universität wien

DISSERTATION

Titel der Dissertation

"The effect of heat-inactivated Lactobacillus rhamnosus and Lactobacillus delbrueckii on maturation, expression of miRNAs and signaling factors in dendritic cells"

Verfasserin

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Angestrebter akademischer Grad

Doktorin der Naturwissenschaften (Dr.rer.nat)

Wien, 2012

Studienkennzahl lt.Studienblatt: A 091 474
Dissertationsgebiet lt. Studienblatt: Ernaehrungswissenschaften
Betreuer: Univ-Prof. Dr.Ibrahim Elmadfa

Dedicated with appreciation to

my husband

Dr. Ali Shahsavari

whose love , invaluable support and unfailing encouragement is the key for my achievement and brings balance, perspective , purpose as well as joy and laughter into my life

my Parents

who guided me in life, provided inspiration and strength at the most appropriate time.

Acknowledgments

First and foremost thanks to God for all the bless that never wanes and makes all the things possible

*

I'm deeply honored to have Professor Ibrahim Elmadfa as my supervisor. I would like to extend my sincere gratitude to him for giving me the chance of doing my PhD at university of Vienna and for encouraging and supporting me to focus on immunonutrition and probiotics which has truly opened to me a door to an interesting field in nutritional science

*

I have to convey my heartfelt appreciation to Dr. Ingrid Singer, for her extra support and assistance from the very beginning till the end. Without her patience and experience this achievement would have never come possible. I've been very fortunate to have such a wonderful angel in the department of nutritional science in Vienna.

*

My Special thanks go to Dr. AbolghasemDjazayery who kindly holds my hand in my scientific life and by his recommendation my PhD goal becomes true.

*

Many thanks to Dr. Petra Klein, who thought me all the basics of cell culture and facilitated the initial setup of my project.

*

I've to acknowledge Dr. Mostafa Hosseini for his sincere assistance in statiscal analysis of data.

*

I wish to acknowledge Dr. Alexander Haslberger for his tremendous advice and constructive guidance with genetic and epigenetic approach. Also his kind working group from whom I've learned a lot during the lab work.

*

I must remember to thank all the blood donors of this project and all the colleagues at department of nutrition particularly Dr. Alexa Meyer, Dr. Verena Nowak and Eva Aumueller for all their assistance and kind behavior that made staying in Vienna more pleasing for me.

*

I want to thank my Iranian friends Dr. Zahra abdolahi, Dr, Haleh Sadrzadeh, and Miss. Mansoore Tajaddod, whose presence was a bless during the days of missing home.

*

Finally my special praise goes to my sole dear sister, *Laila*, for deeply caring about me and helping me to see the lighter sides of life.

Thank you dear Laila, for always being with me by your warm thoughts and positive energy

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Abbreviations

APC	Antigen presenting cell
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
cDNA	complementary DNA
CFU	Colony Forming Unit
CSIF	Cytokine synthesis inhibitory factor
DC	Dendritic Cell
dNTP	Deoxynucleotidtriphosphat
EDTA	Ethylenediamine Tetraaceticacid
ERK	Extracellular Signal Regulated Kinase
FACS	Fluorescence – Activated Cell Sorter
FCS	Fetal Calf Serum
FITC	Fluorescein isothiocyanate
GAPDH	Glycerinaldehyd-3-phosphate Dehydrogenase
GM-CSF	Granulocyte macrophage colony stimulating factor
IFN	Interferon
IκB	Inhibitor of NFκB
IRAK	Interleukin Receptor Associated Kinase
LAB	Lactic acid bacteria
LPS	lipopolysaccharide
MAPK	Mitogen activated Protein Kinase
MHC	Major histocompatibility complex
miRNA	micro RNA
MODC	Monocyte derived dendritic cell

mRNA	messenger RNA
NFkB	Nuclear Factor kappa B
MRS	Mann rogosa sharp
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PBMC	Peripheral Blood Mononuclear cells
PBS	Phosphate buffer saline
PCR	Polymerase Chain Reaction
PE	R-Phychoerythrin
PerCP	peridinin Chlorophyll protein
TNF	Tumor necrosis factor
TLR	Toll Like Receptor
TRAF	TNF Associated Receptor Factor

1. Introduction & objectives

1.1 Introduction

Probiotic bacteria are well known for their supportive effects in human health, mainly boosting immune homeostasis by altering microbial balance and interacting with the immune system (Calder & Kew, 2002; Cummings, et al., 2004; Galdeano, et al., 2007). In spite of considerable body of information concerning the protective effects of probiotics, little is known about their basic molecular mechanisms by which they exert beneficial effects in viable or non-viable state. In fact, understanding the mechanism by which different forms of probiotics govern the type of immune response could be of crucial importance for development of novel approaches to immune therapy and generation of functional foods.

One of the general understandings is that dendritic cells (DCs) as the professional antigen presenting cells play a pivotal role in initiation and regulation of immune response by processing the microbial products and presenting them together with Major histocompatibility complex (MHC) proteins and co-stimulatory molecules to lymphocytes (Drakes, et al., 2004; Q. Huang, et al., 2001; X. L. Huang, et al., 2008; H. K. Lee & Iwasaki, 2007; Steinman, 2001). Therefore, the observed immunomodulatory effects of probiotic bacteria could result from the interaction between probiotic bacteria and DCs where they can provide maturation stimulus, along with secretion of cytokines and chemokines and ultimately determine the direction of immune response toward T helper cell type 1 (TH1), T helper cell type 2 (TH2) or tolerance by targeting DCs (Christensen, et al., 2002)

It is already known that different strains of probiotics can provoke very distinct DC maturation patterns and each strain seems to utilize different mechanisms to generate the immune response in a dose dependent manner. However, probiotics interaction with DCs is believed to lead to an immunoregulatory state rather than aggressive immune response (Foligne, et al., 2005; Smirnov, et al., 2002; Smits, et al., 2005; Smits, et al., 2001).

So far, most studies have focused on the effect and mechanism of action of viable strains or their cell wall components, but there is an increasing interest in the application of probiotics as microbiologically non-viable yet immunologically active products in the food industry and novel supplement production. Producing and transporting the biological products based on dead cells are definitely simpler. Indeed, they would be relatively easy to standardize and would have a long shelf life (Adams 2010).

So our primary focus in the present study was to investigate the adjuvanticity of the heat-inactivated form of two well documented and widely consumed probiotics i.e., *Lactobacillus rhamnosus GG (LGG)* and *lactobacillus delbrueckii* subsp. *bulgaricus (L.delbrueckii)* on immature human monocyte derived dendritic cells (MODCs). Pattern of expression of co-stimulatory molecules, in particular those from B7 family, as phenotypic DC maturation markers, as well as extracellular cytokine production were analyzed to assess the immune response of *LGG* and *L.delbrueckii* on DCs.

Moreover, it is well known that the immune response is initiated by discrimination of pathogens from commensal bacteria and probiotics via certain toll like receptors (TLR) (An, et al., 2002).Based on this initial recognition a network of complex of interacting downstream signaling cascades become activated which ultimately determine the fate of immune response. So the second focus of our study was to investigate if non-viable *LGG*

and *L.delbrueckii* influence expression of TLR4. Moreover, we investigated if the expression of, inhibitor of nuclear transcription factor NFκB (IκB), and P38 mitogen activated protein kinase (MAPK) as representative of signaling regulators in complex network of inflammatory signaling path ways are modified in probiotics- treated dendritic cells.

On the other hand an entirely new paradigm of immune regulation has been proposed after discovery of single-stranded small non-coding RNAs i.e., microRNAs (miRNAs) and identification of their role in immune homeostasis (Bartel, 2004). The regulation of miRNA expression is known to be tightly controlled and altered levels or temporal expression of a specific miRNA clearly affects the function of immune cells. So it was our third particular interest to look if certain miRNAs that participate in the fine regulation of immune response at post transcriptional level are affected by probiotics, which can provide epigenetic explanation for probiotics mechanism(Kuipers, et al., 2010). In this context we analyzed expression of two validated miRNAs for DCs i.e., miR-155 and miR-146a after 12 hours co-incubation with heat-inactivated *LGG* and *L.delbrueckii*.

1-2 Objectives

1-2-1 General objective

To determine if heat inactivated *Lactobacillus Rhamnosus GG (LGG)* and *Lactobacillus delbreuckii (L.delbrueckii)* are able to induce immune regulatory effect as their viable forms, through the putative mechanisms of action including regulation of phenotypic changes, cytokine production, expression of signaling factors and expression of miRNA in dendritic cells.

1-2-2 Specific objectives

- To determine the expression of co-stimulatory molecules involved in DC maturation and activation (CD 11c, CD 14, CD 54, CD 80, CD 83, CD 86, CD 209, HLADR) after being 24 hours treated with *LGG* and *L.delbrueckii*.
- To determine inflammatory cytokine (IL1-β, INF-γ, IL-12, Tumor necrosis factor (TNF-α) and anti-inflammatory cytokine (IL-10) levels produced from DCs after being 24 hours treated with *LGG* and *L.delbrueckii*.
- To determine the expression of major signaling elements (TLR4, IKB,P38) in DCs after 12 hours co-incubation with *LGG* and *L.delbrueckii*.
- To determine the expression of miR146a and miR155 in DCs after 12 hours co-incubation with *LGG* and *L.delbrueckii*.
- To compare the effect of inactivated *LGG* and *L.delbrueckii* in induction of CD 11c, CD 14, CD 54, CD 80, CD 83, CD 86, CD 209, HLADR expression

- To compare the effect of inactivated *LGG* and *L.delbrueckii* in production of inflammatory cytokine (IL1-β, INF-γ, IL-12, TNF-α) and anti-inflammatory cytokine (IL-10)
- To compare the effect of *LGG, L.delbrueckii* and lipopolysacharide (LPS) in inducing expression of TLR4, p38 and IKB.
- To compare the effect of LGG, L.delbrueckii and LPS in inducing expression of miR7i, miR146a and miR155.

1-2-3 Applied objective

- To modify the general accepted definition for probiotics by omitting the necessity of probiotics to be viable for inducing functional effects.
- To provide evidence for administration of heat-killed probiotics which allows longer product shelf life, easier storage, transportation and more convenient and safer consumption in form of dietary supplements and food products

1-3 Hypothesis

Heat inactivated Probiotic bacteria can induce immune-regulatory effect through maturation of dendritic cells, regulating expression of signaling factors and expression of the responsible non-coding RNAs in immune cells as the viable probiotics.

2. Literature Overview

2.1 Probiotics

2.1.1 Discovery and definitions

The word probiotic (pro-"bios",ie. Pro life) was first proposed for "organisms and substances which contribute to intestinal microbial balance (Parker, 1974). However, it was Metchnikoff's theory which gave birth to the concept of probiotics almost a century ago. This Nobelist suggested that the long healthy life of Bulgarian peasants resulted from consumption of fermented milk products. In his famous book "*The prolongation of life"* he stated that "The dependence of the intestinal microbes on food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes" (Caramia, 2008).

Over years many definitions have been proposed for probiotics and ultimately an expert panel commissioned by the Food and Agriculture Organization of the United Nations and the World Health Organization in 2001 characterized probiotics broadly as "***live nonpathogenic microorganisms which, when administered in adequate amounts, confer a health benefit on the host"***. Of course, this definition has not received the universal acceptance, due to open delicate points of discussion related to the site of activity, the viability of the probiotics strain, the concentration of cells necessary to exert the specified probiotics effect, the use of mono– or mixed cultures, the format of intake and its carrier, and finally its functionality beyond the inherent basic nutrition(Reid, et al., 2003).

Probiotic bacteria consist mainly of strains of *Lactobacillus, Bifidobacterium, and Streptococcu*(Klein *et al*., 1998). Lactobacilli are often part of the human intestinal

ecosystem, but highly variable amounts are found. Probiotics have no necessary phylogenic relation to one another and are therefore best defined functionally rather than structurally.

Expected characteristics of potential probiotics strains are listed in table 2.1.1

Table 2.1.1 characteristics of potential probiotics strains

Characteristics of Potential Probiotics Strains
Non toxic and non pathogenic
Accurate taxonomic identification
Normal inhabitant of the targeted species
Capable of survival, proliferation and metabolic activity in the target site, which implies:
Resist to gastric acid and bile
Able to persist, albeit for short periods, in the gastrointestinal tract, adherence potential preferred
Able to compete with the resident flora
Able to produce antimicrobial substances
Antagonism (*in vivo*) towards pathogenic bacteria
Able to modulate immune responses
Able to exert at least one clinically documented health benefit
Genetically stable
Amenability of the strain and stability of the desired characteristics during processing, storage and delivery
Viability at high populations
Desirable organoleptic and technological properties when included in fermentation processes

2.1.2 General Effects and Functions of Probiotics

The remarkable phenotypic and genotypic variability among isolates belonging to a well–established species make it impossible, at present, to generalize probiotics efficacy. Although it is tempting to speculate that one species will mediate several specific effects, and that different strains of a particular species have comparable effects, research results do not support this conclusion. Indeed it is unlikely that single strains of *lactobacilli, Bifidobacterium* and other will bear the multitude of proposed benefits. As head-to-head comparison on strain-, species- or genus level are very rarely done, generalizations about probiotics performance on the genus and species level are impossible to make (Scarpellini, et al., 2008).

The evidence in support of the efficacy of probiotic bacteria varies from circumstantial to convincing (Level 1) using evidence-based medicine techniques. Level I evidence now exists for the therapeutic use of probiotics in infectious diarrhea in children, recurrent *Clostridium difficile* induced infections, and postoperative pouchitis (Hilton, et al., 1997; Kuisma, et al., 2003; Marteau, et al., 2001). Level II evidence is emerging for the use of probiotics in other gastrointestinal infections, irritable bowel syndrome, and ulcerative colitis(Collado, et al., 2009).

The best effect of probiotics is known to be achieved when the microorganisms colonize the intestinal surface mucus layer, however they can then affect the intestinal immune system, displace enteric pathogens, and provide antioxidants, anti-mutagens, and possibly other effects by cell signaling. While most probiotics exert their effects when introduced

into the gastrointestinal tract, some exert their effects through systemically administered components (Borchers, et al., 2009; Castillo, et al., 2011).

Broad reading of the probiotics literature reveals that different effects of probiotics have been associated mostly to their unique capacities to express particular surface molecules (glycolipids, microbe-associated molecular patterns) or to secrete products that have the ability to interact with the various components of the epithelial cells forming the epithelial barrier or cells in the immune system that lie under this barrier. Some of these properties are observable only with viable probiotics while the others are also observable with dead probiotics or components derived from dead organisms (Corthesy, et al., 2007; Elmadfa, et al., 2010; Liong, 2007; Matsuzaki, et al., 2007; Prisciandaro, et al., 2009).

Beneficial health effects of probiotics and the proposed mechanisms are summerized in table 2.1.2

Table2. 1 Beneficia Health l effects attributed to lactic acid bacteria

Health benefit	**proposed mechanism(s)**
Alleviation of lactose intolerance	Bacterial β-galactosidase acts on lactose
Positive influence on intestinal flora	Lactobacilli influence activity of over growth flora, decreasing toxic metabolite production Antibacterial characteristics
Prevention of intestinal tract infections	Adjuvant effect increasing antibody production Stimulation of the systemic or secretory immune response Competitive exclusion Alteration of intestinal conditions to be less favorable for pathogenicity (pH, short chain fatty acids, bacteriocins) Alteration of toxin binding sites Gut flora alteration

	Adherence to intestinal mucosa, preventing pathogen adherence
	Competition for nutrients
Reduction of inflammatory or allergic reactions	Restoration of the homeostasis of the immune system
	Regulation of cytokine synthesis
	Prevention of antigen translocation into blood stream
Anti-colon cancer effect	Mutagen binding
	Carcinogen deactivation
	Alteration of activity of colonic microbes
	Immune response
	Influence on secondary bile salt concentration
Blood lipids, heart disease	Assimilation of cholesterol
	Alteration of activity of bile salt hydrolase enzyme
	Antioxidative effect
Antihypertensive effect	Peptidase action on milk results in antihypertensive tripeptides (angiotensin converting enzyme inhibitors)
	Cell wall components act as angiotensin converting enzyme inhibitors
Urogenital infections	Adhesion to urinary and vaginal tract cells
	Competitive exclusion
	Inhibitor production (H2O2, biosurfactants)
Infection caused by Helicobacter pylori	Competitive exclusion
	Lactic acid production
	Decreased urease activity of *H. pylori* in humans after administration of a supernatant of a *Lactobacillus* culture
Regulation of gut motility (constipation)	
Feeling of well-being	

2. 1.3 *Lactobacillus GG:* Unique Native Bacterium in Human GI Tract

Lactobacillus rhamnosus GG (LGG) is a facultatively heterofermentative lactic acid bacterium and is frequently isolated from human gastrointestinal mucosa of healthy individuals (Verdenelli, et al., 2009). This strain was found in 1983 while Professor Sherwood Gorbach and Barry Goldin were searching for a strain of lactobacillus that could colonize the human intestine and thereby exert the beneficial effects which Metchnikoff had hoped to produce by his yogurt cultures. Finally *Lactobacillus rhamnosus GG* filled for patent on 17th April 1985, by Sherwood Gorbach and Barry Goldin, the 'GG' derives from the first letters of their surnames (Silva, et al., 1987). The patent refers to a strain of "*L. acidophilus GG*" with American Type Culture Collection (ATCC) Accession No. 53103; later reclassified as a strain of *L. rhamnosus*. The patent claims that the LGG strain is acid and bile stable, has a great avidity for human intestinal mucosa cells and produces lactic acid.

Lactobacillus rhamnosus was originally considered to be a subspecies of *L. casei*, but later genetic research found it to be a species of its own(Avlami, et al., 2001). Indeed, the full genome sequence of this strain was reported in 2009 (Morita, et al., 2009). *LGG* is more likely a transient inhabitant, not autochthonous (Walter, 2008).

Today, *LGG* is the best-studied and most extensively documented probiotic lactic-acid bacteria strain in the world. *L. rhamnosus* is used as a natural preservative in yogurt and other dairy products to extend the shelf life. *LGG's* most well known effects in human revolve around its ability to influence digestive tract health by stabilizing human intestinal microflora and hastening the removal of pathogenic microorganisms (Lam, et al., 2007). *Lactobacillus rhamnosus GG* has been studied extensively for its immune-enhancing

properties. *LGG* can influence immune responses both specifically by stimulating antibody production and nonspecifically by enhancing phagocytosis(Forestier, et al., 2001; Guarino, et al., 2008). It also modifies production of cytokines (Cross, et al., 2002; Pessi, et al., 2000). New results are indicating that LGG can affect the immune system in a variety of populations. Moreover, it can affect the health of organs far apart from the intestinal tract (Pena & Versalovic, 2003; Schultz, et al., 2003).

The effect of on e month treatment with *LGG* was elegantly shown by microarray analysis. Up-regulation of 334 g enes and down-regulation of 92 genes involved in inflammation, apoptosis, cell-cell signaling, cell adhesion and differentiation and signal transcription and transduction clearly show the influences of lactic acid bacteria (LAB) on multiple systems (Di Caro, et al., 2005).

While frequently considered a b eneficial organism, *L. rhamnosus* has been discovered to be pathogenic in certain circumstances (Avlami, et al., 2001).

2.1.4 Lactobacillus Delbrueckii:

Lactobacillus delbrueckii are gram positive, facultative anaerobic, non motile and non spore forming, rod shaped members of the industrially important lactic acid bacteria. The bacterium has complex nutritional requirements, including the inability to ferment any sugar except lactose. The *L. delbrueckii* species contains three subspecies: *L. delbrueckii* subsp. *delbrueckii*, *L.delbrueckii* subsp *lactis* , and *L.delbrueckii* subsps, *bulgaricus.*

L.delbrueckii subsps. Bulgaricus was first identified on 1905 b y Bulgarian doctor Stamen Grigorov, is named after Bulgaria, and is utilized as a component of thermophilic starter cultures alongside *Streptococcus thermophilus* in manufacturing a number of fermented dairy products (Hassan, et al., 2001). Now *L.bulgaricus* is one of the economically most

important representatives of the heterogeneous group of lactic acid bacteria with values of more than $2.1 billion only in United States due to its application in producing special yogurt aroma by fermenting milk and producing acetaldehydes.

The complete genome sequence of *Lactobacillus bulgaricus* is also reported (van de Guchte, et al., 2006).

A well documented health benefit of consumption of yogurt containing live *L. bulgaricus* and *Streptococcus thermophilus* is shown in attenuation of lactose intolerance (Mercenier, et al., 2003; Reid, et al., 2003). In addition, immune modulation and diarrhea-alleviating effects have been reported (Adolfsson, et al., 2004), and both *L. bulgaricus* and *S. thermophilus* have been implicated in these effects (Galdeano & Perdigon, 2004; Guarner, et al., 2005). A positive effect of *L.bulgaricus* is reported in prevention of autoimmune conditions (Kano, et al., 2002).

2.2. Dendritic Cells: Gatekeepers and orchestrators of immune response

The term "dendritic cells" was first proposed by Steinman in 1973 for a novel cell type which has been identified in adherent cell populations prepared from mouse peripheral lymphoid organs (spleen, lymph node, Peyer's patch). Though present in small numbers (0.1-1.6% of the total nucleated cells) the cells presents distinct morphological features. The nucleus is large, retractile, contorted in shape, and contains small nucleoli. The abundant cytoplasm is arranged in processes of varying length and width and contains many large spherical mitochondria. In the living state, the cells undergo characteristic movements, and unlike macrophages, do not appear to engage in active endocytosis (Steinman & Cohn, 1973). Further research revealed that these cells with versatile functions are widely distributed as immature cells within all tissues, particularly those that

interface with the environment (*e.g.* skin, mucosal surfaces) and in lymphoid organs (Wakabayashi, et al., 1997).

Focusing on DCs functionality was started in 1979 by Steinman et al. when they obtained sufficient quantity of cells with high degree of purity from spleen. Functional studies became even more feasible since 1992 after development of new methods which enabled generation of large number of DCs from their progenitors in large numbers (Cella, et al., 1997; Chapuis, et al., 1997; Shortman & Caux, 1997; Steinman, et al., 1979).

Although antigens and lymphocytes (B cells, T cells, NK cells) have long been focused as the mediators of immune response, accumulating evidence demonstrate that DCs provide vital link between antigens and all type of lymphocytes. Several investigations persisted in different laboratories lead to the current acceptance that the most distinguishing feature of DC is its extraordinary ability to take up and present peptides and proteins to T and B lymphocytes. Therefore DCs are known as the most efficient antigen-presenting cells (APCS) which take up antigens and pathogens, generate MHC-peptide complexes, and migrate from the sites of antigen acquisition to secondary lymphoid organs and, finally physically interact with and stimulate T lymphocytes. These unique subsets of leukocytes which are equipped with an array of pattern recognition receptors for microbial structures, orchestrate the functional outcome of T cell activation by high expression of various co-stimulatory molecules depending on the type of DC presenting antigen and the physiological conditions in which DC: T cell interactions take place. Indeed, these cells are the only antigen-presenting cells that induce the activation of resting T cells, both in vitro and in vivo. Besides eliciting immune response DCs are equally responsible for central and peripheral immune tolerance, which silences dangerous immune cells and

prevents them from attacking innocuous materials in the body or the body's own tissue and also induce regulatory T cells that suppress the reactions of other immune cells. Thus, dendritic cells initiate adaptive immune responses and determine tolerance (Banchereau & Steinman, 1998; Y. Li, et al., 1997; Thery & Amigorena, 2001; Zanoni & Granucci, 2011). To do so, dendritic cells have developed unique membrane transport pathways. However, the molecular events controlling DC function are not yet fully understood and their transcriptional control of critical genes is an important new area (Connolly & Kusner, 2007; Gatti & Pierre, 2003; Rolph, et al., 2006; Shklovskaya & Fazekas de St Groth, 2007).

2.2.1 Dendritric cells origins and subsets create functional complexity: Puzzels and paradoxes

It was first believed that all DCs are derived from hematopoietic stem cells in the bone marrow, but it is also shown that many individual tissues generate their own DCs locally, from a reservoir of immediate DC precursors, rather than depending on a continuous flux of DCs from the bone marrow. Dendritic cells form heterogenous cell population can be broadly categorized based on the expression patterns of a number of cell surface markers into myeloid-derived, lymphoid-derived, dermal-derived, and plasmacytoid DC (Fig 2.1). DC should also be subdivided into migratory DCs, which reside in peripheral tissues, or lymphoid organ resident DCs (Hart, 1997).

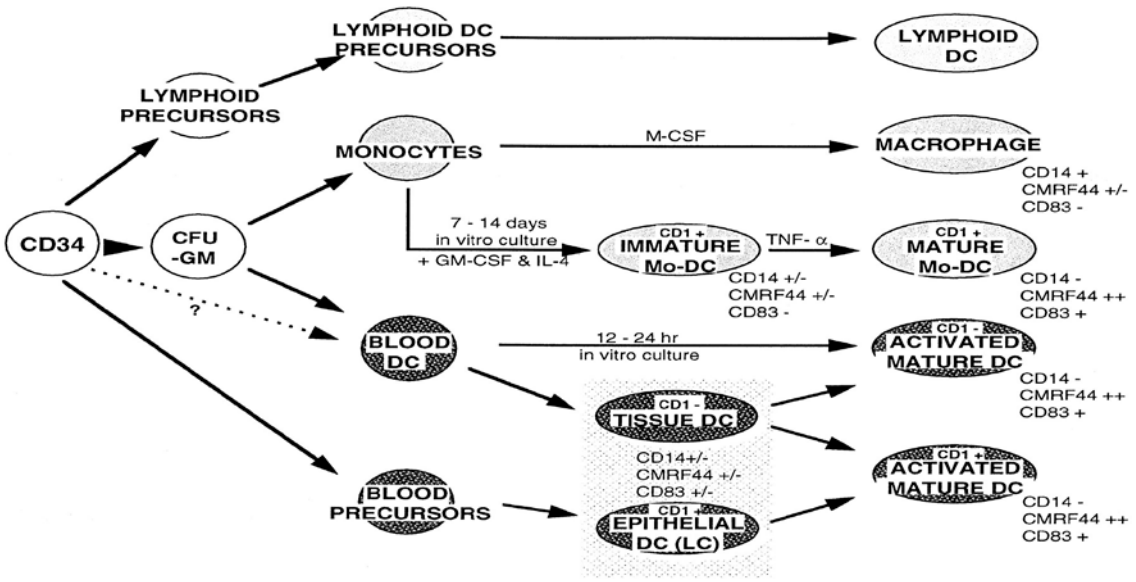

Figure2. 1 A putative hematopoietic differentiation pathway for myeloid and lymphoid DC(Hart, 1997)

The presence of multiple DC subsets may be specialized for certain functions including induction of both immunological tolerance and effective immuno-stimulation as mentioned earlier. However, the clear cut-cut view of functional specializing among DC subsets is unsustainable due to both functional diversity and plasticity of DC subsets. For example CD 8^{+} DCs in mice, display both immunoregulatory or tolerogenic induction ability, but also exhibit the strongest ability to induce T helper1 response by secreting high amounts of IL_12. In addition DCs exhibit great functional plasticity, which is induced by different tissue environments, response to different microbial stimuli or the duration of DC activation. So we have two distinct models, on one hand we have DC lineage model that suggest that phenotypically and functionally defined DC subsets happens early in hemopoesis, so that DC subsets develop as separate lineage with distinct functions and on the other hand functional plasticity of DC subsets represent different activation states or alternative cell fate of a single lineage dictated by local environment. Actually, expression

of a r ange of markers of heterogeneous DCs in human blood reflects differences in maturation or activation state of DCs, rather than delineating separate sub lineages. Functional plasticity of human DC subset when exposed to different cytokine or pathogens makes it d ifficult to assign a fixed function to a particular DC lineage (Ardavin, 2005; Arima, et al., 2010; Ito, et al., 2005).

It should be mentioned that the developmental pathways that lead to the production of DCs are beginning to be understood. The existence of many specialized subtypes of DC has complicated this endeavor, as has the need to distinguish the DCs formed in steady state from those produced during an inflammatory response.

Although the development of DCs from early hematopetic precusors is not fully understood, terminal stages of DC development and their life cycle during an immune response are well defined (Bharadwaj & Agrawal, 2007; Granucci & Zanoni, 2009) .

2.2.2.1 In vitro generation of dendritic cells

There are several models for generating dendritic cells from monocytes or CD34 +human stem cells and also generating plasmacytoid dendritic cells. However, blood monocytes are the most commonly used precursor cells for the generation of human DCs in vitro (Romani, et al., 1994; Thomas & Lipsky, 1994).

It is known that monocytes can differentiate in the presence of the exogenous cytokines granolocyte macrophage colony stimulating factor (GM-CSF) and Interleukin-4 (IL-4) in to immature Dcs after 6 to 8 days (Kim, Hersh et al. 2003). These cells are shown to express characteristic surface markers such as MHC class I and II, CD 1a, CD 11b, CD 40, CD 80, CD 86 and CD 54 (i.e. ICAM-1).

Though, it is hypothesized that monocytes may also comprise a population of DC precursors, especially in inflammatory reactions. Thus, circulating monocytes, once recruited in to inflamed tissue, after taking up antigen, may develop in to DCs (Randolph, et al., 1999). Mast cells at inflammatory sites may provide a possible source for cytokines needed for the monocytes to differentiate (Mekori & Metcalfe, 2000).

However, at this point DCs are easily available for cellular and molecular studies, it is unclear how much differentiation of monocytes into DC occurs *in vivo* compared with the differentiation of Mo-DC induced by cytokines *in vitro* DCs. Indeed the correlation between the DCs generated in culture and naturally occurring DC subsets *in vivo* is not clear.

2.2.3 Dendritic cells transformation from immature to mature state: Initiation of immune response

DCs undergo three or more stages to become fully active APC:

First, DCs are activated by variety of factors via TNF- α receptor super family and Toll-like receptors (C. Brunner, et al., 2000; T. Kaisho & Akira, 2003).

These stimulating factors can be:

- Whole bacteria
- Bacterial-derived antigens (*e.g.* lipopolysaccharide, LPS)
- bacterial CpG DNA
- Inflammatory cytokines and other activation signals received from the surrounding microenvironment
- Ligation of select cell surface receptors (*e.g.* CD40)
- Ligation of viral products (*e.g.* double-stranded RNA).

- Conversion of precursors of ligands for nuclear receptors (PPARɣ , RA receptor, Vitamin D receptor and glucocorticoid receptor) to active hormones by DC itself

Second, initial adhesion and antigen-specific recognition takes place.

Third, full activation of DC via different signaling cascades, including activation of nuclear factor kappa B (NF-κ B), p38 mitogen activated kinase (MAPK) and c-Jun N-terminal kinase (JNK) pathways (J. Y. Lee, et al., 2009; S. Li, et al., 2002). These cascades are followed by costimulatory molecules activity and cytokine production. It should be mentioned that activated DCs have short life span, they undergo apoptosis in order to protect body from over stimulation (Banchereau, 2002) (Fig 2.2 & Figure 2.3).

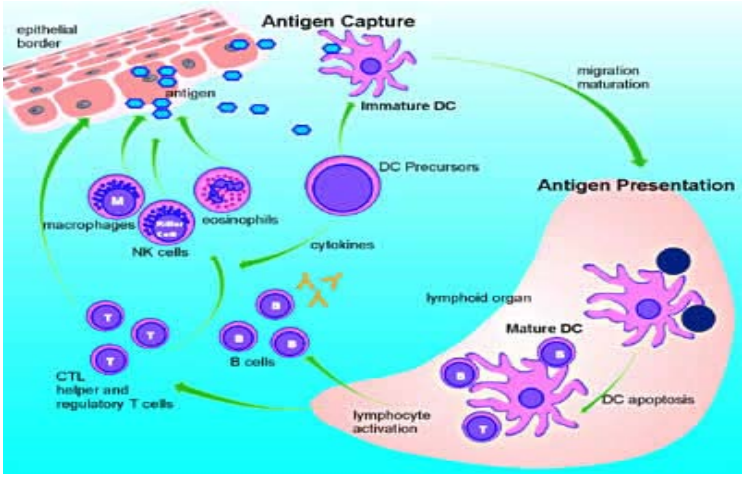

Figure2. 2 Life cycle of dendritic cells. Progenitors in bone marrow give rise to immature DCs with high phagocytotic potential that are recruited to tissues. Upon antigen uptake, cells migrate to lymphoid organs and mature to potent immunostimulatory antigen presenting cells (Banchereau, 2002).

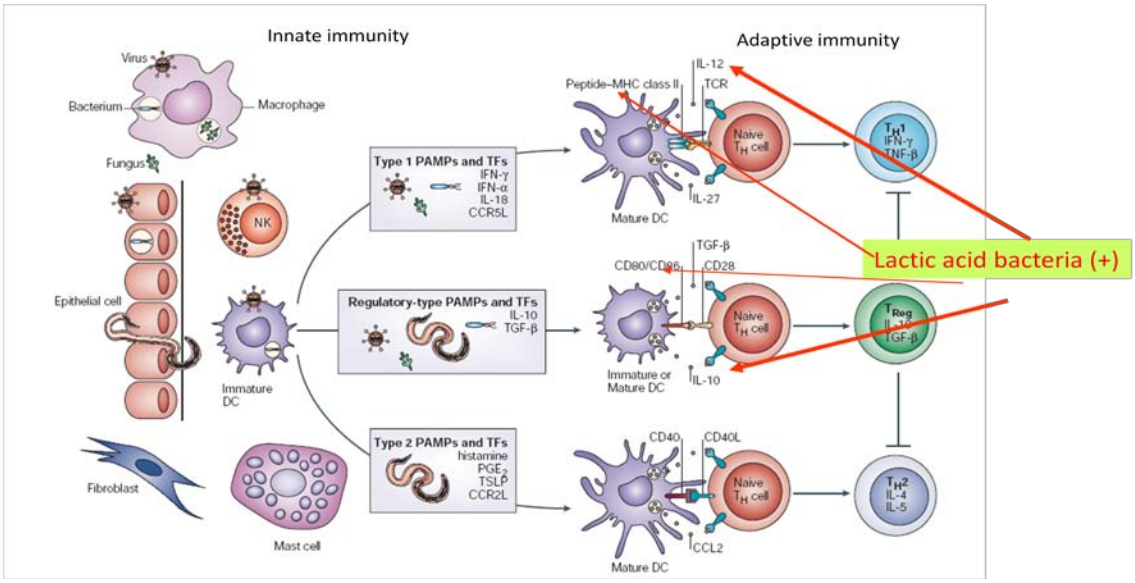

Figure2. 3 The type of signal that leads to DC activation influences the outcome of immune responses

It is now clear that the maturation of DCs is crucial for the initiation and regulation of immunity while in most tissues, DCs are present in a so-called 'immature' state and are unable to stimulate T cells. Although these DCs lack the requisite accessory signals for T cell activation, such as CD40, CD54, CD80 and CD86, they are extremely well equipped to capture Ags in peripheral sites. Immature DCs have features other than phagocytosis that allow them to capture picomolar and nanomolar concentration of Ag, much less than the micromolar levels typically required by other APCs. They also express receptors that mediate adsorptive endocytosis, including lectin receptors like the macrophage mannose receptor and DEC-205, as well as Fcg and Fce receptors which function very effectively in immature DCs. However, once DCs have captured Ags, their ability to capture more Ag rapidly declines (Bharadwaj & Agrawal, 2007; Granucci & Zanoni, 2009; Zanoni, et al., 2009).

DCs are able to produce large amounts of MHC class II-peptide complexes. This is due to the specialized, MHC class II rich compartments (MIICs) that are abundant in immature DCs. During maturation of DCs, MIICs convert to non-lysosomal vesicles and discharge their MHC-peptide complexes on to the cell surface. As mentioned earlier the maturation of DCs is completed only upon interaction with T cells which is characterized by loss of phagocytic capacity and expression of many other accessory molecules such as LFA-3/CD58, ICAM-1/CD54, B7-1/CD80, B7-2/CD86 and CD83 that interact with receptors on T cells to enhance adhesion and signaling. Expression of one or both of the costimulatory molecules B7-1 (CD80) and B7-2 (CD86) on the DCs are essential for the effective activation of T lymphocytes, and for IL-2 production. Other phenotypical and functional changes of DCs during the process of conversion from immature to mature state are summarized in table 2.21.

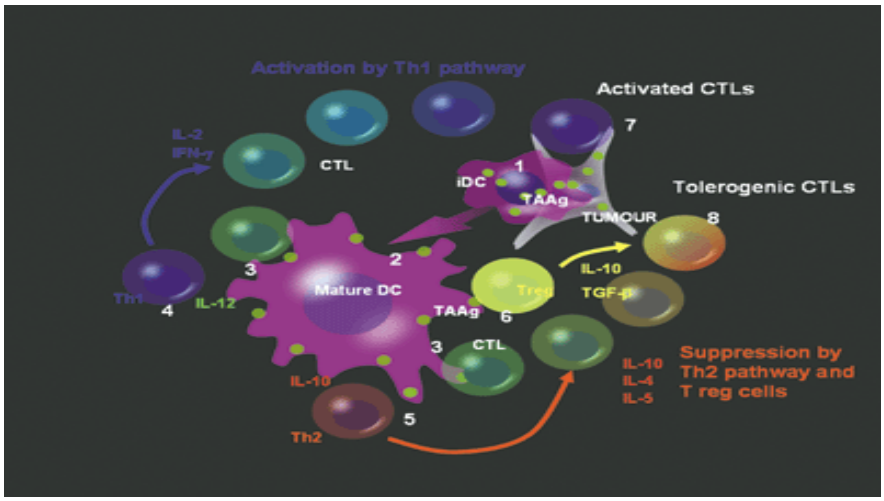

Figure2. 4 Example of Regulation of Immune Responses by Dendritic Cells **1. Immature dendritic cells can endocytose Antigen. 2. Maturation of dendritic cell as it travels toward the secondary lymphoid compartment is heralded by presentation antigen on DC surface. 3. Mature DC presents antigen to naïve T cells, stimulating generation of CTLs 4. Th1 lymphocytes are produced by DCs secreting IL-12. Th1 cells activate CTLs by secreting a range of cytokines, including IL-2 and IFN-.. 5. However, DCs which secrete IL-10 will stimulate production of Th2 cells. Th2 responses are characterised by secretion of IL-10, IL-4 and IL-5. These cytokines down-regulate cell mediated immune responses. 6. Stimulation of Treg cells by DCs further down-regulates cell mediated immunity due to secretion by Treg cells of IL-10 and Transforming growth factor (TGF-ß). 7. Th1 responses are characterised by stimulation of cell-mediated immune responses and enhanced tumour cell killing. 8. Th2 and Treg effects, however, lead to T cell anergy and tolerance of tumour cells (McKechnie, et al., 2004)**

Table2.2 1 Phenotypic and functional changes of DCs in immature and mature state

Characteristics of immature dendritic cells	Characteristics of mature dendritic cells
High intracellular MHC II in the form of MIICs	Morphological change: formation of dendrites
Expression of CD1a	Motility: Active process formation and movement
High expression of chemokine recptors CCR1, CCR2,CCR5,CCR6 and CXCR1	Change in expression of CCR6 and CCR7
Antigen capture: Macrophage mannose receptor (MR),type 1c- type lectin redeptor(DEC-205) type II C-type lectins important for receptor-mediated antigen uptake include DC immunoreceptor, DC-associated C-type lectin-2 and C-type lectin receptor 1	DCIR, MR, and FceR are all down-regulated upon DC maturation, further emphasizing their specific roles in antigen uptake in immature DCs
Active endocytosis for certain particulates and proteins; presence of FcgR and active phagocytosis	
Deficient T cell sensitization in vitro	Antigen presentation: High MHC class I and II expression
Low/absent adhesive and costimulatory molecules (CD40/54/58/80/86)	Abundance of molecules for T cell binding and costimulation, (e.g. CD40, CD54/ICAM-1, CD58/LFA-3, CD80/B7-1 and CD86/B7-2)
Low/absent CD25, CD83, p55, D EC-205, 2A1antigen	Cytokines: Abundant IL-12 production; resistance to IL-10 DC-restricted molecules: p55, CD83, S100b

2.2.4 Dendritic Cell and expression of CD markers

There is no s ingle universally accepted hallmark of dendritic cell activation. Instead a variety of methods are used to identify APCs and assess their activation state. One of these activation measures includes phenotypic methods by assessing the increased expression of surface markers.

The cluster of differentiation (CD) is a protocol commonly used for the identification and investigation of cell surface molecules present on blood cells. CD molecules can act in numerous ways, often acting as receptors or ligands or have other functions, such as cell adhesion. These markers are often used to associate cells with certain immune functions or properties. While using one CD molecule to define populations is uncommon, combining markers has allowed for cell types with very specific definitions within the immune system. CD molecules are utilized in cell sorting using various methods including flow cytometry (Zola, et al., 2007).

Differentiated dendritic cells are $CD80^{+}$, $CD86^{+}$, $CD83^{+}$, $CD54^{+}$, $CD1a^{+}$, $CD11b^{+}$, $CD11c^{+}$, HLA-DR^{+} , $CD34^{-}$, $CD3^{-}$, $CD19^{-}$, $CD14^{-}$, and $CD16^{-}$.

2.2.4.1 CD 11c

CD11c a member of the leukointegrin family, is expressed on the plasma membranes of monocytes, tissue macrophages, NK cells, and most dendritic cells (Ruedl, et al., 1996). As a result of its high level of expression on most DCs, CD11c is considered a marker of these dedicated APC(Metlay, et al., 1990). Although involvement of CD11c in diverse functions of DC is relatively unexplored, its role in antigen presentation has been revealed by some studies (Meunier, et al., 1994).

2.2.4.2 CD 14

CD14 is a surface antigen expressed strongly on monocytes and is anchored to the cell surface by linkage to glycosylphosphatidylinositol(Tobias, et al., 1993). Cytokines such as GM-CSF and IL-4 encourage Cd14+ monocytes to differentiate to dendritic cells (Verhasselt, et al., 1997). CD14 alone is shown to be sufficient and necessary to regulate the dendritic cell life cycle after LPS exposure through nuclear factor of activated T cells (NFAT) pathway (Zanoni, et al., 2009). Indeed, CD14 acts as a co-receptor along with TLR 4 and MD-2 for the detection of bacterial lipopolysaccharide. Apart from binding LPS, CD14 has been shown to bind peptidoglycan of Gram-positive bacteria (Pugin et al, 1994) and other pathogen-associated molecular patterns thus mediate the innate immune response (Heumann et al, 1998). Blocking of CD14 with monoclonal antibodies prevented synthesis of TNF-α by whole blood incubated with LPS (Wright, et al., 1990).

2.2.4.3 CD 54

CD54 also known as Inter-Cellular Adhesion Molecule 1 (ICAM-1) is trans-membrane protein long known for its importance in stabilizing cell-cell interactions and facilitating leukocyte endothelial transmigration. CD54, by virtue of its biological role, is an appropriate candidate as both a phenotypic marker of APCs as well as an indicator of their activation state, thereby strengthening the interface of the immunological synapse between an APC and a T cell (Lebedeva, et al., 2005).

Direct role of CD54 for APC functionality, in terms of activating or priming T cells, is substantiated by a number of murine studies that show an absence of CD54 expression on APCs leads to lowered T cell activation of naïve T cells (Padros, et al., 1992) or

functionally impaired memory T cells. Its expression can be stimulated by IFN-gamma, TNF-alpha, IL-1 beta and LPS. CD 54 possesses binding sites for a number of immune-associated ligands (Sheikh & Jones, 2008).

2.2.4.4 CD 80

Co-stimulatory molecule CD80 also known as B7.1 is a member of the immunoglobulin (Ig) superfamily, was the first ligand to be identified for CD28 (Mochizuki, et al., 1997). CD 80 expression is almost exclusively restricted to lymphoid tissues, primarily on professional antigen presenting cells (Villarroel-Dorrego, et al., 2005).

CD 80 provides similar costimulatory signals as CD 86 for T cell proliferation, cytokine production, and generation of T cells. However, studies have shown that CD80 blockage has negligible effects on T-cell responses while CD86 blockage has detrimental effects on T-cell activation (Dilioglou, et al., 2003; Manickasingham, et al., 1998). Indeed, it has been suggested that the presence of CD80 favors the activation of Th1 lymphocytes, whereas CD86 favors priming of Th2 cells (Freeman, et al., 1995).

2.2.4.5 CD 83

CD83 is a membrane glycoprotein belonging to the Ig superfamily and it has been known for decades to be one of the best and typical markers of fully mature dendritic cells (Zhou, et al., 1992). Central role of CD 83 in cell-mediated immunity has been conserved for over 450 million years of vertebrate evolution (Ohta, et al., 2004).

The fact that CD83 is strongly upregulated together with co-stimulatory molecules such as CD80 and CD86 during DC maturation suggests it plays an important role in the induction of immunostimulatory as well as regulatory effects (Lechmann, et al., 2002). CD 83 is

recognized to be essential molecule for CD 4^{+}T cell generation. Indeed, when CD 83 is up-regulated by activation, the soluble form of CD 83 is released from the activated cells and this soluble form has inhibitory function within the immune reactions. Downstream transcription unit for CD83 is known to be triggered by Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and that this signaling pathway plays a crucial role during the induction of an adaptive immune response (McKinsey, et al., 2000).

2.2.4.6 CD86

CD 86 is a type I membrane protein also known as B7.2. Its expression on hematopoietic progenitor cells is regulated by TNF-α and denotes differentiation towards the macrophage or dendritic cell lineages. Expression of CD 86 on dendritic cells along with CD80 (B7.1), provide the necessary stimuli for priming T cells by binding to CD 28. Up regulation of CD86 expression is a hallmark of dendritic cell activation and maturation. It is shown that IL-10 inserts its inhibitory effect through down-regulation of B7-2 expression at DC surface (Buelens, et al., 1995).

Over expression of CD 86 on de ndritic cells in systematic autoimmune disease such as systemic lupus erythematosus may explain how the peripheral tolerance is broken in these conditions (Decker, et al., 2006)

2.2.4.7 CD 209 (DC-SIGN)

Dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN) is a type II transmembrane protein that based on its structure, belongs to the C-type lectin family. DC-SIGN is a specific adhesion receptor expressed on precursors in blood and on immature and mature DCs in both peripheral and lymphoid tissues (Soilleux, 2003). CD 209 is also

essential in several key functions throughout the life cycle and is central to the unusual trafficking capacity of DCs (Turville, et al., 2003). It takes up, processes pathogens and mediates strong adhesion between DC to ICAM-3 on resting T cells by stabilization of the DC–T cell contact zone, thus is essential for DC–T Cell Clustering and DC-induced T cell proliferation(Geijtenbeek, et al., 2000).

2.2.5 Dendritic Cells and Cytokines Production

Dendritic cells are a major source of many cytokines, namely, IFN-α, IFN-ß, IFN-ɣ , IL-1α, IL-1ß, IL-4, IL-6, IL-7, IL-10, IL-12, IL-15, IL-16, IL-17, TNF-α and also produce macrophage inflammatory protein (MIP1g), all of which are important in the elicitation of a primary immune response. The soluble cytokine profile secreted by DCs varies with the different stages of DC development and maturation and not necessarily simultaneously thus influencing the different effector functions characteristic of immature vs. mature DCs. Also, there is evidence that the cytokine secretion pattern of the plastic-adherent monocyte-derived DCs (grown in GM-CSF and IL-4) can be induced along the Th1 (IL-12) or Th 2 (IL-10) cytokine secretory pathway.

2.25.1) IL-10

IL- 10 also known as human cytokine synthesis inhibitory factor (CSIF), is an anti-inflammatory cytokine. Most of the immunosuppressive effects of interleukin-10 (IL-10) are related to functional inhibition of antigen-presenting cells. It is well known that IL-10 has an important regulatory role on monocytes function and on DC maturation (Corinti, Albanesi et al. 2001). Dendritic cells, grown and matured in vitro, can synthesize IL-10 in

a continuous manner. It is also well established that CD14+ DCs of myeloid origin synthesize IL-10 as do monocytes and macrophages.

Dendritic cells secreting IL-10 exhibit minimal or no stimulatory properties and are markedly inhibitory to T cell proliferation induced by polyclonal activators. Thus, IL-10 producing DCs are functionally and phenotypically inhibitory accessory cells and putatively tolerogenic. Moreover, IL-10 significantly inhibits ability of APCs to synthesize IL-12. It inhibits synthesis of pro-inflammatory cytokines like IFN-γ and TNF-α. However, it is also stimulatory towards certain T cells, mast cells and B cells (Chen, et al., 2007; Corinti, et al., 2001; Demangel, et al., 2002).

2.2.5.2) IL-12

Interleukin 12 (IL-12) is naturally produced by dendritic cells macrophages, monocytes, and B cells and is an important regulatory cytokine that has a function central to the initiation and regulation of cellular immune responses notably by inducing T and NK cells to produce IFN-γ. Though, researchers believe that immunological action of IL-12 must be directed primarily to those cells that are capable of producing IFN-γ. Moreover, IL-12 is responsible primarily for the subsequent production of TNF-α from both NK cells and helper T cells.

IL-12 has the capacity to regulate the differentiation of naive T cells into TH1 cells, which is crucial in determining resistance and the type of response that will be elicited in response to a particular pathogen. It stimulates the growth and function of T cells and alters the normal cycle of apoptotic cell death. The most powerful inducers of IL12 are bacteria, bacterial products, and parasites (Muller-Berghaus, et al., 2005; Wesa & Galy, 2001).

2.2.5.3) IFN-γ

IFN-γ is the sole type II interferon produced by B cells, NKT cells, and professional APCs that participates in the control of the innate and adaptive phases of the immune response (Frucht, et al., 2001). Indeed, IFN-γ production by professional APCs acting locally may be important in cell self-activation and activation of nearby cells. IFN-γ exerts most of its effects on APCs; it activates macrophages and polarizes immature DCs into Th1 cell–promoting effector DCs (Vieira, et al., 2000).

In addition to acting on mature APC functions, interferon γ can orchestrate immune response by switching monocyte differentiation from DCs to macrophages (Delneste, et al., 2003). IFN-γ–activated macrophages exhibit potent bactericidal and antitumor activities.

IFN-γ coordinates a diverse array of cellular programs through transcriptional regulation of immunologically relevant genes. Production of IFN-γ itself is controlled by cytokines secreted by APCs, most notably IL-12 as mentioned earlier. IFN-γ also acts on DCs to augment IL-12 secretion. Thus, IL-12 and IFN-γ comprise a positive feedback system and plays a major role in the clearance of intracellular pathogens Negative regulators of IFN-γ production include IL-4, IL- 10, transforming growth factor and glucocorticoids (Pan, et al., 2004).

2.2.5.4) TNF-α

TNF-α is a cytokine involved in regulation of immune cells. It is able to induce systemic inflammation and apoptotic cell death. TNF-α is a member of a group of cytokines that stimulate the acute phase reaction and is well recognized for its role in mediating innate

immune responses. Also TNF-α appears to have profound effects on DC function, as it contributes to their activation, maturation, migration, accumulation within draining lymph nodes, and significantly reduces IL-10-mediated inhibition of DC development and function. Inhibition of TNFα activity during dendritic cell maturation has been shown to lead to the development of semi-mature cells (van Lieshout, et al., 2005). All these functions indicate the importance of this innate cytokine in activating adaptive immunity to viral challenge. TNF-α is mainly secreted by macrophages, monocytes, neutrophils, T-cells, NK-cells following their stimulation by bacterial lipopolysaccharides (Christoph Brunner, et al., 2000; Trevejo, et al., 2001).

2.2.6 Toll-like receptors (TLRs)

TLRs are germline-encoded receptors with high sequence homology expressed by cells of the innate immune systems that recognize pathogen-associated molecular patterns (PAMPs) (Akira, et al., 2006). Thirteen TLRs (named simply TLR1 to TLR13) have been identified in humans and mice together. It is becoming increasingly evident that TLR regulate all major phases of the immune response. Stimulation of TLRs strongly activates DCs to up-regulate co-stimulatory molecules (CD80 and CD86) and to produce pro-inflammatory cytokines (TNF-α, IL-6, and IL-12) though causes an immediate defensive response (Tsuneyasu Kaisho & Akira, 2001; Thoma-Uszynski, et al., 2000). Accumulating evidence has shown that individual TLRs can activate overlapping as well as distinct signaling pathways, ultimately giving rise to distinct biological effects (Zhang, et al., 2008). The first evidence that show cytokine gene expression is differentially regulated by the various TLR was obtained from studies using mouse macrophages (Hirschfeld et al.,

2001) and human(Re & Strominger, 2001). For instance TLR2 and TLR4 although both led to comparable activation of NFκb and mitogen activated protein kinase family differentialy activate human DCs. Though, the observed patterns of gene induction of TLRs predict differential polarization of adaptive immune responses. Moreover, distinct TLRs act in synergy and cooperate with other PRPs resulting in coordinated sum of signals (Napolitani, et al., 2005; Sato, et al., 2000). However, it is shown that TLR4 can even activate intracellular signaling pathway without the need of other TLRs.(Kokkinopoulos, et al., 2005).

Investigation of TLR gene expression helps to understand how immune cell responses to bacteria are controlled. It is believed that expression of TLR is tightly regulated and differs between DC populations at different anatomical sites (Iwasaki & Medzhitov, 2004). Still there is little known regarding the regulation of TLR gene expression in DCs.

2.2.6.1 TLR4

TLR4 is a r eceptor for Gram_ LPS, respiratory syncytial virus protein F, and other endogenous ligands, such as surfactant protein A and fibronectin fragment. There are controversies with regard to expression of TLR4 in DCs. Some have reported that TLR4 is not expressed in monocyte derived dendritic cells whereas there are reports which indicate some expression levels in these DCS (Muzio, et al., 2000; Thoma-Uszynski, et al., 2000). Indeed, it is shown that TLR4 and its agonists are capable of inducing DC maturation as measured by the up-regulation of several cell surface markers such as CD40, CD86, HLA-DR, and CD83 (Re & Strominger, 2001). TLR4 deficient mice showed hyporesponsiveness to LPS, demonstrating that TLR4 is a critical receptor for LPS

signaling. Indeed, it is shown that LPS stimulation increase TLR4 expression in mouse immature DC significantly (An, et al., 2002)

TLR4 agonist specifically promotes production of the TH-1 inducing cytokine interlukine 12 and the chemokine interferone gamma which is also associated with TH-1 responses (Re & Strominger, 2001).

Inflammatory signaling pathways are induced by activation of NF-kB and MAPK family members after ligation of stimulants to TLR4. On the other hand, it is also shown that inhibition of NFκb and p38 kinase prevented upregulation of TLR-4 gene expression by LPS.

Moreover, it is said that transcription of several chemokine genes stimulated by TLR4 rapidly fades in cells thus TLR4 stimulation leads to transient responses than other TLRs as TLR2 (Re & Strominger, 2001)..

2 .2.7 P38 mitogen-activated protein kinase

Mitogen-activated protein (MAP) kinase pathways are one of the intracellular signaling pathways that mediate cellular behavior in response to extracellular stimuli. In fact, MAP kinases are members of discrete signaling cascades and serve as focal points in response to a variety of extracellular stimuli such as cytokines (TNF-α & IL-1), growth factors, ultraviolet irradiation, heat shock, and osmotic shock, and are involved in cell differentiation and apoptosis(Raingeaud, et al., 1995). This plethora of activators conveys the complexity of the p38 pathway and this matter is further complicated by the observation that activation of p38 is not only dependent on stimulus, but on cell type as well. Four distinct subgroups within the MAP kinase family have been described: (1) extracellular signal-regulated kinases (ERKs), (2) c-jun N-terminal or stress-activated

protein kinases (JNK/SAPK), (3) ERK/big MAP kinase 1 (BMK1), and (4) the p38 group of protein kinases.

We have selected p38 for our study. The downstream targets of the p38 MAPkinases include a w ide array of cytoplasmic and nuclear factors. Cellular response to stress activation depends upon the activity of each specific p38 isoform and cell context (Zarubin & Han, 2005).

P38 has shown different roles in the LPS response of DC. Inhibition of p38 ki nase has been shown to prevent LPS-induced production of a variety of cytokines in DC and suppress LPS- induced TLR-4 messenger RNA (mRNA) increase (An, et al., 2002) .

2.2.8 Iκb

IκB is family of inhibitory proteins which with their multiple forms appear to regulate NF-κ B by distinct mechanisms at primary level. The transcription factor NF-κ B is generally present in an inactive cytoplasmic form, bound to inhibitory IκB proteins. Activation of NF-κ B to move into the nucleus is controlled by the targeted phosphorylation and subsequent degradation of I kappa B(Baldwin, 1996). Cell stimulation causes its dissociation and translocation of active NF-κ B to the nucleus. NF-κ B can be activated by exposure of cells to LPS or inflammatory cytokines such as TNF-α or IL-1, viral infection or expression of certain viral gene products, UV irradiation, B or T cell activation, and by other physiological and non-physiological stimuli.

NFκb activation is essential for the expression of a variety of cytokines in the LPS response. NFκb inhibitors are shown to inhibit LPS-induced NFκB p65 s ub unit nuclear translocation as well as up-regulation of TLR4 mRNA suggesting that NFκb pathway is important in the regulation of TLR expression in DC (Tsuneyasu Kaisho & Tanaka, 2008).

2.2.9 miRNA

micro RNAs (miR) are small non coding single stranded RNAs of about 18-24 nucleotides length that are highly conserved during evolution. They have recently emerged as potent regulators of gene expression after transcription level (Bartel, 2004). Just like other RNAs, miR sequences are anchored in the DNA and get transcribed. Approximately 500 genes encoding miRNAs have been identified in mammals which are shown to be both temporally and spatially regulated. miRNAs play pivotal roles in shaping cellular development and differentiation in various tissues including immune cells. In the immune system, miRNAs act at check points during hematopoietic development and cell subset differentiation, they modulate effector cell function, and they are implicated in the maintenance of homeostasis (Figure 2.5),(Mark A, 2008; Turner, et al., 2011). miRs have the ability to "cleave" to their target-mRNA and in this way block the translation or they elicit target mRNA degradation, thereby the mRNA cannot be translated into the protein structure and degrades. Clearly, this process is a post-transcriptional modification, with the effect that the protein is not built and the cellular response is changed (Chekulaeva & Filipowicz, 2009). No doubt, deregulated miRNA levels can lead to several types of malignancies and autoimmune conditions.

Recently the miRNA content of DC's at different functional states have been profiled, and they can be utilized as potential novel biomarkers. Indeed, distinct miRNA profile can be also used for separation of mature and tolerogenic DCs(Kuipers, et al., 2010).

miR-155 and miR-146a are among the validated miRNAs expressed in DCs , however, their role in dendritic cell biology has not been well studied in depth yet.

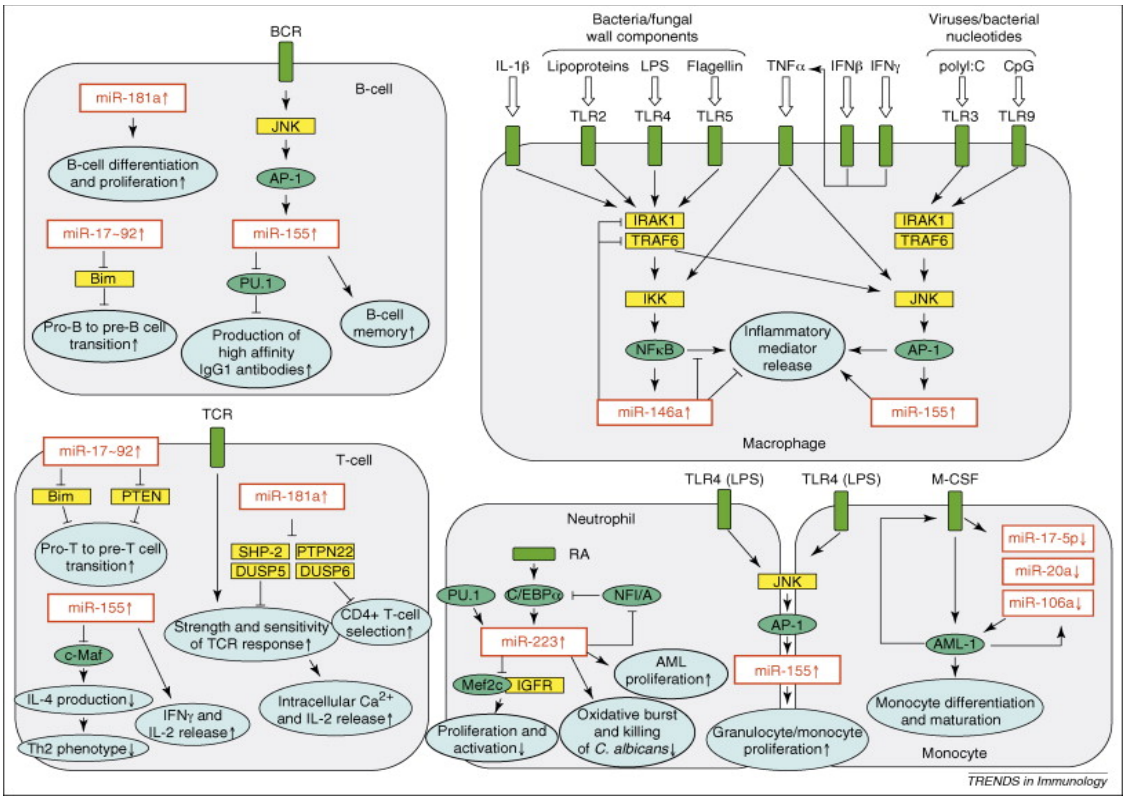

Figure2. 5 Role of microRNAs (miRNAs) in the regulation of the immune response. **The illustration summaries what is know of the mechanism by which individual miRNAs (red boxes) interact with transcription factors (green circles) and other proteins (yellow boxes) to regulate cell responses (blue circles) during immune cell development and the innate (monocytes, macrophages and neutrophils) and acquired (B and T cells) immune response. Stimulation (↑) or inhibition (T) should be determined by following how the changes in the individual miRNA expression (red squares) impacts on the indicated biological response (blue circles). AML, acute myeloid leukaemia; AP-1, activator protein; BCR, B-cell receptor; C/EBP, CCAAT-enhancer binding protein; DUSP, dual specificity phosphatase; IGFR, insulin-like growth factor receptor; IRAK, IL-1 receptor activated kinase; JNK, c-jun N-terminal kinase; M-CSF, macrophage colony stimulating factor; Maf, musculoaponeurotic fibrosarcoma: Mef, myeloid ELF-1 like factor; NF, nuclear factor; PTEN, phosphatase and tensin homolog; PTP, protein tyrosine phosphatase; RA, retinoic acid; SHP, Steroid Receptor Coactivator (Src) homology 2 domain–containing protein-tyrosine phosphatase; TCR, T-cell receptor; TLR, Toll-like receptor; TNF Associated Receptor Factor (TRAF), TNF receptor-associated factor (Mark A, 2008).**

2.2.9.2 miRNA146a

miRNA146a is known as a novel negative regulator that helps to fine-tune the immune response. Its expression is shown in macrophages and alveolar/bronchial epithelial following activation of TLR-2, TLR -4 and TLR -5 or exposure to TNFα and IL-1β. Indeed, miRNA146a is shown to regulate the maturation process and pro-inflammatory cytokine secretion in DCs as well. Later it became clear that miRNA 146a directly control TLR4 and cytokine signaling through NFκB pathway by targeting Interleukin-1 receptor associated kinase (IRAK1), Interleukin-2 receptor associated kinase (IRAK2) and TNF receptor-associated factor 6 (TRAF6), (Konstantin D. Taganov, et al., 2006). Such findings demonstrate that miRNAs levels can be altered by bacterial endotoxin and thereby involved in regulating innate immune responses. Moreover, changes in the expression of miR-146a are found as important event in the pathogenesis of many human diseases, including autoimmune disorders such as rheumatoid arthritis (RA), systemic lupus erythematosus, psoriasis(Pauley, et al., 2008).

2.2.9.3 miRNA 155

In contrast to miR-146a, miRNA-155 seems to positively regulate the release of inflammatory mediators during the innate immune response. Its own expression is a lso induced by inflammatory signals such as exposure to antigen, Toll-like receptor ligands and proinflammatory transcription factors such as NF-κB (O'Connell, et al., 2007). An increase in miR-155 levels during maturation of human monocyte-derived dendritic cells after exposure to LPS is demonstrated. More evidence on the role for MAPK signaling elements

in the regulation of miR-155 levels has been increasingly emerged. Indeed, miR-155 knock-out mice show aberrant immune functions including abnormal function of APCs. In a further study, inhibiting miR-155 has shown to impaire pathogen binding capacity by increasing DCSIGN. So DC-SIGN, a protein of functional importance in the immune system, is regulated indirectly by miR-155 (Rocio T. Martinez-Nunez, et al., 2009).

2.3 Interaction of Lactobacillus with the Immune System

During the 1990s growing interest was focused on the effect of LAB on specific and non-specific immune functions. Certain strains were found to enhance phagocytosis, secrete lysosomal enzymes and reactivate oxygen species (Gill, et al., 2000). Some strains as *Lactobacillus GG* were shown to be able to increase the immunogenicity of an oral rotavirus vaccine (Isolauri, et al., 1995), and several strains of mainly lactobacilli were found to induce pro- or anti-inflammatory response (Hessle, et al., 1999). Gradually strong evidences were found that probiotics adhesion to endothelial cells is not necessary for induction of immune stimulation. Also not only live strains but even heat-killed *L.casei* and *L. fermentum*, lipoteichoic acid (LTA) and crude cell extracts, likewise induced a proinflammatory response in the form of TNF-α in a macrophage cell line (Morita, et al., 2002; Wehkamp, et al., 2004). On the other hand, a mixture of 3 strains, *L.acidophilus, L delbrueckii* ssp *bulgaricus* and *B. bifidum* was reported to induce a predominant anti-inflammatory response in monocytes (Michalkiewicz *et al*., 2003). Oral administration of *L. rhamnosus, L. acidophilus* or *B. lactis* was able to enhance the immunoreactivity of spleen cells and phagocytes, and enhance serum antibody response to orally and systemically administered antigens (Gill, et al., 2000). Oral administration of *L. rhamnosus* GG for 4 weeks to atopic children resulted in a significant elevation of the serum

concentration of IL-10 and was later confirmed in a placebo-controlled trial (Kalliomaki, et al., 2001; Pessi, et al., 2000).

In DCs all tested strains of lactobacilli induced DC maturation and cytokine production (Christensen, et al., 2002). Indeed, VSL#3, a probiotic cocktail containing 8 di fferent strains of *Bifidobacterium* and *Lactobacillus* sp and *St salivarius* ssp *thermophilus* resulted in substantial production of IL-10 in DCs (Drakes, et al., 2004)

The identification of the underlying mechanisms is crucial for new credence for the use of probiotics in clinical medicine.

2.4 The probiotics paradox: live and dead cells both are biological response modifiers

The probiotics paradox is that both live and dead cells in probiotic products can generate beneficial biological responses even if they didn't meet the WHO definition of probiotics as being viable. This has become an ongoing debate as some strains are found to be effective only in viable form as *L.salivarius ssp.salivarius ,* while products based on dead cells or cell fractions have been demonstrated to be effective as well (Kataria, et al., 2009). For example killed *Lactobacillus* has been shown to inhibit adherence of gastrointestinal pathogens to epithelial cells (Coconnier, et al., 1993). Orally administered cell wall preparations stimulate the gut immune system (Sakai, et al., 2011). Killed *Enterococcus faecalis* cells have been shown to stimulate the bone narrow(Hasegawa, et al., 1996). Live and heat treated *Lactobacillus rhamnosus* have been shown to be effective in reducing the inflammatory response in the gastrointestinal tissue (N. Li, et al., 2009). Similarly, heat killed *Bifidobacterium* was effective against ulcerative colitis (Imaoka, et al., 2008).

Moreover, the biological response to dead probiotic cells was shown to be similar to an oral immunization response from a killed vaccine (Adams, 2010). Hence, many effects obtained from viable cells of probiotics are also shown to be obtained from populations of dead cells.

3. Methods & Materials

3.1 Probiotics

3.1.1. Probiotics Strains

Lactobacillus *rhamnosus* GG (ATCC-53103) and Lactobacillus delbrueckii subsp. Bulgaricus (ATCC-11842) are the applied probiotics in this study purchased from LGC standards GmbH (Germany).

3.1.2 Thawing and Preparation of Probiotics

One aliquot (1000µl) of each strains were thawed and centrifuged with 3000* rcf for 5 minutes and were washed three times with Phosphate buffer saline (PBS) in order to remove the glycerol which had been added for freezing.

3.1.3 Culturing and counting strains

Eight dilutions of each strain were made after resuspending them thoroughly in 1000µl of PBS (PAA- laboratories, Austria) and two plates were cultured from each dilution using MRS agar (deMan, Rogsa sharp Agar, LAB 93, U.K) . Plates were incubated (CO_2: 5%, temperature: 37 c) for 48 hrs. After wards, the mean CFU were calculated by counting the CFUs for each dilution at logarithmic phase. The second calculation was performed by culturing three plates from the countable dilutions. The mean of two calculations were considered as the strains population in 1000µl medium.

3.1.4 Heat inactivation

Autoclaving was selected for heat inactivation of probiotics as the strains particularly *lactobacillus delbrueckii* resisted to heat inactivation protocols using either 90 c for 15 min

or 100c for 45 min as mentioned in the available publications.100 µl of each strain were cultured on three plates after applying each protocol of heat inactivation to make sure no colony is growing.

3.1.5 Preparation for co culture

One aliquot of heat inactivated probiotics were thawed and centrifuged (350* g) to remove the PBS and complemented RMPI (RPMI+ FCS 10% + glutamine) was added to reach 1ml volume.

3.2 Dendritic Cells

3.2.1. Blood collection and Peripheral Blood Mononuclear cells (PBMC) isolation

64 ml blood was collected in 8 ml heparin containing tubes from 4 healthy volunteers in fasting state. Bloods were centrifuged for 10 min (623 *rcf, break 0, 18^{c}). Serums were pumped out and the remaining bloods were diluted with sterile warm RPMI (PAA Laboratories, Austria), and were resuspended carefully to become completely homogenized. The diluted bloods were added slowly to ficoll tubes (Ficoll Paque Plus, GE Healthcare, Vienna, Austria) and centrifuged with slower speed (318*g; 30 min; 18 c; brake 0) to reach the buffy ring of lymphocytes (Figure3.1). The ring was harvested carefully and collected in tubes containing RPMI and Centrifuged (318*g; 30 min; 18c; brake 9). After washing the cells for the second time with RPMI the cells were collected in a single tube and counted by invitrogen countess automated cell counter.

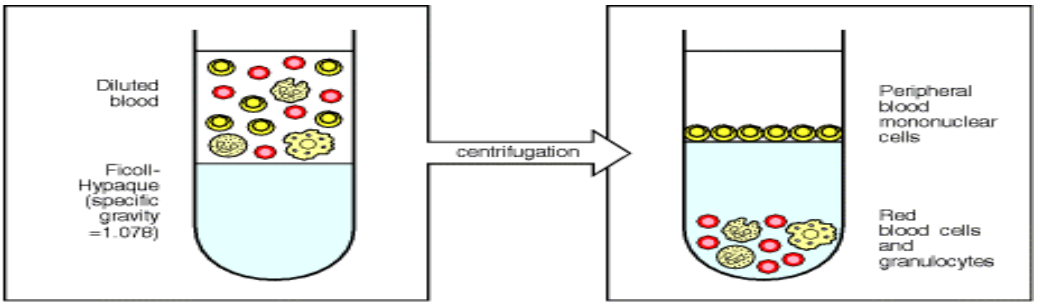

Figure 3. 1 Peripheral blood mononuclear isolated from whole blood by Ficoll-Hypaque centrifugation. Diluted anticoagulated blood (left panel) is layered over Ficoll-Hypaque and centrifuged. Red blood cells and polymorphonuclear leukocytes or granulocytes are more dense and centrifuge through the Ficoll-Hypaque, while mononuclear cells consisting of lymphocytes together with some monocytes band over it and can be recovered at the interface (right panel).

3.2.2 Monocyte isolation using Dyabeads (Invitrogen)

After centrifuging the cells for 10 min and pumping out the supernatant, the cells were washed with isolation buffer (Ca and Mg free phosphate buffered saline supplemented with 0.1% Bovine Serum Albumin (BSA) and 2ml Ethylenediamine Tetraaceticacid (EDTA) and the RPMI was removed. The further steps were performed in 2-8°C according to the cell number following the the Dynabeads untouched Human Monocytes kit protocol (Dynabeads, MyPure Monocyte Kit 2, Invitrogen, Lofer, Austria), (Appendix I).

3.2.5 Differentiation to DC

To generate immature monocyte derived DCs (Mo-DC), negatively isolated monocytes were cultured at a cell density of 5×10^5 cells/ml in cell culture medium, in the presence of recombinant IL-4 (100 ng/ml) (Invitrogen, GmbH) and recombinant GM-CSF (10 ng/ml) (Invitrogen, GmbH) for 6-7 days. Then the cells were distributed in 6 well plates (Iwaki 3810-006, Japan) according to the achieved cell number and 2ml complemented RPMI was added to each well and the cells were incubated overnight. After one night 10 µl of IL-4 and GMCSF were added to each 1ml of cell

suspension. Every other day half of the medium were removed and exchanged with new medium containing the same cytokines until day 7.

3.2.6 Coculturing probiotics with immature DC

On 7th day the immature DCs were scrapped off from the wells using cell scrapper. After DCs were counted they were co cultured with lactobacillus Rhamnosous and delbrueckii in ratio of 10:1 and 100:1 (CFU: DC) and incubated for 24 hours. LPS (Sigma –Aldrich Handels, GmbH) was used as the positive control.

3.3 Fluorescence – Activated Cell Sorter (FACS) analysis

3.3.1 Harvesting and staining of cells for FACS analysis: Cluster of differentiation (CD) markers measurement

The cells were scrapped off the wells, collected in separate tubes and were centrifuged for 30 min at 250 rcf. The supernatants were removed and stored as 500µl aliquots in -20c freezer for cytokine measurement.

Dendritic cells were washed twice with PBS and centrifuged for 5 min at 250*g. As we had 4 antibodies and 100µl of cell suspension was needed for each antibody tube the cells were finally resuspend in 400µl PBS and 5µl of antibodies were added (Table 3.1)

The tubes were vortexed and incubated for 25 min in refrigerator. At the next step the stains were washed twice with 2ml stain buffer and centrifuged for 5 minutes at 3000 *g while avoiding light exposure. The cells were prepared for FACS analysis by adding 200 to 300 µl stain buffer to each tube.

Cells were measured using a 4-color BD FACScalibur system (BD Biosciences GmbH, Schwechat, Austria) and analyzed using BD CellQuest software.

Table 3. 1 FACS antibodies

Tube	Epitopes	Conjugate
A	Mouse IgM Mouse IgG Mouse IgG 2b Muse IgG2a	FITC PE Percp APC
B	CD14 CD86 CD 209 CD83	FITC PE Percp APC
C	CD 80 CD 54 HLADR	FITC PE APC
D	CD 11c Mouse IgG1	PE APC

3.3.2 Cytokine detection by C ytometric bead array (CBA)

BD CBA Flex Set bead-based immunoassay was used for measurement of cytokines by BD FACS calibur. The data generated by BD CBA are actually comparable to ELISA based assays, but in a "multiplexed" or simultaneous fashion (Figure 3.2).

CBA data were analyzed using FCAP Array software

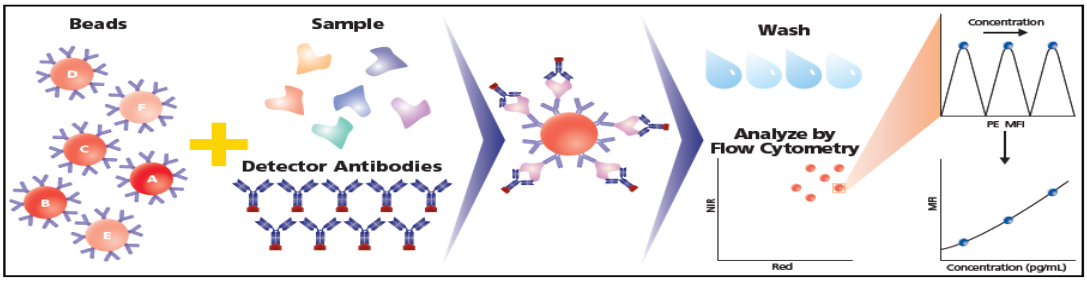

Figure 3. 2 Cytometirc bead array

3.4 Statistical Analyses:

Two way ANOVA with experiments assigned as blocked was applied to reveal significant differences *LGG*, *L.delbrueckii*, positive control (LPS), and negative control relative to capacity to induce co-stimulatory molecules expression and extra cellular cytokine production with two concentrations in 3 independent experiments. Bonferoni criterion was then used to compare the effect of each strain and the ratios with the two control groups. All analyses were performed using STATA (9.0) and the graphs were drawn by SPSS 16. Statistical significance was considered if $p < 0.05$.

3.5 Gene expression analysis using real time Polymerase Chain Reaction (PCR)

For this part of our study the same strains were co-cultred with DCs at ratio of 10:1 (CFU:DC) and incubated for 12 hours. The cells were collected in RNAlater (Ambion, Applied biosystem, USA) and stored in the freezer for further experiments.

All end point PCRs for this study were performed by genXpress (Life Science) and the RT-PCRs by Applied Biosystems (ABI) StepOnePlus Instrument with 96 wells.

3.5.1 mRNA extraction, Reverstranscription & amplification using Syber green

Extraction of total mRNA was performed by RNeasy Mini Kit from Qiagen (Hilden, Germany) according to the instruction (appendix II).

Reverse transcription was followed by 8 µl of mRNA template with the Phusion RT-PCR Kit from Finnzymes (Epoo, Finland) using Oligo(dT) primer, which consist of only

Thymine and bind to the polyA-tail of the mRNA. All the further steps were done according to the manual (apendixIII).

For the RT PCRs, the following optimized reaction set up was applied:

10µl of total reaction volume was prepared consisting:

- 5 µl MasterMix: SensiMix SYBR Kit from genXpress (Wiener Neudorf, Austria)
- 1 µl Primer, the concentration for each primer was considered separately according to the information provided and our inter lab experiments for each primer. All the primers were purchased from biomers (genXpress,Wiener Neudorf, Austria
- 3 µl nuclease free water
- 1 µl complementary DNA (cDNA)

The Magnesiumchloride ($MgCl_2$) concentration for all assays was 3mM. The SensiMix already contains the Sybr Green dye for the real-time analysis.

The temperature conditions applied for this part is shown in table 3.2 and the primers which were purchased from biomers are listed in table 3.3, Further details appendix (IV) :

Table 3. 2 Temperature setting for RT-PCR

	Step	Annealing Temperature	Time
	Hot Start for the Activation of the Polymerase	95°C	10 minutes
40 cycles	Denaturation	95°C	30 seconds
	Annealing	57°C-60°C	40 seconds
	Elongation	72°C	30 seconds
		Melt Curve	

Table 3. 3 Primers sequences

Primer	Sequence5′-3′	Annealing temperature	Concentration
TNFα	Forward:AAGAGGGAGAGAAGCAACTACAGA Reverse:GGTGGAGCCGTGGGTCAG	60°C	0.25pmol/µl
TLR4	AAGCCGAAAGGTGATTGTTG CTGAGCAGGGTCTTCTCCAC	52°C	0.24pmol/µl
IKB	Forward:GCTGTGATCACCACATTACCGTC Reverse: GAGTACATTTGCGCGTTCACG	56°C	0.25pmol/µl
P38	Forward: ACTCAGATGCCGAAGATGAAC Reverse: GTGCTCAGGACTCCATCTCT	51°C	2,5pmol/µl
GAPDH	Forward:CGACCACTTTGTCAAGCTCA Reverse:AGGGGAGATTCAGTGTGGTG	58°C	0.5pmol/µl

3.5.1 miRNA Quantitation by RT-PCR using TaqMan assay

After removal of RNAlater and lysing the cells by 300-600µl Lysis Binding Solution, total miRNA was isolated by *mir*Vana^{TM} miRNA Isolation Kit (Ambion,USA) (appendix V). It has to be mentioned that *mir*Vana isolation kit is designed for specifically purification of miRNA and by immobilization of large RNAs on the fibre filter with low ethanol concentration small-RNA enriched sample can be perfectly eluted.

Afterwards, TaqMan MicroRNA assay(Applied biosystem, California) was applied for reverse transcription and RT-PCR amplification of selected miRNAs from the purified miRNA template according to the protocol. For this experiment TaqMan® Universal PCR Master Mix 2✕, No AmpErase® UNG was used. This kit includes the primers for the target miRNAs and U66 was used as endogenous control (appendix VI)

3.6 Gene expression Analysis

Real time PCR data were analyzed with the StepOne software belonging to the StepOne real time PCR Life Technologies (Carlsbad, California). Gene expression data were normalized to the expression of the housekeeping gene Glycerinaldehyde-3-phosphate Dehydrogenase (GAPDH) & miRs U66. Mean ± standard deviation (SD) of independent experiments performed in triplicate is demonstrated in figures. One sample t- test and two sample t te st were applied to reveal significant differences of treatments relative to capacity to regulate gene expressions in three independent experiments at various time points. Statistical analysis were performed using OriginPro 8G (OriginLab,Northampton, USA). The figures have been also made by OriginPro 8G. Results were considered significant if $p<0.05$.

3.6 Table of Variables

No	variable	Type of variable		quantitative		qualitative		Scientific definition	Method of measurement	scale
		independent	dependent	continuous	Non continuous	nominal	ordinal			
1	Lactobacillus Rhamnosus (LGG)	*				*		A strain of lactic acid bacteria used as natural preservative in dairy product	Counting colonies on MRS culture	Colony forming Unit (CFU)
2	Lactobacillus Delbrueckii (l. delbrueckii)	*				*		A gram positive, facultative anaerobic, non motile and non spore forming, rod shaped Probiotic and a members of industrially important lactic acid bacteria	Counting colonies on MRS culture	CFU
3	LPS	*				*		Lipopolysacharide a major cell wall constituents of gram negative bacteria	weighting	µg
4	IL-1β		*	*				Maturation factor for DC and an important mediator of the inflammatory responses	Flow cytometry	Pg/ml
5	IL-10		*	*				Anti-inflammatory cytokine with regulatory role on DC maturation	Flow cytometry	Pg/ml
6	IL-12		*	*				Inflammatory cytokine produced by DC , initiate and regulate cellular immune responses notably by inducing T and NK cells to produce IFN-ɣ	Flow cytometry	Pg/ml
7	TNF-α		*	*				Inflammatory cytokine involved in regulation of immune cells	Flow cytometry	Pg/ml
8	IFN-ɣ							Type II interferon, polarizes immature DCs into Th1 cell–promoting effector DCs	Flow cytometry	Pg/ml
8	CD 86		*	*				Co-stimulatory molecule on DC involves in T cell activation	Flow cytometry	MFI
9	CD 83		*	*				Typical marker of fully mature dendritic cell	Flow cytometry	MFI
10	CD 80		*	*				Co-stimulatory molecule on DC involves in T cell activation	Flow cytometry	MFI

12	CD 54		*	*				Trans membrane protein, phenotypic marker of DC and indicator of DC activation state	Flow cytometry	MFI
13	CD 14		*	*				Surface antigen that regulate dendritic cell response and lifecycle after exposure to LPS	Flow cytometry	MFI
14	CD 11c		*	*				a type I transmembrane protein found at high levels on most human DC	Flow cytometry	
15	mRNA TNF-α		*	*				inflammatory cytokine	RT-PCR	2^-ddct
16	mRNA TLR4		*	*				LPS specific receptor for cell signaling	RT-PCR	2^-ddct
17	mRNA P38		*	*				Mitogen-activated protein kinase	RT-PCR	2^-ddct
18	mRNA Iκb		*	*				of nuclear inhibitor factor kappa b	RT-PCR	2^-ddct
19	miR7i		*	*				microRNA targeting TLR	RT-PCR	2^-ddct
20	miR146a		*	*						2^-ddct
21	miR155		*	*					RT-PCR	2^-ddct

4. Results

4.1 Expression of co-stimulatory molecules

4.1.1 CD14

Expression of Cd14 which is a marker of monocytes and get reduced during differentiation of monocytes to dendritic cells remains at low levels in all samples. LPS didn't have notable stimulating effect in regulation of CD 14 (9.3± 2.0) in differentiated monocytes. Neither the Statistical difference between groups after adjustment for dilution nor the differences between concentrations in each group reached statistical significance (Figure 4.1.1).

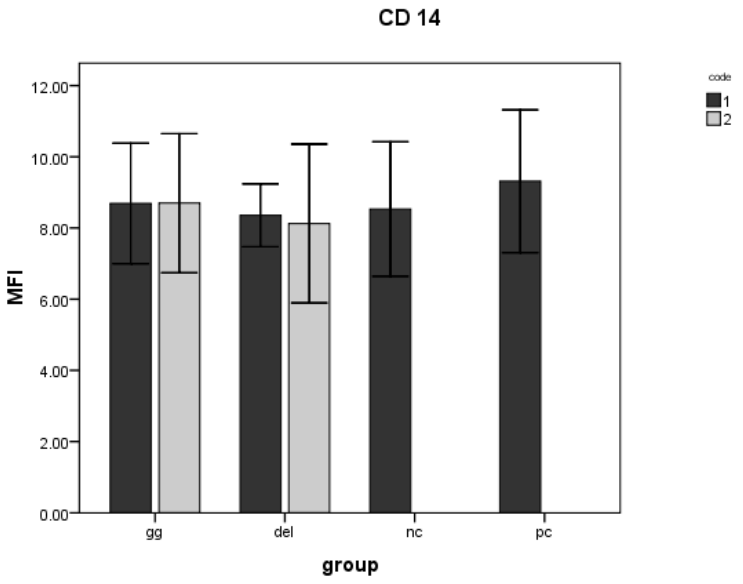

Figure 4.1.1 Expression of CD14 by DCs treated with Lactobacillus Rhamnosus GG (gg), Lactobacillus Delbrueckii (del), LPS (pc) and no stimulants (nc) for 24 hours with bacteria:DC ratio of 10:1 (code1) and 100:1(code 2) . Data are shown as means (± SD) and are representative of 3 independent experiments.

41.2 CD 11c

Remarkable expression of CD11c, a well known marker of APCs was observed in all cultures. *L.delbrueckii* (10:1) increased expression of CD11c 5.3 fold comparing to none treated DCs. While increasing concentration of *L.delbrueckii* (100:1) doesn't lead to higher levels of CD11c and the observed up regulating effect remained only 2.8 times more than non- treated cells.

Moreover, treatment of DCs with *LGG* changed expression of CD11c 0.2 fold respecting to negative control and this effect is increased to 2.4 fold by 10 time increasing the concentration of *LGG.*

Unlike probiotics LPS shows little effect on expression of this trans-membrane protein (68.4± 13.3 MFI vs. 71.1±30.5 MFI), (figure4.1.2)

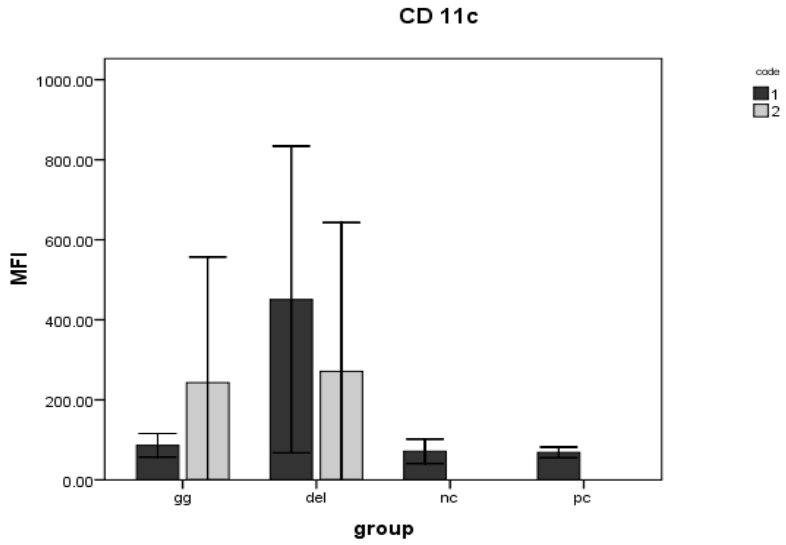

Figure 4.1.2 Expression of CD11c by DCs treated with Lactobacillus Rhamnosus GG (gg), Lactobacillus Delbrueckii (del), LPS (pc) and no stimulants (nc) for 24 hours with bacteria:DC ratio of 10:1 (code1) and 100:1(code 2) . Data are shown as means (± SD) and are representative of 3 independent experiments

4.1.3 CD 86

CD 86, maturation marker of DCs was significantly up-regulated among cultures after adjustment for concentrations *(P<0.05)*. However, *LGG* seemed to be a stronger inducer of CD 86 than *L.delbrueckii* (104.1 ±107.9 MFI vs. 92.2± 77.2 MFI). Though comparing to negative control, up regulation of CD 86 only achieved statistical significance by *LGG* (about 7.3 fold, p≤ 0.05). LPS was shown to have higher (192.6± 58.9 MFI) up regulating effect than probiotics with regard to this co-stimulatory molecule.

Increasing concentration of probiotics didn't lead to higher expression of CD86 in neither of groups (figure 4.1.3)

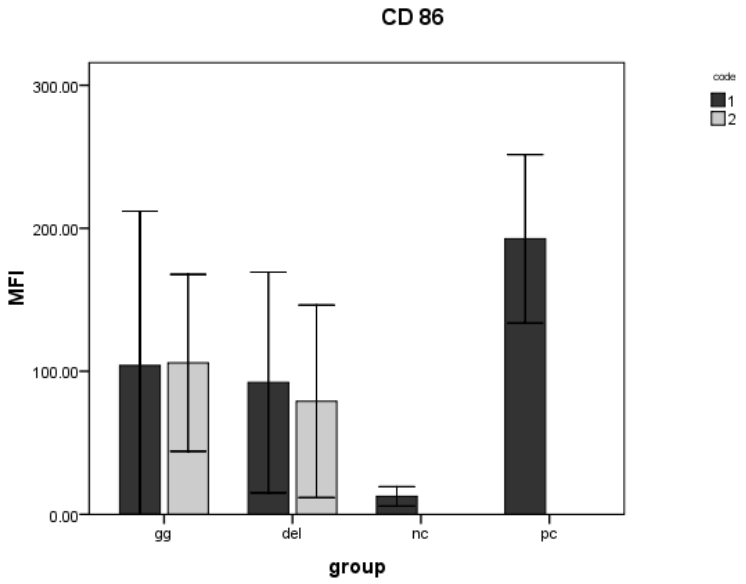

Figure 4.1.3 Expression of CD86 by DCs treated with Lactobacillus Rhamnosus GG (gg), Lactobacillus Delbrueckii (del), LPS (pc) and no stimulants (nc) for 24 hours with bacteria:DC ratio of 10:1 (code1) and 100:1(code 2) . Data are shown as means (± SD) and are representative of 3 independent experiments

4.1.4 CD 83

CD 83, is expressed 2.3 MFI, 1.9 MFI and 2.1 MFI more comparing to negative control by LPS, *LGG* and *L.delbrueckii* respectively. However, these differences do not reach statistical significance after adjustment for concentrations. Indeed higher concentration of probiotics didn't cause a significant up regulation in CD 83 expression in neither of groups (Figure 4.1.4).

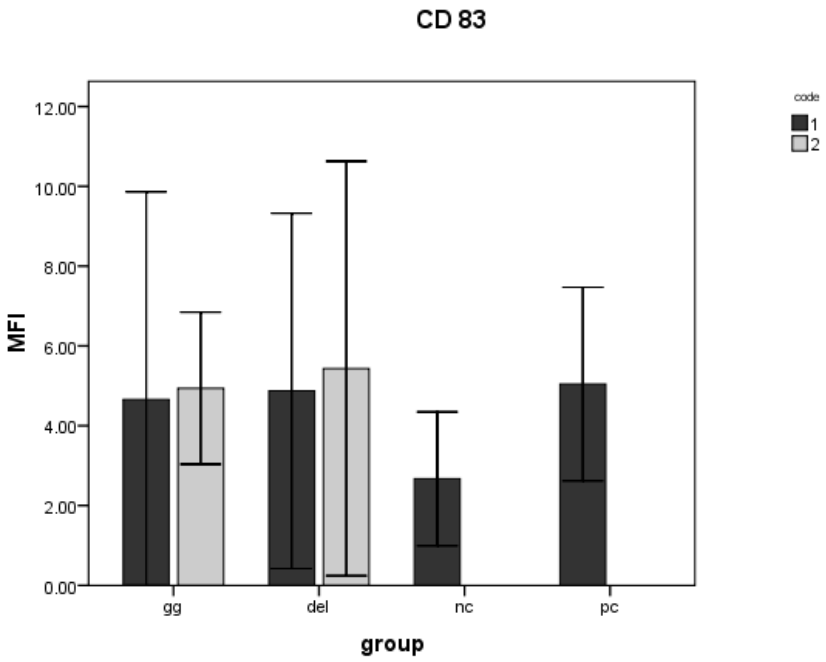

Figure 4.1.4 Expression of CD83 by DCs treated with Lactobacillus Rhamnosus GG (gg), Lactobacillus Delbrueckii (del), LPS (pc) and no stimulants (nc) for 24 hours with bacteria:DC ratio of 10:1 (code1) and 100:1(code 2) . Data are shown as means (± SD) and are representative of 3 independent experiments

4.1.5 CD 80

LGG up regulated CD80 to 21.2±2 MFI and 16.4±7.4 MFI at 10:1 and 100:1 concentrations respectively. Whereas, *L.delbrueckii* didn't seem to be much effective (11.1 ±4.5 MFI and 15.1±5 MFI) in induction of CD 80 c omparing to the non-treated DCs (11.6± 1.9 MFI). Although, LPS led to higher expression of CD80 than probiotics, neither the difference among cultures after adjustment for concentration nor within each group was found to be significant, (Figure 4.1.5)

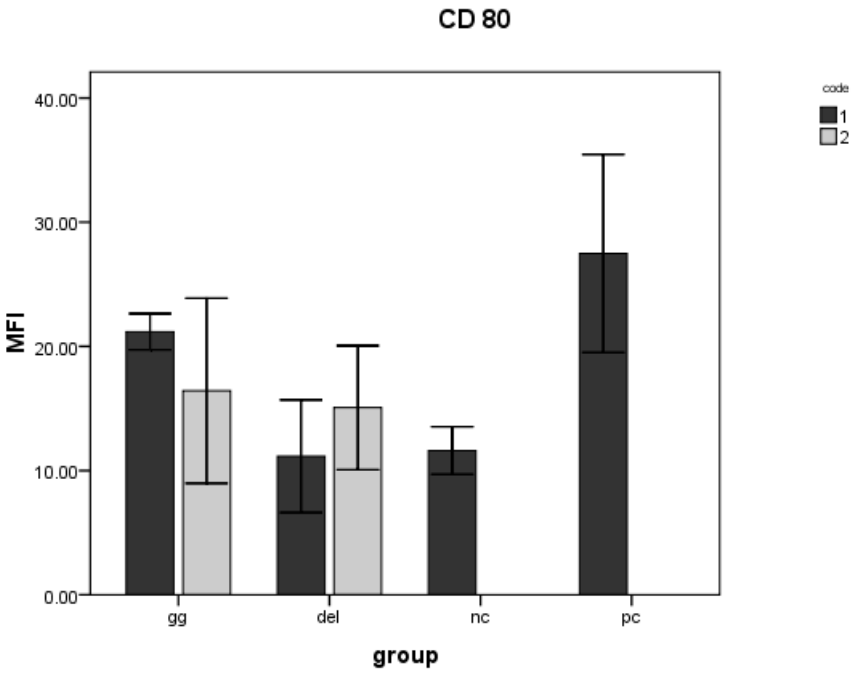

Figure 4.1.5 Expression of CD80 by DCs treated with Lactobacillus Rhamnosus GG (gg), Lactobacillus Delbrueckii (del), LPS (pc) and no stimulants (nc) for 24 hours with bacteria:DC ratio of 10:1 (code1) and 100:1(code 2) . Data are shown as means (± SD) and are representative of 3 independent experiments

4.1.6 CD 209

The expression of CD 209, the important receptor for endocytosis and DCs life cycle was significantly different among groups after adjustment for concentrations (p= 0.003). CD 209 was significantly down regulated in *LGG* (100:1, p= 004) and *L.delbrueckii* (10:1, p= 0.006; 100:1 p= 0.002) treated cultures. Evidently, *L.delbrueckii* (10:1) was about 3.6 times more efficient in negative stimulation of CD 209 comparing to *LGG(10:1)*. The negative stimulation was rather equally increased 27% by *L.delbrueckii* (124.7±34.0 MFI vs. 91.4± 24.0 MFI) and 28% by *LGG* (241.1±71.2 MFI vs. 173.3± 51.0 MFI) when their concentration was 10 times increased (Figure 4.1.6).

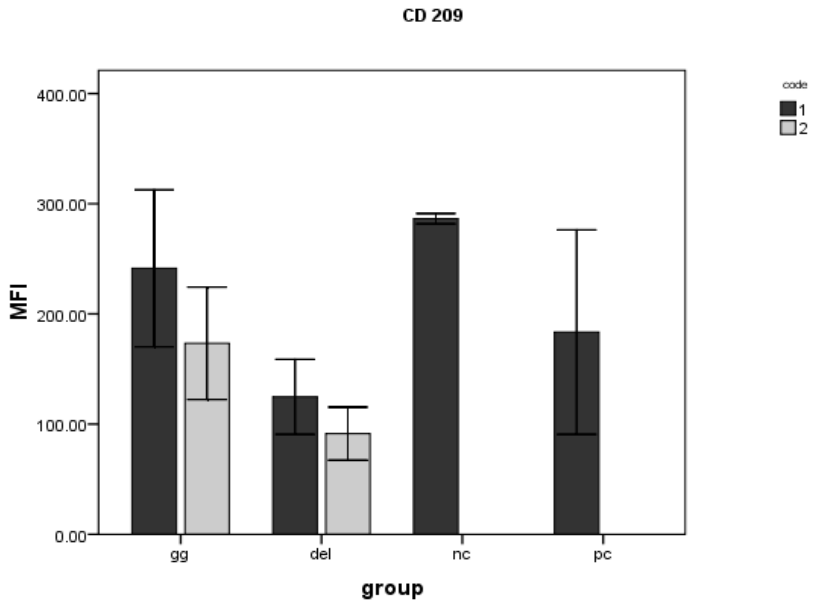

Figure 4.1.6 Expression of CD209 by DCs treated with Lactobacillus Rhamnosus GG (gg), Lactobacillus Delbrueckii (del), LPS (pc) and no stimulants (nc) for 24 hours with bacteria:DC ratio of 10:1 (code1) and 100:1(code 2) . Data are shown as means (± SD) and are representative of 3 independent experiments

4.1.7 CD 54

Expression of CD 54 was increased 73% (833.3± 154.3 MFI) by *LGG* (10: 1) and 12% (539.2± 284.5 MFI) by *L.delbrueckii* (10:1) comparing to non treated DC (479.7±399.5 MFI). Surprisingly, 10 times increasing concentration of *LGG* and *L.delbrueckii* reduced expression of CD54, to 5 % (452.8± 267.7 MFI) and 26% (353.0±202.0 MFI) respectively comparing to negative control.

Neither the difference among groups after adjustment for concentrations nor difference in concentrations within each group achieved statistical significance (Figure 4.1.7).

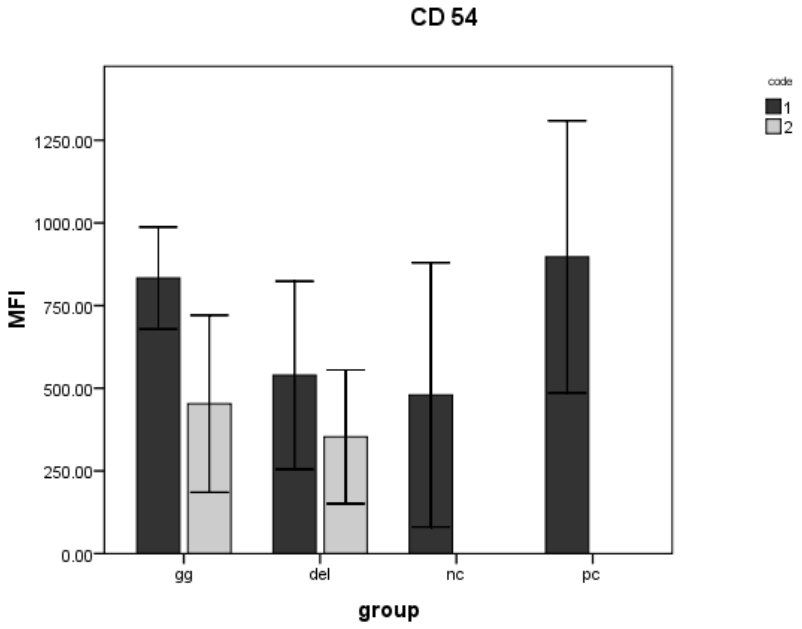

Figure 4.1.7 Expression of CD14 by DCs treated with Lactobacillus Rhamnosus GG (gg), Lactobacillus Delbrueckii (del), LPS (pc) and no stimulants (nc) for 24 hours with bacteria:DC ratio of 10:1 (code1) and 100:1(code 2) . Data are shown as means (± SD) and are representative of 3 independent experiments

4.2 Cytokine levels

4.2.1 IL-1 β

Expression of IL-1β, an inflammatory cytokine, was increased by *L.delbrueckii* (77.4±38.3 pg/ml) and *LGG (*99.5±47.7 pg/ ml) comparing to negative control at low concentration (10:1). Moreover, the expression of IL-β was increased 5 times, by LGG (413.1±263.9 pg/ml) and 1.7 times (169.1±98.3 pg/ml), by *L.delbrueckii* when their concentration was 10 times increased. Hence, the difference between groups after adjustment for concentrations was statistically significant *(p= 0.009)*. Also there was a difference between concentrations in each group, although the significance level was borderline *(p= 0.055*), (Figure 4.2.1).

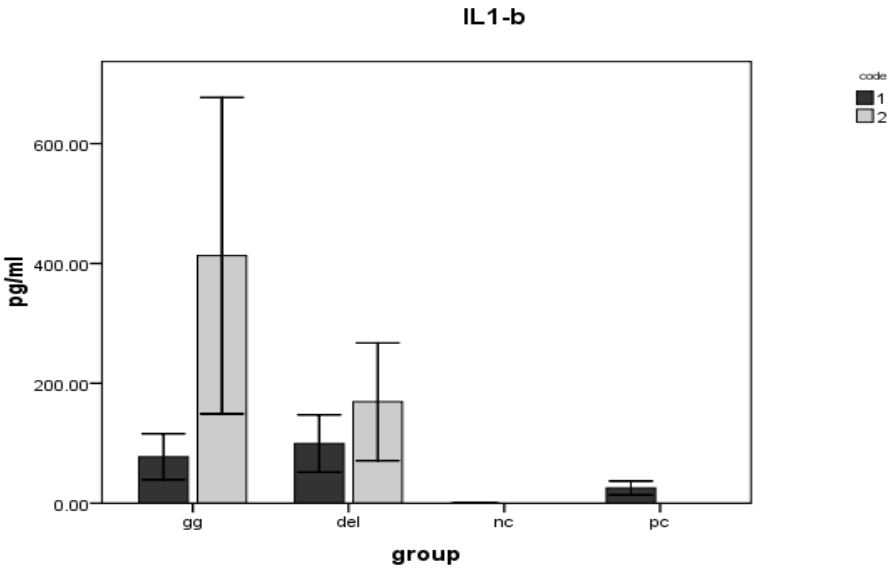

Figure 4.2.1 Level of IL-1β in DCs treated with Lactobacillus Rhamnosus GG (gg), Lactobacillus Delbrueckii (del), LPS (pc) and no stimulants (nc) for 24 hours with bacteria:DC ratio of 10:1 (code1) and 100:1(code 2) . Data are shown as means (± SD) and are representative of 3 independent experiments

4.2.2 TNF-α

Both probiotics stimulate TNF-α secretion. Obviously *L.delbrueckii* had the most significant stimulatory effect in induction of TNF-α at t 10:1 ratio (26.5 fold,*p= 0.006)* and even higher at 100:1 ratio (50.2 fold, *p= 0.0002*) comparing to negative control.

LGG also increased TNF-α level 2.5 fold at 10: 1 ratio which was not statistical significant but ten times increasing the concentration of LGG led to significantly higher (21.6 fold) expression of TNF-α comparing to negative control *(p= 0.05*).

Accordingly, the difference between groups after adjustment for concentrations was statistically significant *(p= 0.0001)*. Also there was a significant difference between concentrations in each group *(p= 0.03*), (Figure 4.2.2).

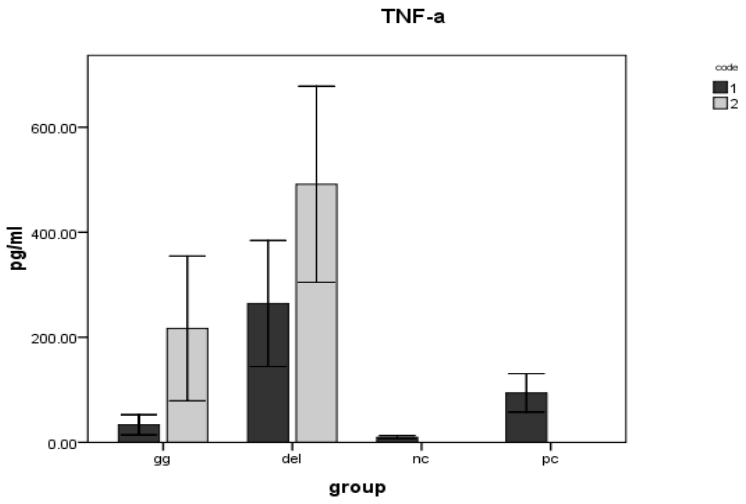

Figure 4.2.2 Level of TNF-α in DCs treated with Lactobacillus Rhamnosus GG (gg), Lactobacillus Delbrueckii (del), LPS (pc) and no stimulants (nc) for 24 hours with bacteria:DC ratio of 10:1 (code1) and 100:1(code 2) . Data are shown as means (± SD) and are representative of 3 independent experiments

4.2.3 IL-10

Expression of IL-10, an anti-inflammatory cytokine was increased 13.9 fold (134.4 pg/ml) by *LGG* (p=0.02) and 17.4 fold (170.1pg/ml) by *L.delbrueckii (p=0.002)* at 10: 1 ratio. Moreover, IL-10 level was noticeably raised when the concentration of probiotics was10 times increased. Actually at 100:1 ratio IL-10 level was increased 38.6 fold (391.3 pg/ml) by *LGG (p=0.0006)* and 21.7 fold (215.6 pg/ml) by *L.delbrueckii (p= 0.005*).

A significant difference was observed between groups in increasing IL-10 level after adjustment for concentrations *(p= 0.01)*.However, the difference between concentrations in each group wasn't found to be significant, ((Figure 4.2.3).

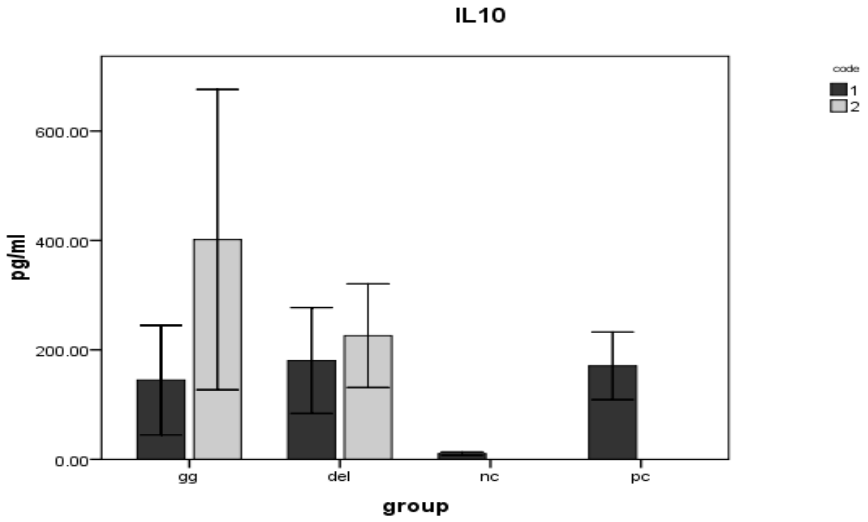

Figure 4.2.3 Level of IL-10 in DCs treated with Lactobacillus Rhamnosus GG (gg), Lactobacillus Delbrueckii (del), LPS (pc) and no stimulants (nc) for 24 hours with bacteria:DC ratio of 10:1 (code1) and 100:1(code 2) . Data are shown as means (± SD) and are representative of 3 independent experiments

4.2.4 IL-12

Expression of IL-12, the pro inflammatory cytokine was reduced (0.1 pg/ml) by *LGG* at low ratio (10:1) where as there was a significant increase in IL-12 level at cultures treated with higher ratio of *LGG* (1.7 pg/ml, p =0.01). In *L.delbrueckii*- treated DCs, there was a minor increase (0.4 pg/ml) in IL-12 level at low ratio but there was a significant rise (1.6 pg/ml, p=0.01) in DCs treated with higher ratio of *L.delbrueckii*. So IL-12 level was only significantly increased at high ratio of probiotics to DCs (100:1). Moreover, there was a significant difference among groups in increasing IL-12 level after adjustment for concentrations *(p= 0.006).* Also, the difference between concentrations within each group achieved statistical significance, *(p=0.01)*, ((Figure 4.2.4).

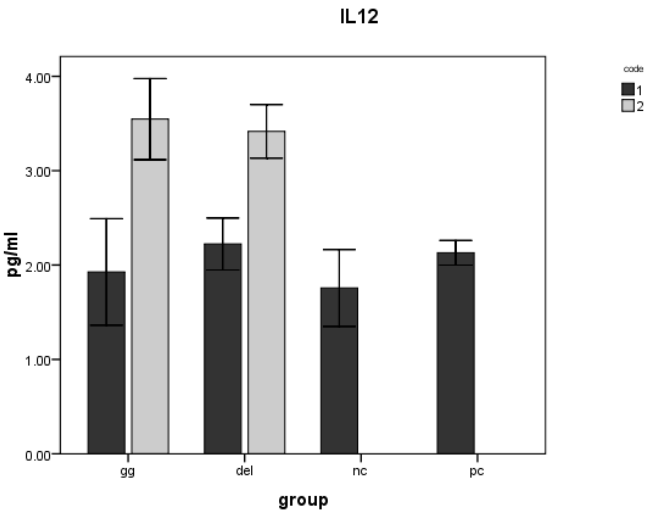

Figure 4.2.4 Level of IL-12 in DCs treated with Lactobacillus Rhamnosus GG (gg), Lactobacillus Delbrueckii (del), LPS (pc) and no stimulants (nc) for 24 hours with bacteria:DC ratio of 10:1 (code1) and 100:1(code 2) . Data are shown as means (± SD) and are representative of 3 independent experiments

4.2.5 INF-γ

Level of INF-γ was increased l.6 pg/ml in *LGG* (10:1) treated DCs and 1.3pg/ml in *L.delbrueckii* (10:1) treated DCs. Ho wever, at ratio of 100:1 these levels were significantly increased to 9.2pg/ml *(p= 0.005)* and 5.8pg/ml *(p= 0.01)* by *LGG* and *L.delbrueckii* respectively. Accordingly after adjustment for concentrations there was a significant difference in INF-γ level between groups *(p= 0.002)* and between concentrations in each group *(p= 0.003)*, (Figure 4.2.5).

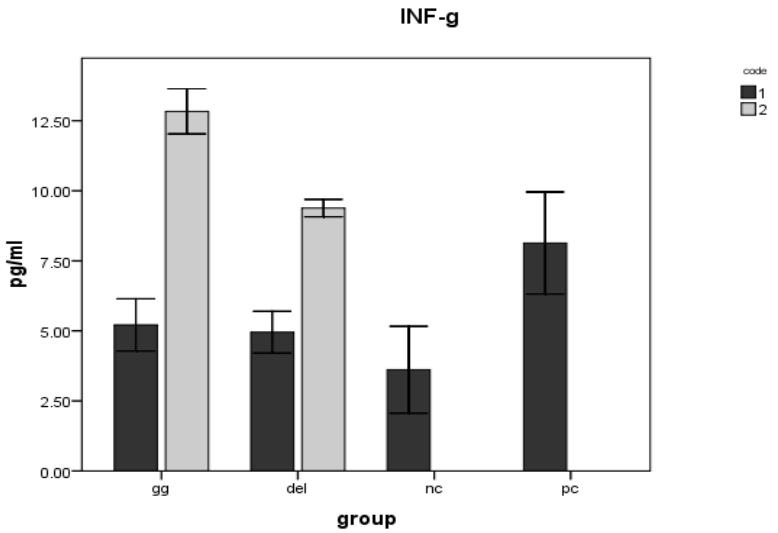

Figure4.2.4 Level of INF-γ in DCs treated with Lactobacillus Rhamnosus GG (gg), Lactobacillus Delbrueckii (del), LPS (pc) and no stimulants (nc) for 24 hours with bacteria:DC ratio of 10:1 (code1) and 100:1(code 2) . Data are shown as means (± SD) and are representative of 3 independent experiments

4.3 Gene expression experiments

4.3.1 TLR4 mRNA

Gene expression of TLR4 was shown to be down regulated by *LGG* (0.86±0.32,p=0.5) and more strongly by *L.delbrueckii* (0.52±0.39, *p=0. 2*) in the same direction that it was influenced by LPS (0.71±0.04,p=0.005) after 12 hours (figure4.3.1).

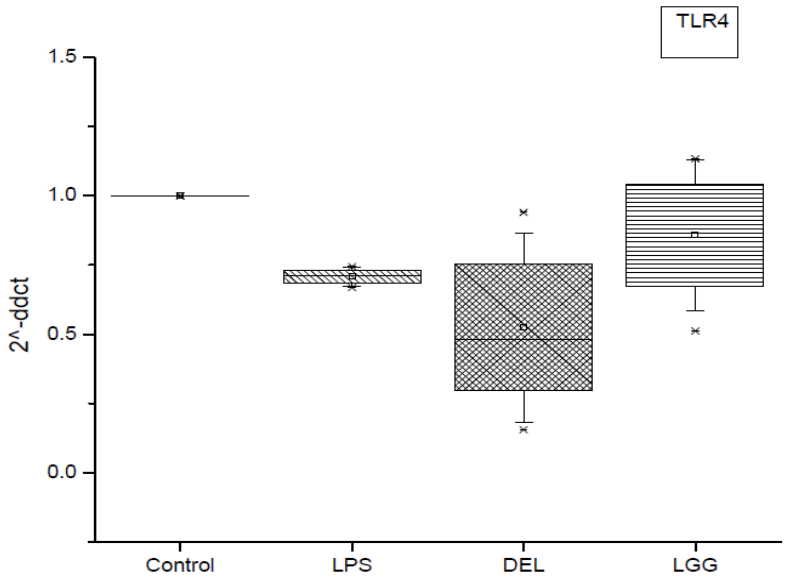

Figure 4.3.1 Expression of TLR4 mRNA in DCs treated with Lactobacillus Rhamnosus GG (gg), Lactobacillus Delbrueckii (del) and LPS (pc) for 12 hours with bacteria:DC ratio of 10:1

4.3.2 P38 mRNA

Expression of P38 was down regulated by both strains as well as by LPS. However, only *LGG's* down regulatory effect on p38 (0.47±0.1, *p=0.01*) was shown to be statically significant (Figure4.3.2).

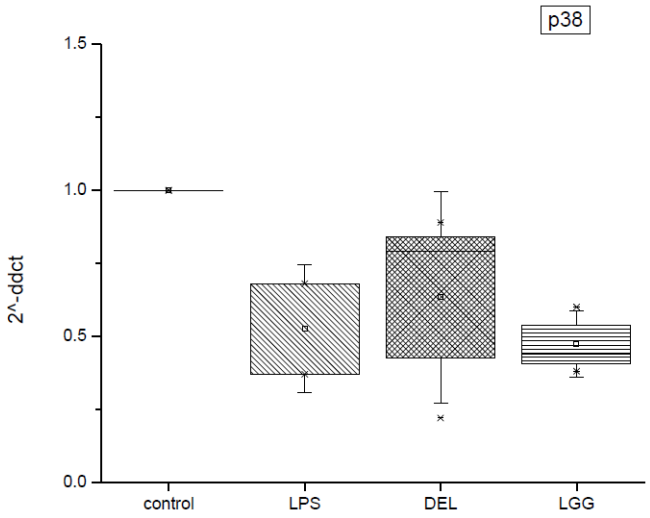

Figure 4.3.2 Expression of p38 mRNA in DCs treated with Lactobacillus Rhamnosus GG (gg), Lactobacillus Delbrueckii (del) and LPS (pc) for 12 hours with bacteria:DC ratio of 10:1

4.3.3 IKB mRNA

At our measuring time point (12 hours after incubation) *L.delbrueckii* seemed to have significantly influenced the signaling pathway through inhibition of Ikb expression (0.37±0.2, *p=0.03*), while *LGG* showed quite no effect on IKb expression (Figure 4.3.3).

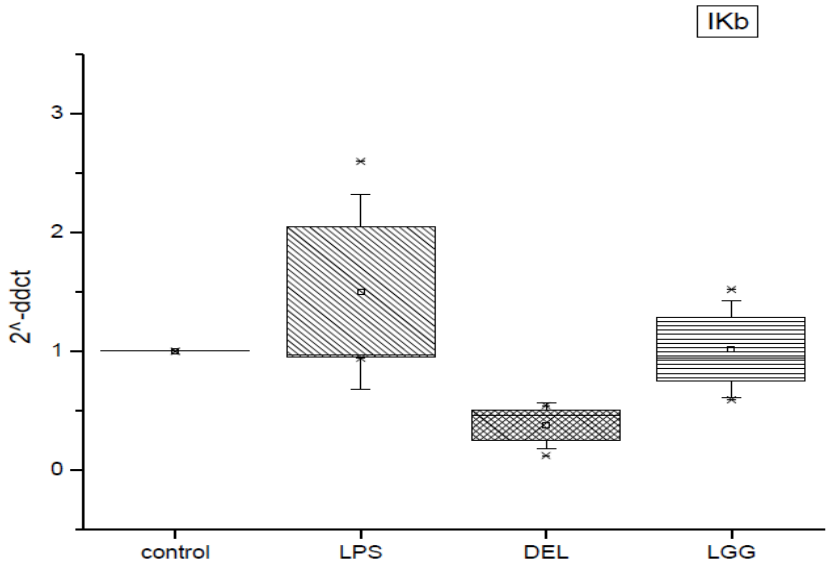

Figure 4.3.3 Expression of IκB mRNA in DCs treated with Lactobacillus Rhamnosus GG (gg), Lactobacillus Delbrueckii (del) and LPS (pc) for 12 hours with bacteria:DC ratio of 10:1

4.3.4 TNF-α mRNA

Only LPS led to a significant up- regulation in TNF-α gene expression (2.28±0.3,*p=0.02*) after 12 hour s. Neither inactivated form of *LGG* (1.12±0.3, p=0.6) nor *L.delbrueckii* (0,9±0.4,p=0.7) affect TNF-α mRNA expression in our experimental setting (bacteria: DC. 10: 1, after 12 hours) Figure 4.3.4).

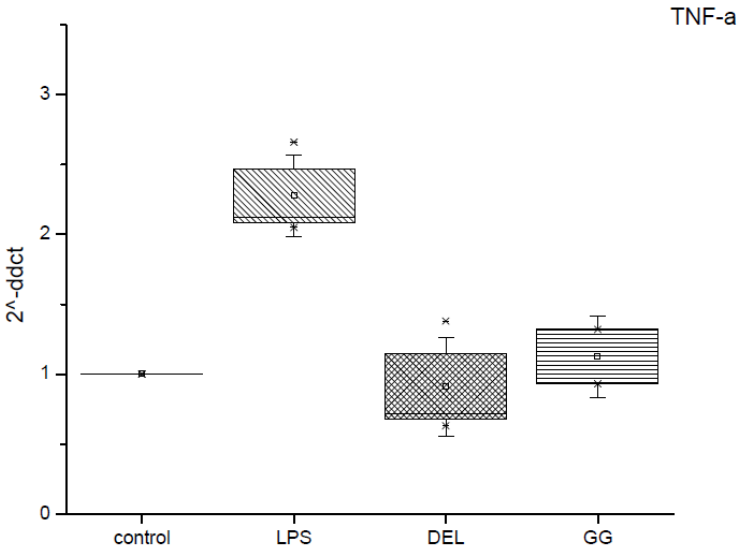

Figure 4.3.4 Expression of TNF-α mRNA in DCs treated with Lactobacillus Rhamnosus GG (gg), Lactobacillus Delbrueckii (del) and LPS (pc) for 12 hours with bacteria:DC ratio of 10:1

4.3.5 miRNA 146a

Expression of miRNA146a was significantly down-regulated by *LGG* (0.6±0.01, p=0.02) where as LPS (1.21±0.3, p=0.3) and *L.delbrueckii* (1.29±0.2, p=0.2) were not particularly changing the expression level of this miRNA (Figure4.3.6)

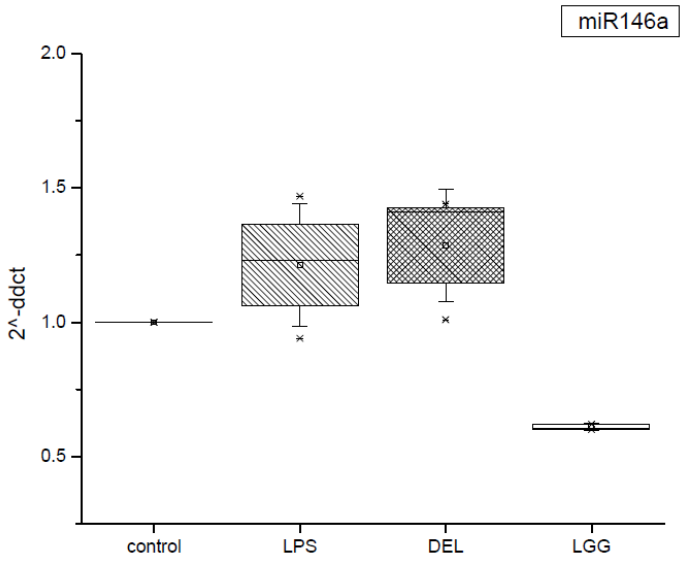

Figure 4.3.6 Expression of miRNA146a in DCs treated with Lactobacillus Rhamnosus GG (gg), Lactobacillus Delbrueckii (del) and LPS (pc) for 12 hours with bacteria:DC ratio of 10:1

4.3.6 miR155

Expression of miR155 showed to be mostly influenced by LPS (1.57±0.1,*p=0.02*) and *LGG* (1.53±0.4, p=0.1). In DCS treated with *L.delbrueckii* no recognizable effect was found on miR155 expression (1.18±0.2,p=0.3), (figure 4.3.7).

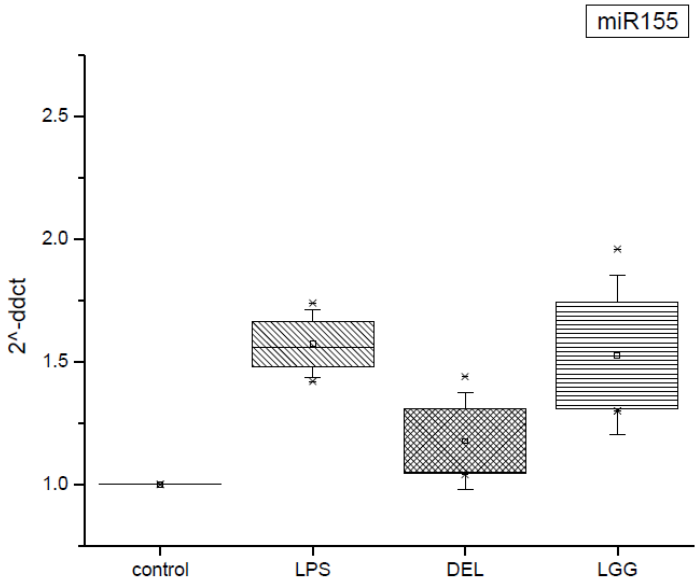

Figure 4.3.4 Expression of miRNA 155 in DCs treated with Lactobacillus Rhamnosus GG (gg), Lactobacillus Delbrueckii (del) and LPS (pc) for 12 hours with bacteria:DC ratio of 10:1

4. Discussion

Immuno-modulatory effect of probiotic bacteria including lactobacilli is widely emphasized in literature (Borchers, et al., 2009; Corthesy, et al., 2007; Elmadfa, et al., 2010). However, the absence of comprehensive mechanistic data makes the interpretation of the vast array of available findings rather conflicting. It remains unclear to what extent, via which mechanisms or which components of probiotics (the whole viable bacteria, cell wall component, soluble compounds, metabolites or heat resistant components) are responsible for the immune modifications which favor anti-inflammatory or regulatory responses. Unlike most studies that have investigated only the effect of viable probiotics or cell wall extracts this study also examined whether nonviable *LGG* and *L.delbrueckii* can provoke immune modulatory effect by targeting DCs that are the key cells responsible for orchestrating further immune responses.

Our results clearly demonstrated that non viable *LGG* had considerable capacity to up regulate expression of co-stimulatory molecule CD86 (B7.2), the hallmark of dendritic cell activation and maturation (Dilioglou, et al., 2003; Nguyen, et al., 2002), along with CD80 (B7.1) and CD83. So we can say that *LGG* is able to provide the necessary stimuli for priming T cells. On the other hand, CD86 and CD83 were noticeably up regulated by *L.delbrueckii* while no effect was observed in induction of CD80.

The dose of lactobacilli didn't however influence the expression of these co-stimulatory molecules. Based on the fact that CD80 favors the activation of TH1 lymphocytes, CD86 favors priming of TH2 cells and the relative amounts of CD86 and CD80 on DC influence the type of T cell response, it is therefore thought that *LGG* and *L.delbrueckii* stimulate different immune responses (Drakes, et al., 2004; Zheng, et al., 2004). This divergence in

stimulatory effect of *LGG* and *L.delbrueckii* was not confined to CD80, CD86 and CD83. Actually, *L.delbrueckii* was also able to up-regulate CD11c, a marker of myeloid DC, more than *LGG* especially at lower ratios (10:1) whereas CD54 was up-regulated more by *LGG*. Increasing the ratio of probiotics diminished the stimulatory effect of *LGG* and *L.delbrueckii* on CD54 expression.

On the other hand, expression of dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN; CD209) was reduced after 24 hours by *L.delbrueckii* and *LGG* in dose dependent manner. It is known that binding of pathogens to DC-SIGN leads to Ag processing and DC–T Cell clustering and DC-induced T cell proliferation (Geijtenbeek, et al., 2002). Subversion of this process can induce infection by some pathogens. In addition some viruses are reported to use DC-SIGN to establish infection in the host (Rappocciolo, et al., 2006). This result is not persuasive enough to describe inactivated *LGG* and *L.delbrueckii* as stimulants which weaken T cell responses in pathogenic conditions. This study had no inflammatory stimuli whose existence and binding to CD209 in the cultures may have possibly changed the above findings.

Additionally, unique cytokine production profile of MODCs after treatment with *LGG* and *L.delbrueckii* was analyzed. The results indicated both strains werre not only able to maturate DCs but also to yield considerable cytokine production in their non viable form. *LGG* was found to augment the secretion of immunosuppressive cytokine IL-10 which inhibits activation and function of effector T cells (Pena & Versalovic, 2003). This finding is in accordance with in vivo results demonstrating generation of IL-10 in atopic children after oral administration of *LGG.* Negative effect of IL-10 in TH2 cell differentiation and expansion and the shift from TH2 response toward long lasting TH1 response lead to the

idea of using probiotics, such as *LGG*, as a novel therapeutic strategy for treatment of allergic disease (Kalliomaki, et al., 2001; Vissers, et al., 2011). Production of IL-10 by *LGG* stimulated DCs might also lead to differentiation and function of T regulatory cell (Treg) subsets (Calcinaro, et al., 2005; Yu, et al., 2010). Nevertheless secretion of IL-1β and, to a lesser extent TNF-α, was also found to increase after incubation of DCs with *LGG* especially at ratio of 100:1 of bacteria to DC. IL-12 as well as INF-γ also increased when DCs were stimulated with higher concentration of *LGG*. The moderate secretion of pro-inflammatory cytokines by probiotic bacteria stimulated MODCs has been explained by induction of low grade inflammation, maintenance of the balance between TH1 and TH2 responses and maintenance of Treg cells that might in turn protect allergy prone people from development of allergy (Fink, 2010; Marschan, et al., 2008). Also IL-12 is known to limit establishment and maintenance of TH2 type responses mainly by enhancing INF-γ production. This can explain why the presence of lactic acid bacteria in in-vitro models is associated with reduction of TH2 cytokine release (Meijerink & Wells, 2010).

This study found that DCs treated with *L.delbrueckii* secreted more INF-γ, IL-12, IL-10 and IL1-β than non treated DCs. At probiotic:DC ratio of 10:1, *L.delbrueckii* demonstrated the same capacity as *LGG* for producing all cytokines except TNF-α. *L.delbrueckii* led to significantly higher expression of TNF-α than *LGG* at both measured concentrations. The orientation of the immune response by *L.delbrueckii* may differ from LGG especially at higher concentrations, because *LGG* led to higher production of IL-10, IL1-β and INF-γ where as *L.delbrueckii* markedly increased TNF-α after 24 hours incubation. Probiotic bacteria which induce a pattern of maturation of DC characterized by

release of TNF-α and IL-12 with increased levels of IL-10 are said to inhibit generation of pro-inflammatory TH1 cells.

Administration of heat killed of other strains of Lactobacilli, such as *L.casei shirota*, had also down regulated existing TH2 allergic response and pulmonary inflammatory response in subcutaneous and air way allergy challenges (Lim, et al., 2009). Different species of UV exposed lactobacilli have also yielded identical results in producing T reg cells, indicating that regulatory DC priming does not depend on viability of the bacteria. It is supposed that probiotics have heat–stable anti-proliferative components which are located in the cytoplasm and not in the cell walls and are responsible for immuno-suppressive effects (Pessi, et al., 1999).

In the above findings, the strain specific capability of lactobacilli to up-regulate surface markers didn't correlate with the cytokine induction profile.

Probiotics not only interfere with generation of mature DCs but also influence the survival of DCs. Future studies should examine the duration that probiotics can stay within DCs by monitoring immune responses and how they affect DCs viability and whether or not the remains of probiotic-killed DCs can be processed by viable DCs in the environment.

Additionally, we know that sensing of bacterial products by the immune system is mediated by Toll-like receptors. TLR4, the first of the TLRs described, has been the focus of particular interest since its recognition as the receptor for LPS and is widely studied on both enterocytes and immune cells. In this study we have reported that the expression of TL4 is influenced by *LGG* and *L.delbrueckii* as it is influenced by LPS. Although LPS is known to have positive stimulatory effect on T LR4 we have to consider that this

stimulation follows a time kitenetic pattern and the expression level do not remains constant at transcriptional level for a long time during exposure to LPS or other ligands.

Several recent studies have reported that the signaling pattern of gram negative bacteria slightly differs from that of gram positive bacteria, and most cases Gram-positive probiotics bacteria are shown to induce TLR2 expression via interactions with lipoteichoic acids but not TLR4 expression. H owever,a recent study by Votan et al (2007) demonstrated that Gram-positive *L.crispatus* increases TLR2 and reduces TLR4 mRNA in clonic mucosa of mice. Also *Lactobacillus casei CRL 431* administration to healthy mice increased the expression of TLR2, TLR4 and TLR9 and improved the production and secretion of TNFα, IFNγ and IL-10 in the inductor sites of the gut immune response Peyer's patches. Although probiotics and pathogenic bacteria may share TLR signaling, their downstream substrates and outcomes could be different. For example, both *L.rhamnosous GG* and *Streptococcus pyogenes* enhance TLR2 expression and the subsequent NFkB activation depends on this expression. However, in addition to enhanced TLR2 gene expression *s.pyogenes* also up-regulates TLR3 and TLR7 expression resulting different response (Miettinen 2008). In human cells, *L.rhamnosus GG* upregulates TLR2 and TLR9 transcription levels as well as protein levels of TLR2 and TLR5 (Vizoso et al., 2009). So we have to notice that there is a cross talk between different TLRs which can indirectly influence activation of one another. In this study the observed down regulatory effect of *LGG* and *L.delbrueckii* on TL4 might be the result of an indirect effect of ligation of particles of these strains to other TLRs.

Furthermore, activation of TLR4 signal is related to induction of various signaling pathways including NFκB and P38 MAPK, and release of inflammatory cytokines. We

have observed that both strains of lactobacilli were able to influence p38 a nd IKB expression. However, *LGG's* effect was more significant on dow n regulating p38 expression and *L.delbrueckii* induced significant inhibitory response in IKB expression. Infact optimal' NF-κB activity plays a significant role in maintaining normal immune homeostasis. The inflammatory responses involve the inhibitor of kB (IkB)/nuclear factor (NF)kB complex. NFkB is usually bound in the cytoplasm to the inhibitory molecule IkB, but in response to certain stimuli, phosphorylation, ubiquitination, and proteolysis of IkB occur, resulting in NFkB nuclear translocation and proinflammatory mediation. Inhibititon of IkB degradation thereby prevents NFkB translocation. The observed down regulation in IKB mRNA expression might be the reason of high level of IKB at protein level at the measuring point which could have consequently inhibited NFKb response and ultimately inhibited the production of inflammatory cytokines. In other studies it has been shown that that live and UV-inactivated *LGG* mechanisms of action in down regulating inflammatory products in inflammatory induced conditions merge at the IkB and NFkB steps. Actually, some probiotics strains and even their cellular components such as *L. casei* are reported to activate both NF-kB and p38 M APK signaling pathways (kim 2005). In a human and murine inflammatory model, VSL#3 DNA inhibited IL-8 secretion, reduced p38 mitogen-activated protein kinase activation, delayed nuclear factor kappaB activation, stabilized levels of IkappaB, and inhibited proteasome function . Similarly, *S. boulardii* prevented enterohemorrhagic *Escherichia coli* infection by interfering with the transduction pathways that control tight-junction structure as well as inhibiting NF-κB and MAPK signaling pathways leading to the production of IL-8.

The novel approach of our study was to look for the epigenetic mechanisms which probiotics might be involved. Though, we looked for the effect of our strains on expression of miRNAs which have been validated for DCs. Although it is concluded that miRNAs participate in the regulation of immune system there is still no published result with regard to the effect of probiotics on miRNA expression. We have observed for the first time that *LGG* significantly down- regulate miRNA146a, which is known to directly control TLR4 and regulate the maturation process in dendritic cells . However, LPS and *L.delbrueckii* showed a little up-regulatory effect on expression of this miRNA. Previous studies have shown that LPS- induced regulation of miRNA146a is cell-type-specific and it depends to the cell environment (Moschos, et al., 2007). Indeed it has been shown that only the ligands of TLRs that recognize bacterial constituents and reside on the cell surface are able to induce miR146 expression(K. D. Taganov, et al., 2007). Whereas the noteworthy finding of our study is that our tested strains have modified miR146 expression in spite of being autoclaved which their surface components have been defiantly denaturated.

Until now we just know that aberrant miRNA146 expression is related to systemic autoimmune disorders and chronic inflammation. Thus determination whether probiotics as immune modulating agents can be used to modulate miRNA in vivo represents important further research task.

Finally we have also found that miRNA 155 is considerably up-regulated in LPS and *LGG* treated DCs. In a recent study highly up-regulation of miR155 during dendritic cell maturation has been reported and it is shown that miR155 modulates TLR/IL-1 signaling pathway in activated monocyte derived dendritic cells. More importantly, it has been reported that the top ranked pathway whose genes are mostly up-regulated upon miR155

inhibition are the p38MAPK pathway (Ceppi, et al., 2009). This finding support our results that show significant down-regulation in p38 expression in *LGG* treated DCs that induce higher expression of miR155 at the same time. In fact, in resting DCs, low miR155 levels may enable the activation of p38 MAPK pathway, while in activated DCs induction of miR155 and miR146 can lead to decreased p38 MAPK pathway and consequently, limit the over production of inflammatory cytokines. In addition to the regulatory role of miR155 in TLR4 signaling, it was recently shown that miR155 also regulates human dendritic cell function by modulating their pathogen binding ability, through down regulation of DC-SIGN, resulting in impaired recognition and binding of pathogens (R. T. Martinez-Nunez, et al., 2009). In accordance to this finding we have seen significant down-regulation of DC-SIGN in DCs treated with *LGG* and *L.delbrueckii.* These findings provide genetic and epigenetic mechanistic explanations for the proposed anti-inflammatory effect of *LGG* in inflammatory conditions such as inflammatory bowel disease.

5. Conclusion

By combining the results of the current in-vitro study we can suggest that nonviable *LGG* and *L.delbrueckii* possess heat stable components that influence functional ability of DCs to generate specific immune responses depending on the level of co-stimulatory molecule expression and cytokine production profile.

We have provided evidence for the first time that autoclaved probiotics are not only able to influence key immune signaling nodes at transcriptional level but also interact with miR146 and miR155. Thereby, target genes responsible for DCs maturation, pathogen binding capacity such as DC-SIGN, and cytokine signaling pathways including TLR4 and p38MAPK at post- transcriptional level.

These effects in autoclaved probiotics strains are shown to be absolutely strain specific as in live strains. Therefore, lactobacilli viability may not be required for regulating DC priming and immuno-modulation.

Additionally, dose-dependent responses of probiotics indicate involvement of different signaling pathways at higher doses.

Continuation of studies using probiotics loaded DCs is a fertile field for investigating the mechanism of action of probiotics which is hoped to lead us to a new paradigm for development of desirable immune responses.

6. Limitation

Due to the nature of in *vitro* experimental setting it won't be precise to extrapolate the results of *in vitro* immuno-modulatory properties of probiotics to *in vivo* conditions. However, some researchers have shown that *in vitro* immune profiling of strains can be predictive of their *in vivo* protective effect in mice models. So the first and most important limiting factor is the method of DCs differentiation *in vitro* by GMCSF and IL-4. No doubt, DCs development *in vivo* differs in cytokine dependency, and the micro environment at the differentiating stage has a great impact on fine complex properties of the differentiated cell.

The second prominent fact is that we have looked for the effect of each strain individually to find their individual effects, while practically we are never exposed to a single strain and actually each single strain is going to induce a response which is indirectly influenced by a cross talk with immense variety of commensal strains.

Third point is that all the biologic responses and events in signaling pathways specifically at transcriptional level follow a conscious time kinetic response that for better understanding of the effect of our stimulation it would have been better that we could have selected different time points. However, due to limitations in producing sufficient number of DCs for each experiment that was not feasible in this study.

Forth, the high standard deviation in expression of some CD markers would have been narrowed in case of having the possibility to run more experiments.

So it is believed that investigating the effect of probiotics on immune system, in viable or nonviable condition is worth to be done with a more systemic approach considering the

well explained network of responses which take place in conscious timing in a setting which resembles the *in vivo* condition as much as possible.

7. Summary

Strain specific immunomodulatory effect of probiotics is among their key health benefit. It is believed that probiotics determine the fate of an immune response by targeting and polarizing dendritic cells. Therefore we studied the effects of a heat-inactivated form of *Lacto bacillus rhamnosus GG (LGG)* and *Lactobacillus delbrueckii (L.delbrueckii)* on maturation pattern and extracellular cytokine production profile of monocyte derived dendritic cells. Expression of specific maturation markers and induction of extracellular cytokines were detected by flow cytometry. Up regulation of CD86, CD80, and CD83 and down regulation of DCSIGN was observed after 24 hours co-incubation. In addition, *LGG* induced secretion of high levels IL-10, INF-γ and IL-1β whereas. *L.delbrueckii* seemed to be more potent in induction of TNF-α in a dose dependent manner. These results indicated that non-viable forms of both tested strains are able to induce divergent immune regulatory effects via the induction of phenotypic changes and cytokine production of dendritic cells.

Moreover, as the immune response is initiated by recognition through toll like receptors and activation of network of downstream signaling pathways, we investigated the effect of autoclaved *LGG* and *L.delbrueckii* on expression of TLR4 and signaling factors such as P38MAPK and IκB at transcription level. Our findings demonstrated that even autoclaved probiotics strains can affect TLR4 expression in a down regulatory direction as LPS after 12 hours. Indeed, *LGG* significantly down regulated expression of P38 while IκB expression was significantly reduced in *L.delbrueckii*- treated DCs.

Finally, we found the tested strains could even influence immune response at post transcriptional level by modifying miRNAs expression. Based on our findings *LGG* induced significant down regulatory effect on miRNA146a expression which is known as a

novel fine negative regulator of immune response that targets NFκB. On the other hand, miRNA 155 was up-regulated by *LGG* which is consistent with down-regulation of p38 and DC-SIGN expression in *LG*G- treated dendritic cells. These findings provide genetic and epigenetic explanations for the responsible underlying mechanisms by which probiotics influence immune response by targeting dendritic cells.

Zusammenfassung

Einer der wichtigsten gesundheitlichen Wirkungen von Probiotika sind stammspezifische immunmodulatorische Effekte. Man geht davon aus, dass Probiotika die Art der Immunantwort durch das Ansprechen und Polarisieren von dendritischen Zellen regulieren. Daher untersuchten wir die Effekte von Hitze-inaktivierten *Lactobacillus rhamnosus GG* (*LGG*) und *Lactobacillus delbrueckii* (*del*) auf die Reifung und das Profil der extrazellulären Zytokinproduktion von dendritischen Zellen, die aus Monozyten gewonnen wurden. Die Expression verschiedener Reifungsmarker und die Induktion extrazellulärer Zytokine wurden mit Durchflusszytometrie gemessen. Nach 24 Stunden Ko-Inkubation wurden ein Anstieg von CD86, CD80 und CD83, sowie ein Absinken von DCSIGN beobachtet. Zusätzlich induzierte *LGG* die Sekretion von hohen Levels IL-10, INF-γ und IL-1β, während *L.del* größeres Potenzial in der dosisabhängigen Induktion von TNF-α aufzeigte. Diese Resultate lassen vermuten, dass abgetotete Formen beider getesteten Stämme fähig sind, unterschiedliche immunregulatorische Effekte über die Induktion von phänotypischen Änderungen und die Zytokinproduktion von dendritischen Zellen hervorzurufen.

Desweiteren untersuchten wir die Effekte von autoklavierten *LGG* und *del* auf die Expression von TLR4 und Signaltransduktionsfaktoren wie z.B. P38MAPK und IκB, da die Immunantwort über toll-like Rezeptoren und die Aktivierung von Netzwerken der weiterführenden Signaltransduktion initiiert wird. Unsere Ergebnisse zeigten, dass auch autoklavierte, probiotische Stämme die TLRR4-Expression herabregulierend beeinflussen können wie LPS nach 12 Stunden. Die Expression von P38 wurde durch *LGG* signifikant

herabreguliert, während die IkappaB-Expression in *del*-behandelten DZs signifikant reduziert war.

Außerdem stellten wir fest, dass die getesteten Stämme die Immunantwort sogar post-transkriptional durch die Modifikation der miRNA-Expression beeinflussen konnten. Unsere Ergebnisse zeigten, dass *LGG* ein signifikantes Absinken der Expression der miRNA146a induziert, die bekanntermaßen die NFκB -vermittelte Immunantwort negativ reguliert. Andererseits wurde miRNA 155 durch *LGG* hinaufreguliert, was mit dem Absinken der p38 und DC-SIGN-Expressionen in *LGG*-behandelten dendritischen Zellen einhergeht. Diese Ergebnisse bieten genetische und epigenetische Erklärungen für die verantwortlichen zugrundeliegenden Mechanismen, durch welche Probiotika die Immunantwort über DZs beeinflussen.

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Protocols

Appendix I Dynal® Monocyte Negative Isolation Kit

http://tools.invitrogen.com/content/sfs/manuals/113.09_10%20Dynal_Monocyte_Negative_Isolation_Kit.pdf

Dynabeads Washing Procedure

1. Resuspend the Dynabeads in the vial to a homogenous suspension.

2. Transfer the desired volume of Dynabeads to a tube.

3. Add the same volume of Buffer 1, or at least 1 ml, and mix.

4. Place the tube in a magnet for 3 min and discard the supernatant.

5. Remove the tube from the magnet and resuspend the washed Dynabeads in the same volume

of Buffer 1 as the initial volume of Dynabeads (step 2).

Preparation of MNC from Buffy Coat to Obtain Low Platelet Numbers

1. Dilute 10 - 18 ml buffy coat with Buffer 2 to a total volume of 35 ml at room temperature.

2. Add the diluted buffy coat on top of 15 ml of Lymphoprep™.

3. Centrifuge at 160 x g for 20 minutes at 20°C. Allow to decellerate without brakes.

4. Remove 20 ml of supernatant to eliminate platelets.

5. Centrifuge at 350 x g for 20 minutes at 20°C. Allow to decellerate without brakes.

6. Recover MNC from the plasma/Lymphoprep interface and transfer the cells to a 50 ml tube.

7. Wash MNC once with Buffer 1 by centrifugation at 400 x g for 8 minutes at 2-8°C.

8. Wash MNC twice with Buffer 1 by centrifugation at 225 x g for 8 minutes at 2-8°C and resuspend the MNC at 1 x 108 MNC per ml in Buffer 1 (keep cold).

Isolation of Human Monocytes from MNC

This protocol is based on 1 x 107 MNC. It can be scaled up from 1 x 107 - 5 x 108 cells

1. Transfer 100 µl (1 x 107) MNC in cold Buffer 1 to a tube.

2. Add 20 µl Blocking Reagent.

3. Add 20 µl Antibody Mix.

4. Mix well and incubate for 20 min at 2-8°C.

5. Wash the cells by adding 2 ml Buffer 1. Mix well by tilting the tube several times and centrifuge at 300 x g for 8 min at 2-8°C. Discard the supernatant.

6. Resuspend the cells in 900 µl Buffer 1.

7. Add 100 µl pre-washed Depletion Dynabeads and mix well.

8. Incubate for 15 min at 2-8°C with gentle tilting and rotation.

9. Resuspend the bead-bound cells by gently pipetting 5 times using a pipette with a narrow

tip opening (e.g. a 1000 µl pipette tip or a 5 ml serological pipette).

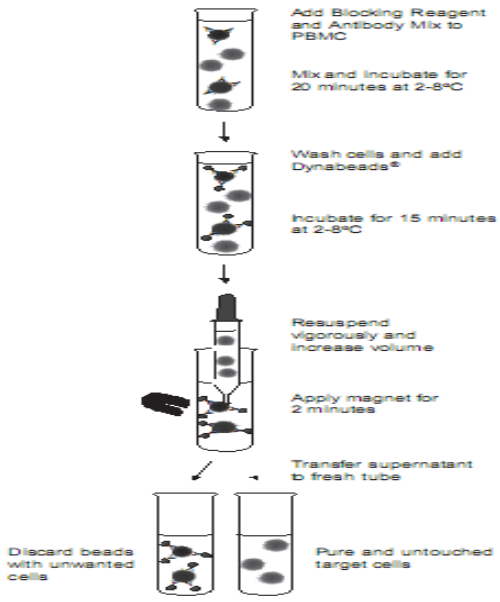
10. Add 1 ml Buffer 1.

11. Place the tube in the magnet for 2 min.

12. Transfer the supernatant to a new tube.

13. Repeat step 10-12.

The supernatant contains the untouched human monocytes.



Appendix II Protocol Purification of Total RNA from Animal Cells

*www.**qiagen**.com/hb/**rneasymini***

Harvest cells according to step 1

1. Cells grown in suspension (do not use more than 1 x 107 cells): Determine the number of cells. Pellet the appropriate number of cells by centrifuging for 5 min at 300 x g in a centrifuge tube (not supplied). Carefully remove all supernatant by aspiration.

2. Disrupt the cells by adding Buffer RLT. For pelleted cells, loosen the cell pellet thoroughly by flicking the tube. Add the appropriate volume of Buffer RLT . Vortex or pipet to mix

Number of pelleted cells	Volume of Buffer RLT (µl)
<5 x 10^6	350
5 x 10^6 – 1 x 10^7	600

3. Homogenize the lysate for 30 s using a rotor–stator homogenizer.

4. Add 1 volume of 70% ethanol to the homogenized lysate, and mix well bypipetting. Do not centrifuge.

5. Transfer up to 700 µl of the sample, including any precipitate that may have formed, to an RNeasy spin column placed in a 2 m1 collection tube (supplied). Closethe lid gently, and centrifuge for 15 s at _8000 x g (_10,000 rpm). Discard theflow-through. Reuse the collection tube in step 6.

If the sample volume exceeds 700 µl, centrifuge successive aliquots in the same RNeasy spin column. Discard the flow-through after each centrifugation

6. Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at _8000 x g (_10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 7.

7. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 15 s at _8000 x g (_10,000 rpm) to wash the spin column membrane. Discard the flow-through. Reuse the collection tube in step 8.

8. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid gently, and centrifuge for 2 min at _8000 x g (_10,000 rpm) to wash the spin column membrane.

9. Optional: Place the RNeasy spin column in a new 2 m1 collection tube (supplied),and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min. Perform this step to eliminate any possible carryover of Buffer RPE, or if residual

flow-through remains on the outside of the RNeasy spin column after step 8.

10. Place the RNeasy spin column in a new 1.5 ml collection tube (supplied). Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid gently, and centrifuge for 1 min at _8000 x g (_10,000 rpm) to elute the RNA.

11. If the expected RNA yield is >30 µg, repeat step 10 using another 30–50 µl RNasefree water, or using the eluate from step 10 (if high RNA concentration is required). Reuse the collection tube from step 10.

Appendix III *Phusion RT-PCR Kit Finnzymes* Protocol for cDNA synthesis

*www.**finnzymes**.fi/**rt-pcr/Phusion_rt-pcr_kit**.html*

1. Thaw template RNA, 10x RT buffer, Deoxynuclteotidtriphosphat (dNTPs) and primers. Mix the individual solutions to assure homogeneity and centrifuge briefly before pipetting.

2. Combine the following components in reaction tubes:

Template RNA x µl (up to 1 µg) 10 mM dNTP mix 1 µl

Oligo(dT) primer* 1 µl

RNase-free H2O Add to 10 µl

3. Incubate at 65°C for 5 minutes to predenature the RNA.

4. Place the reaction tubes on ice and add to each tube

10x RT buffer 2 µl

RT enzyme mix 2 µl

RNase-free H2O 6 µl

5. Program a thermal cycler:

Primer extension 25°C 10 min

cDNA synthesis 40°C 30 min

Reaction termination 85°C 5 min

Cooling of the sample 4°C Hold

6. Place the tubes in the cycler and start the program.

Appendix IV QuantiMir RT Kit Small RNA Quantitation System

www.genycell.es/images/productos/protocolos/sbra420a-1__43.pdf

II. Protocol

A. QuantiMir™ RT Reaction Setup

It is important to start with total RNA that includes the small RNA fraction.

Start: **In a thin-walled PCR tube or PCR-compatible plate well, combine:**

STEP 1: PolyA Tail

5	µl	Total RNA (10 pg – 10µg)
2	µl	5X PolyA Buffer
+ 1	µl	25mM $MnCl_2$
1.5	µl	5mM ATP
0.5	µl	PolyA Polymerase
10	µl	Total in tube

↓

Incubate for 30 min. at 37°C

↓

STEP 2: Anneal Anchor dT Adaptor

Add: + 0.5 µl Oligo dT Adaptor

↓

Heat for 5 min. at 60°C
Let cool to room temp for 2 min.

↓

STEP 3: Synthesize cDNAs

Add: 4	µl	5X RT Buffer
2	µl	dNTP mix
+ 1.5	µl	0.1M DTT
1.5	µl	RNase-free H_2O
1	µl	Reverse Transcriptase
20.5	µl	Total in tube

↓

Incubate for 60 min. at 42°C
Heat for 10 min. at 95°C

Done!

*The QuantiMir™ cDNAs can be stored at -20°C.

How much cDNA template per qPCR reaction?

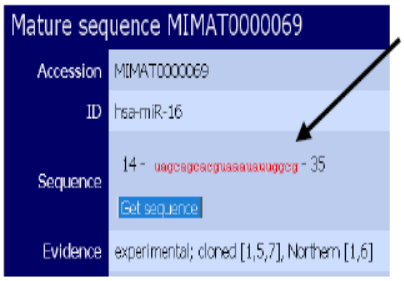
Starting RNA	QuantiMir cDNA per qPCR reaction
10pg -100 ng	0.5ul to 1ul
100ng – 1 µg	Dilute 1: 100
> 1 µg	Dilute 1: 1000

B. Primer Design Considerations for QuantiMir™ cDNA Detection and Quantitation

The user needs only to design the forward "sense" orientation primer to perform the end-point or qPCR reactions. MicroRNAs typically range in size from 19 – 24 nt. We recommend using the exact sequence of the miRNA or siRNA being studied when designing the forward primer. If the miRNA under study is known and documented, using the miRBase database can be an easy starting point:
(http://microrna.sanger.ac.uk/sequences/search.shtml).

An example of the known and documented miRNA, Human miR-16, is shown below.

Hsa-miR-16



Simple: Directly use sequence of mature miRNA as forward primer in oligo design.

The mature miRNA sequence 5' – uagcagcacguaaauauuggcg – 3' can be simply converted to a DNA sequence and used directly as the forward primer for end-point and qPCR analysis.

Forward primer for hsa-miR-16 (included in kit):
5' – TAGCAGCACGTAAATATTGGCG – 3'
Tm= 58.9°C, 45% GC and length = 22 bases.

If the user is developing a new assay for a novel miRNA, follow the guidelines of the example above. Design the primer to have a Tm of at least 50°C and to have a length of at least 18 bases. If the primer being designed for the miRNA to be studied has a Tm below 50°C, lower the annealing temperature in your cycling conditions for end-point and qPCR instrument settings. An example of successfully using this approach is demonstrated in **Figure 5**.

D. Real-time qPCR Reactions using QuantiMir™ cDNAs

For end-point PCR reactions, please refer to the **Section C** above, "End-point PCR Reactions using QuantiMir™ cDNAs".

1. qPCR Reaction Setup

To determine the expression profile for your miRNA under study, mix the following per well:

For Single well determination:

	15	µl	2X SYBR Green qPCR Mastermix buffer
	1	µl	User-designed Forward Primer (10 µM)
+	0.5	µl	Universal Reverse Primer (10 µM)
	1	µl	Diluted QuantiMir™ cDNA (see p.# 7)
	12.5	µl	RNase-free water
	30	µl	Total / well

The Mastermix contents can be scaled up or down depending upon on your experimental needs. Once reagents are loaded into the wells, cover the plate with the optical adhesive cover and spin briefly in a centrifuge to bring contents to bottom of wells. Place plate in the correct orientation (well A1, upper left) into the Real-time qPCR instrument and perform analysis run.

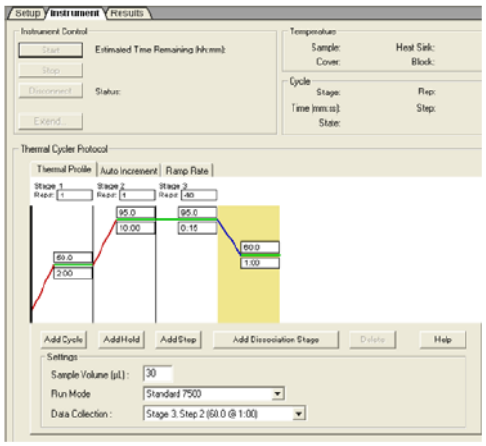
2. Real-time qPCR Instrument Parameters

Follow the guidelines as detailed for your specific Real-time instrumentation. The following parameters tested by SBI were performed on an Applied Biosystems 7300 Real-time PCR System but can also apply to an ABI 7500 or an ABI 7900 96-well system. The details of the thermocycling conditions used in testing at SBI are shown below. A screenshot from SBI's instrument set up is shown on the right. Default conditions are used throughout except for those cases where the Forward Primer's Tm is below 55°C, then the annealing temperature in Step 2 of Stage 3 is lowered from 60°C to 50°C.

qPCR cycling and data accumulation conditions:

1. 50°C 2 min.
2. 95°C 10 min.
3. 95°C 15 sec.
4. 60°C 1 min.

(40 cycles of steps 3 and 4), data read at 60°C 15 sec. Step (gold rectangle)



An additional recommendation is to include a melt analysis after the qPCR run to assess the Tm of the PCR amplicon to verify the specificity of the amplification reaction. Refer to the User Manual for your specific instrument to conduct the melt analysis and the data analyses of the amplification plots and Cycle Threshold (Ct) calculations. In general, Cycle thresholds should be set within the exponential phase of the amplification plots with software automatic baseline settings.

Appendix V *mir*Vana™ miRNA Isolation Kit

http://www.ambion.com/techlib/prot/fm_1560.pdf

1. **Add 1.25 volumes 100% ethanol, and mix thoroughly:** Add 1.25 volumes of room temperature 100% ethanol to the aqueousphase (e.g. if 300 µL was recovered in step E.3, add 375 µL ethanol).

2. **Pass the lysate/ethanol mixture through a Filter Cartridge:** Pass the lysate/ethanol mixture through a Filter Cartridge

a. For each sample, place a Filter Cartridge into one of the Collection Tubes supplied.

b. Pipet the lysate/ethanol mixture (from the previous step) onto the Filter Cartridge. Up to 700 µL can be applied to a Filter Cartridge at a time, for samples larger than this, apply the mixture in successive applications to the same filter.

c. Centrifuge for ~15 sec to pass the mixture through the filter: Centrifuge at RCF 10,000 x g (typically 10,000 rpm). Spinning harder than this may damage the filters. Alternatively, vacuum pressure may be used to pass samples through the filter.

d. Discard the flow-through, and repeat until all of the lysate/ethanol mixture is through the filter. Reuse the Collection Tube for the washing steps.

3. Apply 700 µL miRNA Wash Solution 1 (working solution mixed with ethanol) to the Filter Cartridge and centrifuge for ~5–10 sec or use a vacuum to pull the solution through

the filter. Discard the flow-through from the Collection Tube, and replace the Filter Cartridge into the same Collection Tube.

4. Wash the filter twice with 500 µL Wash Solution 2/3:

a. Apply 500 µL Wash Solution 2/3 (working solution mixed with ethanol) and draw it through the Filter Cartridge as in the previous step.

b. Repeat with a second 500 µL aliquot of Wash Solution 2/3.

c. After discarding the flow-through from the last wash, replace the Filter Cartridge in the same Collection Tube and spin the assembly for 1 min to remove residual fluid from the filter.

5. Elute RNA with 100 µL95°C Elution Solution : Transfer the Filter Cartridge into a fresh Collection Tube (provided with the kit). Apply 100 µL of pre-heated (95°C) Elution Solutionto the center of the filter, and close the cap. Spin for ~20–30 sec at maximum speed to recover the RNA. Collect the eluate (which contains the RNA) and store it at –20°C or below.

F.II. Enrichment Procedure for Small RNAs

This variation of a traditional glass-fiber filter RNA purification yields RNA that is significantly enriched for small RNAs. This enrichment is accomplished by first immobilizing large RNAs on the filter with a relatively low ethanol concentration and collecting the flow-through containingmostly small RNA species. More ethanol is then added to this flow-through, and the mixture is passed through a second glass filter where

the small RNAs are immobilized. This second filter is then washed a few times, and the small-RNA enriched sample is eluted.

1. Add 1/3 volume 100% ethanol, and mix thoroughly: Add 1/3 volume of 100% ethanol to the aqueous phase recovered from the organic extraction (e.g. add 100 µL 100% ethanol to 300 µL aqueous phase). Mix thoroughly by vortexing or inverting the tube several times.

2. Pass the sample through a Filter Cartridge, and collect the filtrate:

a. For each sample, place a Filter Cartridge into one of the Collection Tubes supplied.

b. Pipet the lysate/ethanol mixture (from the previous step) onto the Filter Cartridge. Up to 700 µL can be applied to a Filter Cartridge at a time. For sample volumes greater than 700 µL, apply the mixture in successive applications to the same filter.

c. Centrifuge for ~15 sec to pass the mixture through the filter. Centrifuge at RCF 10,000 x g (typically 10,000 rpm). Spinning harder than this may damage the filters. Alternatively, vacuum pressure can be used to pull samples through the filter. d. Collect the filtrate. If the lysate/ethanol mixture is >700 µL, transfer the flow-through to a fresh tube, and repeat until all of the lysate/ethanol mixture is through the filter. Pool the collected filtrates if multiple passes were done, and measure the total volume of the filtrate.

3. Add 2/3 volume 100% ethanol and mix thoroughly: Add 2/3 volume room temperature 100% ethanol to filtrate (i.e.flow-through). For example, if 400 µL of filtrate is recovered, add

266 µL 100% ethanol. Mix thoroughly.

4. Pass the mixture through a second Filter Cartridge, and discard the flow-through:

a. For each sample, place a F ilter Cartridge into one of the Collection Tubes supplied.

b. Pipet the filtrate/ethanol mixture (from the previous step) onto a second Filter Cartridge. Up to 700 µL can be applied to a Filter Cartridge at a time. For sample volumes greater than 700 µL, apply the mixture in successive applications to the same filter.

c. Centrifuge for ~15 sec to pass the mixture through the filter. Centrifuge at RCF 10,000 x g (typically 10,000 rpm). Spinning harder than this may damage the filters. Alternatively, vacuum may be used to pass samples through the filter.

d. Discard the flow-through, and repeat until all of the filtrate/ethanol mixture is through the filter. Reuse the Collection Tube for the washing steps.

5. Wash the filter with 700 µL miRNA Wash Solution 1:

Apply 700 µL miRNA Wash Solution 1 (working solution mixed with ethanol) to the Filter Cartridge and centrifuge for ~5–10 sec or use vacuum to pass the solution through the filter. Discard the flow-through from the Collection Tube, and replace the Filter Cartridge into thesame Collection Tube.

6. Wash the filter twice with500 µL Wash Solution 2/3:

a. Apply 500 µL Wash Solution 2/3 (working solution mixed with ethanol) and draw it through the Filter Cartridge as in the previous step.

b. Repeat with a second 500 µL aliquot of Wash Solution 2/3.

c. After discarding the flow-through from the last wash, replace the Filter Cartridge in the same Collection Tube and spin the assembly for 1 min to remove residual fluid from the filter.

7. Elute RNA with 100 µL 95°C Elution Solution or Nuclease-free Water: Transfer the Filter Cartridge into a fresh Collection Tube (provided with the kit). Apply 100 µL of pre-heated (95°C) Elution Solution or nuclease-free water to the center of the filter, and close the cap. Spin for ~20–30 sec at maximum speed to recover the RNA. Collect the eluate (which contains the RNA) and store it at –20°C or colder

Appendix VI TaqMan® Small RNA Assays

http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_042167.pdf

Prepare the RT reaction

1. Thaw the 5✕ RT primer and RNA template on ice. Before use, vortex the RT primer tubes to mix, then centrifuge briefly.

4. preparing single-stranded RNA, prepare the total RNA template:

a. For each 15-µL RT reaction, combine RT master mix with total RNA in the ratio of: 7 µL RT master mix : 5 µL total RNA (1 to 10 ng per reaction)

b. Mix gently. Centrifuge to bring the solution to the bottom of the tube.

c. Before opening the RT primer tubes, thaw the tubes on ice and mix by vortexing, then centrifuge them. d. For each 15-µL RT reaction, add 12.0 µL of RT master mix containing total

RNA into a 0.2-mL polypropylene reaction tube (the RT reaction tube) or into a well of a 96-well reaction plate.

e. Add 3 µL of 5✕ RT primer from each assay set into the corresponding RT reaction tube or plate well.

5. Seal the tube or reaction plate and mix thoroughly by inverting the solution. Centrifuge to bring the solution to the bottom of the tube or well.

6. Incubate the tube on ice for 5 minutes and keep it on ice until you are ready to load the thermal cycler.

1. Use the following parameter values to program the thermal cycler:

Step	Time	Temperature
Hold	30 minutes	16 °C
Hold	30 minutes	42 °C
Hold	5 minutes	85 °C
Hold	∞	4

2. Set the reaction volume to 15.0 µL.

3. Load the reaction tube or plate into the thermal cycler.

4. Start the RT run.

Perform the qPCR amplification

Thaw and mix the reagents

1. Thaw on ice, resuspend completely by gently vortexing, then centrifuge briefly:

• TaqMan® Assay (20✕)

• Complementary DNA (cDNA) samples

Calculate the number of reactions Calculate the number of reactions that you need for each assay.

• A small RNA assay for each cDNA sample

• Endogenous control assay(s)

• No template controls (NTCs) for each assay on the plate to evaluate background signal.

The recommended reaction volume is 20 µL. Prepare the plate so that each qPCR reaction contains the components as listed below.

To prepare the qPCR reaction mix:

1. Obtain a sterile 1.5-mL microcentrifuge tube for each sample (to be run in triplicate).

3. Pipet the following components into each tube:

Component	Volume per 20-µL Reaction	
	Single reaction	Three replicates§
TaqMan® Small RNA Assay (20X)	1.00 µL	3.60 µL
Product from RT reaction*	1.33 µL	4.80 µL
TaqMan® Universal PCR Master Mix II (2X), no	10.00 µL	36.00 µL
Nuclease-free water	7.67 µL	27.61 µL
Total volume	20.00 µL	72.01 µL

3. Cap the tube and invert several times to mix.

4. Centrifuge the tube briefly.

Prepare the PCR reaction plate

1. Transfer 20 µL of the complete qPCR reaction mix (including assay and RT product) into each of three wells on a 48-, 96-, or 384-well plate.

2. Seal the plate with the appropriate cover.

3. Centrifuge the plate briefly.

4. Load the plate into the instrument.

Set up the experiment or plate document and run the plate

1. In the real-time PCR system software, create an experiment or plate document on your real-time PCR system using the following parameters:

• Run Mode: Standard

• Sample Volume: 20 µL

• Thermal Cycling Conditions:

PCR		Enzyme Activation	Optional AmpErase® UNG activity*	Step
CYCLE (40 cycles)		HOLD	HOLD	
Anneal/extend	Denature			
60 °C	95 °C	95 °C	50 °C	Temperature
60 seconds	15 seconds	10 minutes	2 minutes	Time

2. . If the reaction plate is not already loaded, load the plate into the instrument.

3. Start the run.

Curriculum vitae

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Name: Ladan Giahi

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I. Education:

- **1999 - 2003 BSc.; Nutritional Science,** Faculty of Nutrition and Food Technology, **S**hahid Beheshty University of Medical Sciences, Iran
- **2003-2006 MSPH.; Health Science in Nutrition,** Department of Nutrition and Biochemistry, School of Public Health and Institute of Public Health Research, Tehran University of Medical Sciences, Iran (Graduation Rank: Top student with a GP of 18.97/20)
- **Since 2009 PhD; Life Sciences (Immunonutrition),** Department of Nutritional Sciences**,** Faculty of Life Sciences, University of Vienna, Austria

II. Certificates:

- Certificate of European Union Nutrigenomics organization, *Polymorphisms and Responsiveness to diet*, Wageningen Univesrtiy, Neatherland. 2011
- Certificate from European Union Nutrigenomics organization, *Molecular Nutrition and Genomics*, Wageningen Univesrtiy, Neatherland. 2011
- Certificate of European Union Nutrigenomics organization, *Nuclear Receptors in Nutrition*, Wageningen Univesrtiy, Neatherland. 2011

- Certificate of Leadership Group on the Consultative Meeting on Leading Iranian Nutrition Sciences and Research "One Focus, One Voice Through Collaborative and Participatory Dialogue, Tehran, 2011
- Certificate of European Union Nutrigenomics organization, *Proteomics*, Wageningen Univesrtiy, Neatherland. 2010
- Certificate of *scientific writing,* Tehran University of Medical Sciences, Iran .2006
- Certificate of *Nutrition and Diet Therapy in Renal disease*. Tehran University of Medical Sciences, Iran. 2004
- Certificate of *Diet Therapy in Gastrointestinal Disease*. Institute of Nutrition and Food Technology, **S**hahid Beheshty University of Medical Sciences, Iran, 2004
- Certificate of *Practical Disciplines in Children's Nutrition (Infancy till 19Yrs)* Institute of Nutrition and Food Technology, **S**hahid Beheshty University of Medical Sciences, Iran, 2004

III. Skills:

A. Languages:

- English advanced level
- French advanced level
- German elementary level
- Arabic elementary level

B. Practical Laboratory skills:

- Cell culture techniques
- Gene expression (RNA extraction, PCR)
- Flow cytometry
- ELIZA, IRMA and Biochemical Tests.

C. Clinical Nutrition counseling:

- Nutrition counseling in diabetes, transplant, growth failure, CVD, metabolic syndrome, Renal disease, spinal cord injury, obesity and eating disorders

IV. Experiences:

A. Scientific & Clinical Nutrition Counseling:

- Senior nutrition consultant of directorate of nutrition, Ministry of Health, Tehran, Iran, since 2011.

- Permanent editorial board and consultant of World of Nutrition and Health Culture Institute, since 2007

- Nutrition counseling (Diabetes, transplant, growth failure, CVD, metabolic syndrome, Renal disease, spinal cord injury, obesity, eating disorders), Iran. 2007-2010

B. Researches:

- Effect of Heat Inactivated Probiotics on Dendritic Cell Maturation, Supervised by Professor Ibrahim Elmadfa, Department of nutritional Science, Faculty of Life Sciences, University of Vienna, 2009-2011.

- Toward Understanding Strain specific molecular mechanisms in the regulation of dendritic and epithelial cells by probiotics, Supervised by Professor Ibrahim Elmadfa, Department of nutritional Science, Faculty of Life Sciences university of Vienna, since 2011.

- Effect of Omega 3 Fatty Acid Supplementation on the Serum Levels of Leptin, Adiponectin and Inflammatory Mediators and Their Association with Anthropometric and Biochemical Indices in the Diabetic and Non-diabetic Overweight Men", supervised by Dr. A. Djazayery and Dr. B Larijany at and EMR,2005-2006

- Evaluation of Nutritional Care Status in Cardiac Surgery Unit at Modares Hospital, Tehran. 2003.

C. Congresses:

1. Oral Presentations:

- Heat inactivated probiotics strains specifically stimulate NFK-b, MAPkinase pathways, miRNAs and maturation in CACO2 endothelial cells and in dendritic. Ladan Giahi, Eva Aumueller, Manuela Nestlberger, Ibrahim Elmadfa, Alexander Haslberger. 6th Probiotics & probiotics new foods conference, Rome 2011.

- Viable and non-viable forms of identical probiotics strains induce differential response in human dendritic cells. L. Giahi; I. Elmadfa; P. Klein. Annals of Nutrition and Metabolism Vol 58, Suppl 3. p 10. 11th European nutrition Conference (FENS) Madrid, 2011.

- Gut Microbiome in Health and Disease, Faculty of Life Sciences university of Vienna, 2011.

- Nutrigenomics & Development of Personalized Diet, Faculty of Life Sciences university of Vienna, 2010.

- Effect of Probiotics in immune system, Iran Nutrition association, Institute of Nutrition and Food Technology, **S**hahid Beheshty University of Medical Sciences, Iran, 2010

- Effect of Omega-3 Fatty Acids Supplement on Serum level of PRO- and Anti-Inflammatory Adipokines in Over Weight Diabetic Men, The 6th International Scientific Conference for Medical Students in the GCC Countries, Al Ain, Emirate, 2009.

- Missing Chains in Treatment of Rheumatoid Arthritis. 15th Congress of Physical therapy. Tehran 2004

- Leptin and Role of Dietary Composition on Regulating its Serum Concentration**,** Tehran University of Medical Sciences, 2004.

- Leptin and Metabolic Disturbances, Is There a Relation? Department of Nutrition and Biochemistry Tehran University of Medical Sciences, 2004.

- The Effect of Vitamin E, C and Folic Acid in Development and Progression of Alzheimer's disease. Institute of Nutrition and Food Technology, **S**hahid Beheshty University of Medical Sciences, 2002.

2. Poster Presentations:

- Differential regulation of immune responses by non-viable probiotics at transcription and post- transcription level, Ladan Giahi, Alexander Haslberger, Dragana kremenovic, Ibrahim Elmadfa. 2nd international symposium Microbes for health, Paris, 2011.

- The Effect of Heat Inactivated Lactobacillus Rhamnosus and Lactobacillus Delbrueckii on Human Dendritic Cell Maturation and Cytokine Profile, Pharmanutrition Conference in Amsterdam, 2011.

- Comparing Serum Level of Leptin and its Association with Body Fat Mass, HbA_1c and CRP in Diabetic and Nondiabetic Patients. 9th Congress of Nutrition, Tabriz, Iran, 2007.

- Nutritional Status and Related Factors in Primary School Aged Children, Hotk, Kerman. 9th congress of Nutrition, Tabriz, Iran, Sep 2007.

D. Teaching Experiences:

- Nutritional care of School age children, Shushtar Health Center, 2008.

- Nutritional care of kindergarten and preschool children, Shushtar Health Center, 2008.

- Nutritional care from birth till 2yrs of life (Breastfeeding and complementary nutrition), Shushtar health center, 2008.

- Cell culture techniques, gene extraction, Department of nutritional Science, Faculty of Life Sciences university of Vienna, 2011.

V. Publications:

A. Full Articles:

- Ladan Giahi, Ibrahim Elmadfa, Mostafa Hosseini, *Heat-inactivated Lactobacillus Rhamnosus and Lactobacillus Delbrueckii Induce Efficient Maturation and Differential Cytokine Production in Human Monocyte Derived Dendritic cells*, International Journal of Immunology, Accepted for publication "Food and agricultural immunology"

- Ladan Giahi, Eva Aumueller, Alexander Haslberger, Ibrahim Elmadfa. *Regulation of TLR4, P38 MAPkinase, IκB and miRNAs by inactivated strains of lactobacilli in human dendritic cells.* Submitted for review

- Ladan Giahi, Manuela Nestelberger ,Eva Aumueller, Alexander Haslberger, *Specific probiotics strains differentially induce differential expression of inflammatory cytokines by modulating transcription factors and miRNAs expression in pre-stimulated CACOII cells.* Submitted for review

- Ladan Giahi, Bagher Larijani, Abolghasem Jazayeri, Abbas Rahimi, *Evaluation of the Correlation between Adiponectin, Percent of Body Fat Mass and Insulin Sensitivity in Overweight Men*. Journal of Diabetes and Metabolic Disorders. 2006,6 (9) 183-188;

- Ladan Giahi, Abolghasem Jazayeri, *The Necessity of Considering Adipose Tissue as an Endocrine Organ With Inflammatory Property in Modern Nutritional Research*, Proceedings of the 9th National Congress of Nutrition, Tabriz, Iran. Sep 2007.

- Ladan Giahi, Mohammad Reza Hadian, *Missing Chains in treatment of Rheumatoid Arthritis*. Journal of the Iranian Physiotherapy Association, 2004,39(1).

B. Abstracts:

- L. Giahi; I. Elmadfa; P. Klein. *Viable and non-viable forms of identical probiotics strains induce differential response in human dendritic cells.* Annals of Nutrition

and Metabolism V ol 58, Suppl 3. p 10. 11t h European nutrition Conference (FENS) Madrid 2011.

- Effect of Omega-3 Fatty Acids Supplement on Serum level of PRO- and Anti-Inflammatory Adipokines in Over Weight Diabetic Men, Journal of Medical Sciences Vo.2, Isuue 1 (suppl.), 2009.

C. Books:

- Mohamad Reza Hadian, Ladan Giahi, *Spinal Cord Injury, Guidelines for Treatment and Care*. Tehran University of Medical Sciences, Tehran, 2004.

- Ladan Giahi, *Prescription of Healthy and Permanent Weight Reduction*. Iranian Nutrition Society, 2005.

D. Book Translations:

- Hasse Jeanette, *Comprehensive Guide to Transplant Nutrition*, Translated by : Ladan Giahi, Saeed Hosseini, Vista, 2008

- Amanda Ursell, *The Complete Guide Healing Foods,* Translated by: Ladan Giahi, World of Nutrition and Health Culture Insitute, under publication.

E. Book Chapter:

Ladan Giahi, *Insulin Resistance, Adipokines the missing link between insulin resistance and inflammation*, InTech, under writing