neurite-like extensions on KG-1 cells after 3 days (3-5 differentiated cells per randomly selected field of vision) and cell viability was 90% at the end of the culture period. Extension of the culture period to 5 days did not result in any further cell death, and this differentiation

protocol was selected for further study. This effect of PMA + Ionomycin is in agreement with the findings of Teobald *et al*., 2007 and Berges *et al*, 2005. Other combinations (10 ng/ml PMA + 10 ng/ml TNF-α and 20 ng/ml + 200 ng/ml Ionomycin) produced extensive cell death and were therefore discarded as a possible model for generating DLCs.

For further analysis of the efficacy of the differentiation protocol, KG-1 cells were analyzed for cell surface marker expression, using markers chosen based on their usefulness for determining DC subset and their relevance for DC function. The cell surface markers analyzed were CD83, CD11c, CD123, CD86, HLA-DR and DC-SIGN. In the initial stages of experimentation only expression of CD83 and CD86 or CD80 was examined. This was because CD83 is a DC-specific marker and CD86 is a co-stimulatory molecule, important for DC activation of T cells. Analysis for CD80 expression was discontinued in the later assays because CD80 is usually not found on unstimulated APCs and also its upregulation is much slower than CD86 (Paul, 2005). CD83 expression appeared low on both 10 ng/ml PMA and 20 ng/ml PMA Differentiated KG-1s, but the entire KG-1 population in the culture wells was used to assay cell surface markers (Fig 13). DLCs are loosely adherent, requiring EDTA treatment for effective detachment from the culture plate surface. However, it was apparent that not all of the cells in the differentiated KG-1 cultures were adherent- rather a proportion remained in suspension, while others adhered to the surface of the plate. To determine whether these two populations represented differentiated and non-differentiated KG-1s, the samples were divided into adherent (bottom portion) and non-adherent (top portion) samples. The adherent cells were treated with 1μM EDTA to

slough them off the culture wells, and the two samples were analyzed separately by immunofluorescence and flow cytometry. Analysis of KG-1 cells differentiated with 10 ng/ml PMA + 100 ng/ml Ionomycin at 3 and 5 days showed an increased MFI value for both CD83 and CD86 on day 5 compared to day 3 (Fig 15). For this reason, a 5 day differentiation period instead of the 3 day period suggested by St. Louis (St. Louis *et al.*, 1999) was adopted.

To improve yields of adherent DLC, the initial KG-1 culture concentration was raised from 5 x10^4 cells/ml to 8 x 10^4 cells/ml. Analysis of the adherent and the non-adherent populations of 10 ng/ml PMA + 100 ng/ml Ionomycin-differentiated KG-1 cells did not show conclusive results using only CD83 and CD86 as cell surface markers. To gain a clearer picture of the phenotype of the differentiated KG-1 cells, a battery of cell surface markers was used. Expression of CD11c and CD123 (specific markers for mDC and pDC respectively) was elevated on PMA + Ionomycin- differentiated KG-1 cells relative to expression on KG-1 cells from untreated control cultures. When compared to unstimulated control KG-1s, expression of CD83 was found to be slightly but significantly raised on the PMA + Ionomycin differentiated KG-1 cells, showing 47.6% CD83 positive cells with an MFI of 128.

In a quest to obtain an ideal differentiating agent, atRA, a known stimulator of intestinal DCs (Hengesbach, 2004) was included among a battery of stimulators. Although atRA-treated KG-1 cells expressed elevated CD123 levels, they had very low CD83 expression. Furthermore the viability of the atRA-treated cultures decreased to

86% compared to the control culture viability of 90%. This led to the elimination of atRA as a useful differentiation agent. This result is consistent with an earlier study by Douer and Koeffler who observed that atRA at a concentration of 1µM/ml was lethal to KG-1 cells (Douer and Koeffler, 1981). They attributed the decreased viability of KG-1s in the presence of atRA to an unknown irreversible cytotoxic effect within the cell.

Probiotics have known immunomodulatory effects in the intestine (Mileti *et al*., 2009) and for this reason, strains of *Lactobacillus* were analyzed for their DC-differentiation capacity using the KG-1 model. The two strains tested were *Lactobacillus rhamnosus* R0011 and *Lactobacillus helveticus* R0052, due to their previously reported immunomodulatory activity (Wallace *et al*., 2003; Wood *et al*., 2007; Fiander *et al*., 2005). Cultures were initially carried out using either strain at a concentration of 2 $x10^8$ CFU/ml co-cultured with 5 $x10^4$ cells/ml of KG-1 cells for 5 days. *Lactobacillus helveticus* R0052 was eliminated from further study at this point, as the viability of KG-1 cells cultured with this strain was decreased to nearly 50% on day 1 and 12.3% after day 3. Viability of KG-1 cells treated with *Lactobacillus rhamnosus* R0011 however was 90% after day 5 in the initial set of results, making them an ideal choice as a differentiation agent. The DLCs obtained after KG-1 co-culture with strain R0011 showed increased CD83 expression along with a very high elevated levels of CD123 expression, suggesting a pDC phenotype. However, expression of the mDC marker CD11c was also increased along with HLA-DR, while DC-SIGN expression was very low (Data not shown for reasons discussed below). Analysis of the effects of the same strain of *Lactobacillus* at $2x10^4$ cells/ml in a later

series of experiments revealed decreased viability on day 3 prompting the reduction of the concentration of *Lactobacillus* to 1x 10^4 CFU/ml in subsequent co-cultures. Tests with *Lactobacillus rhamnosus* R0011 at 1 x10^4 CFU/ml after day 5 showed KG-1 cells with a viability of 92%. The cell surface marker expression of KG-1 cells treated with this lower concentration of R0011 remained high, apparently unaffected by the reduction in the number of probiotic bacteria added. This finding is in agreement with observations of Drakes *et al*., 2003 who reported that probiotic *Bifidobacterium longum, Bifidobacterium infantis, Bifidobacterium breve, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus delbreuckii subsp. bulgaricus, Lactobacillus plantarum and Streptococcus salivarius subsp. thermophilus* at 10^7 CFU/ml induced a marked rise in cell surface marker expression on bone marrow-derived DC after 5 days, a finding that lends further support to the utility of the KG-1 model as a useful alternative for freshly isolated human DC. The MFI for CD83 was 115.3 on R0011-treated KG-1 cells, while control KG-1 cells showed a MFI of 81.2 for CD83 expression. The Overton subtraction indicated R0011-treated KG-1 DLC were 76% positive for CD83 expression (Figure 23). The other cell surface marker that significantly increased in expression was CD123 with an MFI of 778.2 on R0011-treated KG-1 cells compared to an MFI of 612.3 on control KG-1 cells. Expression of CD11c and CD86 was not much different between 1 x10^4 CFU/ml R0011-treated and control KG-1 cells. Statistical analysis of cell surface marker expression by R0011-treated KG-1 cells indicated that expression of CD83 and CD123 and was significantly increased relative to untreated KG-1 cells.

Based on the cell surface marker expression the phenotype of DLCs obtained from PMA + Ionomycin differentiated KG-1s and also that of *Lactobacillus rhamnosus* R0011-differentiated KG-1s resembled that of pDCs, as CD123 expression was found to be higher than CD11c expression, as shown in Figures 21 and 18 (Della Bella *et al.*, 2008). To further confirm that the KG-1 DLCs are indeed of the pDC type, it would be useful to determine their ability to produce IFN-α. Detection of IFN-α can be done by ELISA or intracellular staining; however costs of anti-IFN antibodies were prohibitive. An alternative approach would be to examine IFN-α mRNA expression by RT-PCR. It has been reported that the KG-1 DLCs obtained by PMA + Ionomycin stimulation were immature DLC as they expressed low levels of CD83 on their surface (Teobald *et al.*, 2008; Berges *et al.,* 2005). These authors suggested that in order to further differentiate the cells into fully mature DC, stimulation with GM-CSF or TNF-α or a combination of both was required. Based on this observation, the KG-1 DLCs derived by either PMA + Ionomycin treatment or by R0011 treatment appear to be representative of immature human DCs.

Cytokine production by DLCs was also measured to further identify the subtype of DC generated, and to compare responses of PMA + Ionomycin-differentiated KG-1s to those differentiated by R0011. Production of IL-12, IL-10 and TNF-α by KG-1 DLCs generated by either PMA at 10 ng/ml + Ionomycin at 100 ng/ml or by *Lactobacillus rhamnosus* R0011 and stimulated with TLR-agonists were minimal. Cytokine production was tested at both 4 and 16 hours after addition of TLR agonists. While it is possible that longer incubation periods might be needed, this does suggest that the differentiated KG-

1s do not produce significant amounts of these cytokines. Although minimal, some IL-10 production was detected after TLR-agonist stimulation of R0011- differentiated KG-1s, and was highest at 4 hrs after LPS (0.1 μg/ml) or flagellin (10 ng/ml) addition. Interestingly, these results are consistent with earlier reports of induction of IL-10 production and CD83 expression following treatment of human blood monocyte-derived DC with Lactobacilli (Hart *et al.,* 2011).

IL-12 production was detected only when PMA + Ionomycin was used to differentiate into KG-1 DLCS, and was highest in response to stimulation with Poly I:C (TLR3 agonist), flagellin (TLR5 agonist), or LPS (TLR4 agonist). No IL-12 production was observed in response to the TLR7 agonist imiquimod. Analysis of undifferentiated KG-1 cells revealed minimal production of IL-12 and TNF-α in response to any of the TLR agonists tested. These findings are similar to the observations of Teobald *et al.,* (2007), although they did not examine the response to TLR agonists. Based on only IL-12 production, it could be deduced that the PMA + Ionomycin derived KG-1 DLCs are mDCs. This result is not conclusive however, as TNF-α was not detected in this group of KG-1 DLCs. Further analysis for IFN-α production would help in better classifying DC lineage. In addition, preliminary results indicate TGF-β production is induced by both PMA+ Ionomycin and R0011 differentiated KG-1s, a novel finding, as TGF-β production by KG-1 cells has not been reported elsewhere. This suggests that KG-1 DLC may provide a useful system to study effects of probiotics and TLR agonists on TGF-β production by DC, an aspect that is of interest in light of current research highlighting the

importance of this in DC regulatory activity, and in DC "conditioning" by the intestinal microbiota.

In conclusion, these results support the ability of the *Lactobacilli* to modulate surface molecule expression by KG-1 DLC and also influence their cytokine production ability. These results are in agreement with findings published by others examining effects of probiotics on human monocyte-derived DC (Hart *et al*., 2011; Drakes *et al*., 2003). Correlations can also be drawn between KG-1 DLC activity *in vitro* and *in vivo* DC activity based on cytokine production. *In vivo,* DCs appear to be activated by the gut microbiota and by probiotics, often inducing a regulatory phenotype. Certain strains of probiotic lactic acid bacteria have been observed to enhance IL-10 production and also up regulate CD86 expression by DCs, thereby skewing responses away from Th1 production (Hart *et al*., 2004; Rook and Brunet, 2004; Coombes and Powrie, 2008). DCs also regulate adaptive T cell and B cell responses in the intestine. PP DCs produce TGF-β, which promotes differentiation of naive B cells to IgA- producing plasma cells. TGF-β is released in an inactive (latent) form, which is activated by tissue plasminogen activator produced by $CD103^+$ DC found in the PP (Massacand *et al*., 2008). The results obtained here with respect to cell surface molecule expression, IL-10 and TGF-β production suggest that further study of the effects of *Lactobacillus rhamnosus* R0011 on KG-1 DLCs can provide a useful model to examine effects of probiotics and gut microbes on human intestinal DCs- a cell population that is ordinarily extremely difficult to obtain for study.

Suggestions for future study

In the future, analysis of cytokine production by differentiated KG-1 cells could be expanded to fully determine the effect of TLR-agonists on DLCs obtained by either PMA + Ionomycin or *L. rhamnosus* R0011 treatment. Determination of the TLR expression profile of differentiated KG-1 cells could also be carried out by immunofluorescence and RT-PCR. This would further clarify the classification of the DC phenotype of differentiated KG-1s to determine if they do fit into a typical mDC or pDC profile, or whether they may more accurately represent an intestinal DC phenotype. Furthermore the reasons behind the minimal production of cytokines should be assessed. It is possible that the time duration for cytokine production was too short, although this seems counter to reported times for cytokine production by DCs in response to TLR agonists in the literature. Analysis of IFN-α production would be valuable; however the cost of reagents for measurement by ELISA is prohibitive. Alternative strategies to determine if differentiated KG-1 DLCs do produce IFN-α could potentially be carried out using intracellular staining for IFN-α or by analysis of IFN-α gene expression by RT-PCR. Another goal of the development of the KG-1 DLC model is to use them to examine effects of bacterial components and products of lactic acid bacteria on DC activity, including effects of soy and milk ferments. The KG-1 DLC model can also be utilized to determine effects of probiotics or fermented products in simulated microgravity conditions using the High Aspect Ratio Vessel (HARV). It would also be interesting to compare the activity of the KG-1 DLCs with DCs isolated from human blood although this process poses several challenges. Finally the relative ease of handling and maintaining KG-1 cells together with their proven differentiation to DLCs by either

probiotics or chemicals has the potential to provide large quantities of DCs *in vitro* that are readily available for further manipulation and study.

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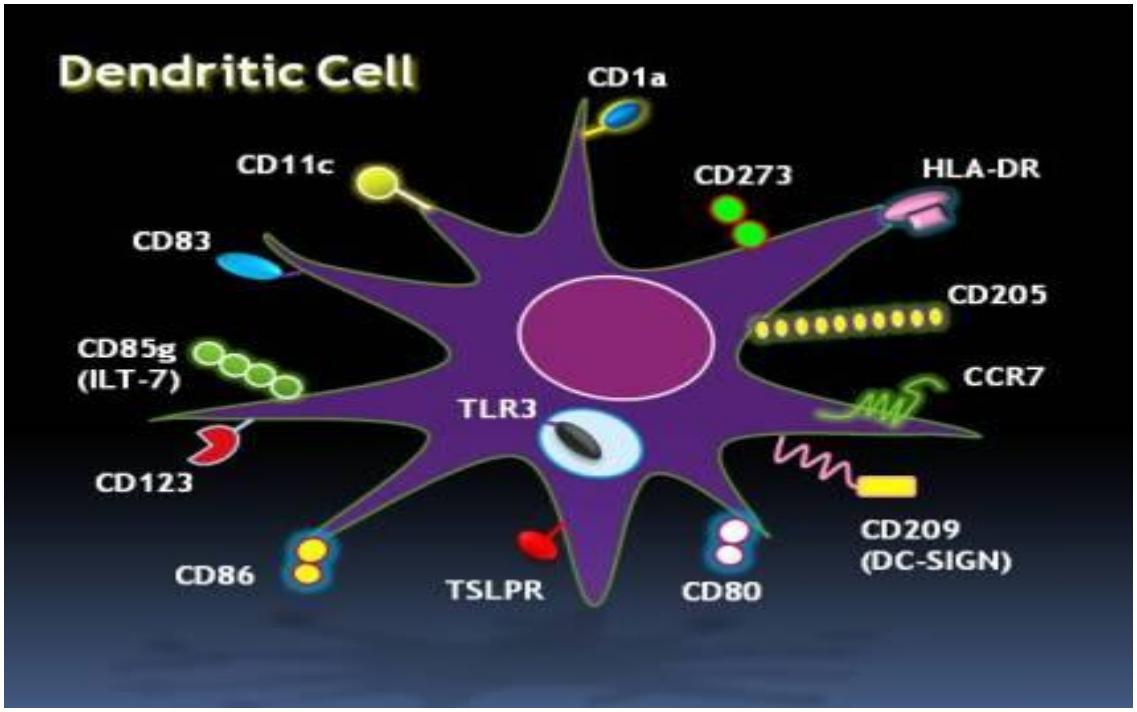
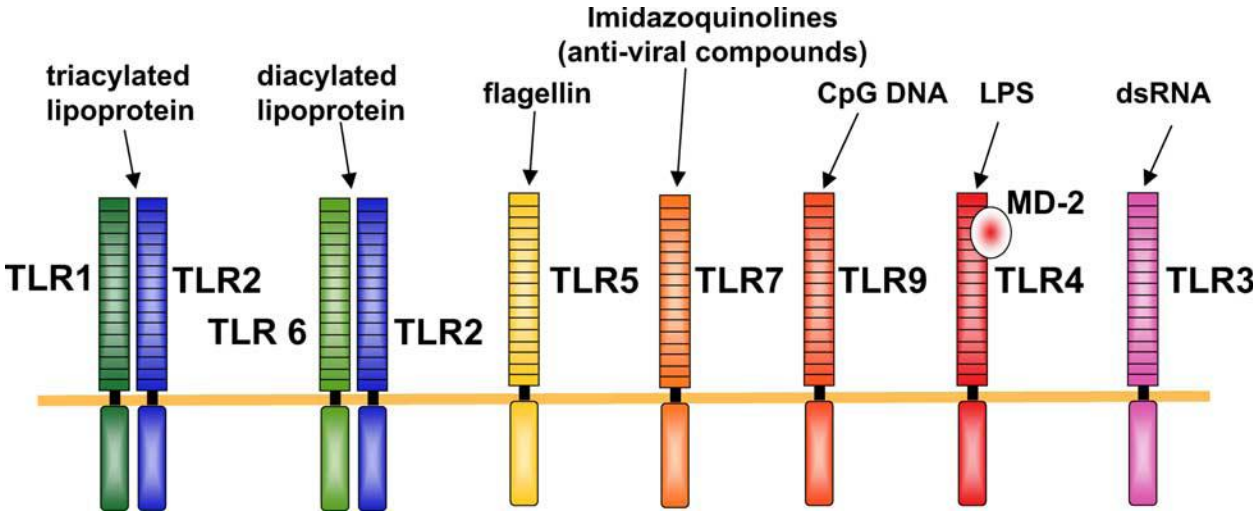
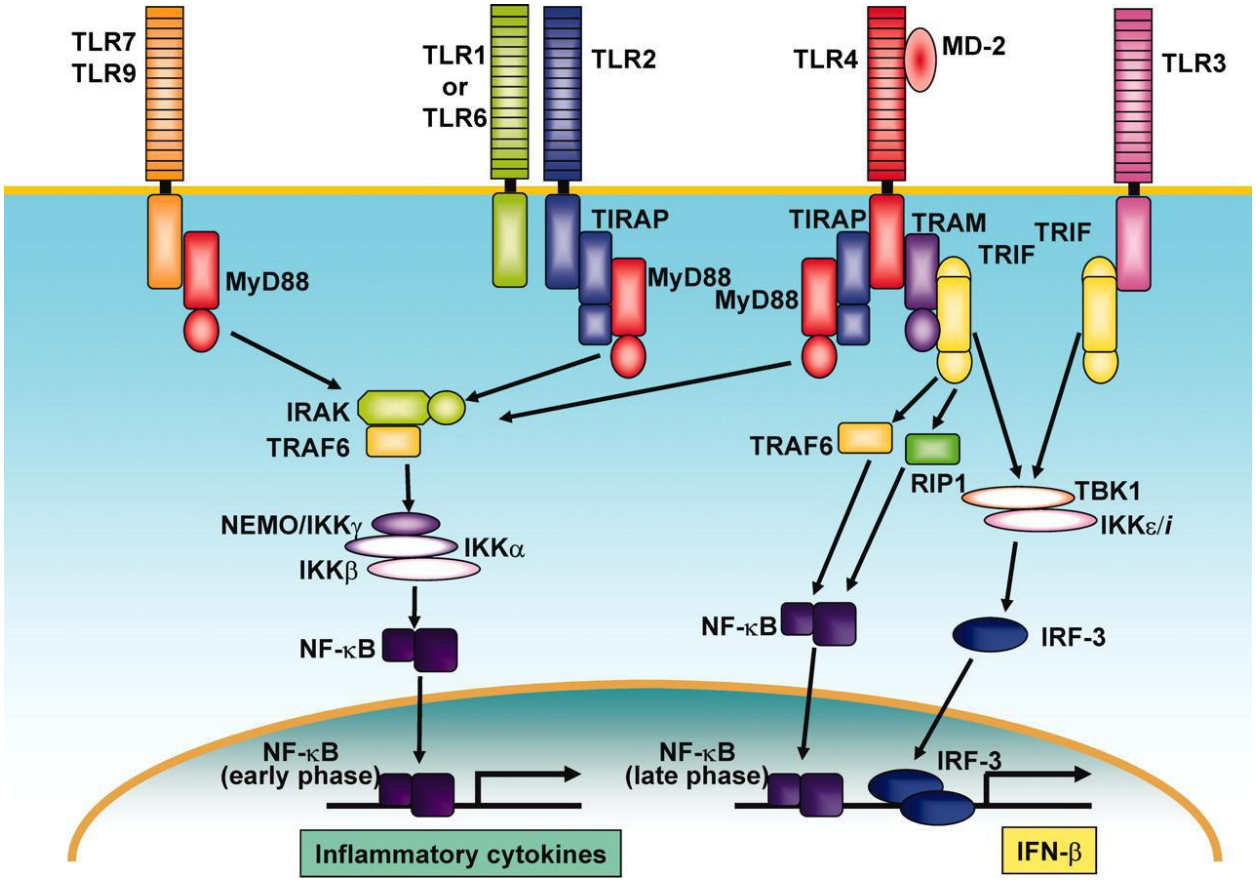
APPENDIX



Dendritic cell surface markers. http://www.biolegend.com retrived August 23, 2011



TLRs and their ligands. (Adapted from: Takeda and Akira, 2004)



TLR signalling. http://www.bioscience.org/2009/v14/af/3397/ retrived August 23, 2011