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Modulating Immune Responses of Langerhans Cells against Microbial Associated Molecular Patterns

A Dissertation Presented

by

Sivaraman Prakasam

to

The Graduate School

in Partial Fulfillment of the

Requirements

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Doctor of Philosophy

in

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Abstract of the Dissertation

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Langerhans cells (LCs) are dendritic cells that reside in the epidermis, but whose predominant function in immunity is still enigmatic. The overarching objective of the doctoral dissertation is to analyze responses of LCs to oral microbial associated molecular patterns (MAMPs) like the LPS of putative periodontal pathogen *Porphyromonas gingivalis* (PGLPS) and the importance of the cytokine micro-environment in determining the outcome of immune response

(immunostimulatory vs. anergy / tolerance) mediated by LCs, especially in the context of oral inflammatory diseases like periodontitis.

Most studies of Langerhans cells in the context of periodontal disease are histo-morphometric analysis of healthy and diseased tissue documenting the trafficking of Langerhans cells. Here we report on the immunological and immuno-stimulatory capabilities of oral mucosal equivalent Langerhans cells and our ability to modulate them in-vitro. Human IL-10 (hIL-10), an immuno-modulatory cytokine has been shown to exert immuno-modulatory effect on immune cells. A viral equivalent of hIL-10 is secreted by Epstein Barr Virus, and is called viral IL-10 (vIL-10). vIL-10 exerts its immuno-modulatory effects on different immune cells without the negative effects of hIL-10 i.e., it's immuno-stimulatory and growth factor like effects. Thus we explore here the immuno-modulatory effects of both hIL-10 and vIL-10 on LC response to MAMPs.

CD40L, expressed by T cell and traditionally used as an equivalent for T cell mediated activation of dendritic cells has been reported to be the signal necessary for final maturation of dendritic cells. Thus we explored the immuno-stimulatory effects of CD40L on LC and their response to MAMPs.

The effects of hIL-10 and vIL-10 have been shown to be transient due to the extreme short half life of IL-10 molecules. Thus we wanted to develop a system which would continuously secrete high amounts of IL-10 into the micro-environment and thus may sustain the immune-modulation of the LC.

Thus the aims of this thesis were to determine the ability of human CD34+ derived Langerin+ LCs to respond to different pattern recognition receptors (PRRs) through the MAMPs that they express by undergoing maturation and stimulating a T-cell mediated immunoproliferative response and moreover, to determine the ability of cytokine micro-environment to modulate this response. This is the one of the first few studies to look into immune responses of these LCs especially in the context of oral MAMPs. The studies also establish a model for immunomodulatory, viral-IL10, secreting epithelial microenvironment.

Langerhans cells were generated from CD34+ cord blood derived hematopoietic stem cells and highly purified by positive selection with the help of antibody to langerin a specific cell surface marker for Langerhans cells through fluorescence associated cells sorting. The phenotype of LC were further characterized and confirmed by flow cytometric analysis by the presence of cell surface markers like CD1a, HLADR, DEC205, E-cadherin, CLA etc.,

The purified LC were then challenged with different doses of four different MAMPs namely, TLR2/TLR-4 ligand PGLPS, TLR4 –ligand (*Escherichia coli* LPS) ECLPS; NOD1/NOD2 ligand Peptidoglycan (PGN) and DEC1/TLR2 ligand Zymosan for 24 hours. The resultant LC activation was determined by analyzing for co-stimulatory molecules CD86/CD80), activation markers (CD83) and HLADR through flow cytometric analysis. Cytokine response was measured through a flow cytometry based cytometric bead assay.

The expression of PRRs were analyzed with help of real time PCR. Immuno-stimulatory capabilities of activated LC was measured with the help of CFSE based T cell proliferation assay.

LC were then pre-conditioned with hIL-10, subsequently challenged with four different MAMPs and again the immune and immuno-stimulatory responses were measured as described above. Genetically engineered viral IL-10 secreting epithelium was generated with the help of retro-virus mediated transduction using a MMLV viral vector carrying the vIL-10 gene. The ability of this conditioning micro-environment on LC immune response was also determined as described above.

The results suggest that, LCs express surface markers consistent with mucosal and epidermal LCs. LCs are capable of recognizing various MAMPs like TLR2&4-activating Porphyromonas *gingivalis* lipopolysaccharide, TLR2-activating peptidoglycan, TLR2 & DEC1-activating zymosan, and TLR4-activating Escherichia *coli* lipopolysaccharide. LC up regulate TLR4, NOD1 and NOD2 in response to various MAMPs but do not up regulate DEC-1 and TLR2. LCs also up-regulate co-stimulatory molecules and activation markers, including CD83, in response to these MAMPs. They also elicit a robust pro-inflammatory cytokine response against these MAMPs. Activated LCs are able to stimulate a proliferative response in both allogeneic and autogeneic $CD4^{+}$-T-lymphocytes. Thus, contrary to current opinion, LCs are able to mount an immunostimulatory

response. When LCs are pre-conditioned with recombinant human-IL10/viral-IL10, the immunostimulatory responses to MAMPs are abrogated i.e. up-regulation of co-stimulatory and activation markers are abrogated along with lack of a cytokine response. The conditioned LCs fails to induce a proliferative response in allogeneic and autogenous T-lymphocytes. This effect was also observed when the LC are co-cultured with a viral-IL10 secreting epithelial micro-environment.

CD40L conditioning produced unexpected and interesting result CD40L conditioning by itself produces a strong cytokine response from the LCs, but only produces a weak co-stimulatory effect. LC pre-conditioning with CD40 did not provide any additional effect to the T cell proliferating capabilities of LC. In fact CD40L reduced the cytokine secretory response as well as the co-stimulatory response of LCs to MAMP challenge.

Thus, in summary we show here that both hIL-10 and its viral equivalent, secreted by Epstein Barr virus, viral IL-10 (vIL-10) are able to down modulate the immune response of LC and make them unresponsive to activation to MAMPs both by themselves as well as in the context of an epithelial micro-environment. IL-10 immuno-modulation also abolishes immuno-stimulatory capabilities of MAMP activated LC on naïve T cells.

Moreover, we show that CD40L activation of LC and subsequent attempt activation by MAMPs result in them either undergoing an endotoxin tolerance or make them semi-mature and hence resistant to further activation by LCs.

These results shed light on the immunobiological functions of LCs and on the importance of the cytokine micro-environment in initiation and, progression / quiescence of chronic inflammatory diseases. Moreover, it opens up the possibility of taking advantage of immuno-modulatory properties of viral-IL10 in periodontal therapy and treatment of other inflammatory conditions.

Dedicated to my parents

Mr. S. N. Prakasam and Mrs. P. Maniammal

&

To my beloved wife

Anju

Their love, support, encouragement, and sacrifices

have made everything possible.

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List of Abbreviations

ATPase: Adenosine Triphosphatase

DC: Dendritic Cells

$E_{(ref)}$: efficiency of amplification for the reference gene (B-Actin)

G-CSF: Granulocyte-Colony stimulating factor

GM-CSF: Granulocyte Macrophage-Colony stimulating factor

IL: Interleukin

IL-10: Interleukin - 10

IL-1RA: Interleukin 1 receptor agonist

IRAK: IL-1R-associated kinase

LIF: Leukemia inhibitory factor

M-CSF: Macrophage-Colony stimulating factor

NOD: Nucleotide Oligomerization Domain

PRR: Pattern Recognition Receptor

T_H: T Helper cells

TNF: Tumor Necrosis Factor

TR cells: T regulatory cells

APC: Antigen presenting Cell

CD: Cluster of Differentiation

cDNA: Compliment of Deoxy- ribonucleic Acid

CFSE: Carboxy fluorescein diacetate succinimidyl ester

CLA: Cutaneous Lymphocyte Antigen

CSIF: Cytokine synthesis inhibitory factor

CT_{ref}: thresh-hold of cycle of amplification for reference gene

CT_{target}: thresh-hold cycle of amplification for target gene

DC: Dendritic cells

DC-MM: Dendritic cell Maintenance Medium

DCSIGN: Dendritic Cell-Specific Intercellular adhesion molecule

DEC205: Dectin 205

DMEM: Dulbecco's Modified Eagle Medium

DMSO: Dimethyl sulfoxide

ECLPS: Lipopolysaccharide of Escherichia coli

ELISA: Enzyme Linked Immunosorbent Assay

ERK: Extracellular Signal Regulated Kinase

E_{target}: efficiency of amplification for the target gene

FACS: Fluorescence Activated Cell Sorting

GFP: Green Fluorescent Protein

GP: Glycoprotein

hIL-10: human Interleukin-10

HLADR: Human Leukocyte Antigen DR

IFN: Interferon

IgM: Immuno globulin M

IKKγ: I-κB kinase-gamma

ITAM: Immuno-receptor tyrosine-based activation

LC: Langerhans cells

LPS: Lipopolysaccharide

LTR: Long Terminal Repeat

mAB: monoclonal antibodies

MACS: Magnetic Associated Cell Sorting

MAMPs: Microbe Associated Molecular Patterns

MAPK: Mitogen Activated Protein Kinase

MFI: Mean Fluorescence Intensity

MHC: Major Histo compatibility

mIL-10: Mouse IL-10

MOI: Multiplicities of Infection

NE: Normalized Expression

NF-κB: Nuclear Factor Kappa B

PAF: Platelet activating factor

PBMC: Peripheral Blood Monocytes

PBS: Phosphate Buffered Saline

PBSA: Phosphate Buffered Saline with Bovine Serum Albumin

PCR: Polymerase Chain Reaction

PDTC: Pyrrolidine dithiocarbamate

PE: Phyco-Erythrin

PGE2: Prostoglandin E 2

PGLPS: Porphyromonas gingivalis LPS

PGN: Peptidoglycan

PHA: Phytohemagglutinin

rhIL10: Recombinant human IL-10

RNA: Ribo Nucleic Acid

RT: Reverse Transcriptase

SAPK: Stress-Activated Protein Kinase

SOCS: suppressor of cytokine signaling

STAT: Signal Transducer and Activator of Transcription

SYK: Spleen Tyrosine Kinase

TGF-β: Tumor Growth Factor Beta

TLR: Toll Like Receptor

TLR2: Toll Like Receptor 2

TLR-4: Toll Like Receptor 4

TLR-6: Toll Like Receptor 6

vIL-10: Viral Interleukin 10

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Introduction

Oral mucosa is an immune privileged site:

The oral mucosal epithelium comprises masticatory and lining mucosa. The oral mucosal epithelium is constantly exposed to millions of various species of micro-organisms including commensals and opportunistic pathogens. Paster et al estimated that there are at least 500 different bacterial species present in the oral cavity. They report that at least 415 species are present in subgingival plaque alone [1]. The oral mucosa consists of not only a stratified squamous epithelium which acts as a physical barrier but also has integrated innate and adaptive immunological barriers that help prevent invasion by pathogenic organisms [2]. Oral mucosa is highly vascular, more permeable and has indistinguishable papillary and reticular dermis. This enables frequent contact of antigenic elements from pathogens to come in contact with local immune cells [3].

In fact even keratinocytes, which were traditionally considered as cells that are involved primarily in barrier function, have been shown to recognize and respond to various conserved structures present on micro-organisms called microbe associated molecular patterns (MAMPs) through various germ line encoded receptors called pattern recognition receptors (PRRs) [4, 5] just like many other immune cells. Thus keratinocytes are themselves considered to be mediators of inflammation [6]. More importantly, they act in cooperation with

resident antigen presenting cells called Langerhans cells which under steady state conditions play a sentinel role at this front line of defense [7].

Thus the oral mucosal epithelium with its integrated immune elements must strike a balance between uptake of different antigens under state conditions without mounting an immuno-inflammatory reaction and the simultaneous prevention of entry of harmful pathogens [3]. In fact such cross talk between epithelial cells and dendritic cells is thought to mediate intestinal immune homeostasis [8]. In the intestine pressure from the micro-flora is translated into tolerogenic signals that are sent to immunocompetent cells and perturbation in this process results in an immediate, localized, and finely tuned response that occurs sub-clinically [9].

A similar situation exists in the oral cavity so much so that periodontal disease and other oral inflammatory diseases occur only in utmost 20-30% individuals of the population. Therefore oral mucosa can be considered as a site where immune tolerance predominates. Antigen Presenting cells, such as Langerhans cells, dendritic cells and different T cell subtypes, serve as key players in this induction and maintenance of oral mucosal tolerance [3]. Epithelial cells and cytokine milieu also play significant roles in this process [10]. The predominance of tolerance in the light of the substantial antigenic challenge that oral mucosa faces in steady state indicates that oral mucosa is a immune privileged site [3, 11] and disease pathogenesis primarily occurs when this

immune privilege and tolerance is disrupted [12-15]. This theme will be expanded in subsequent sections with specific regards to the role that Langerhans cells play and the roles that IL-10 plays in this delicate balance.

Disruption of Oral mucosal immune homeostasis and Pathogenesis of Oral inflammatory diseases including chronic periodontitis:

G. J Seymour, as early as, in 1986 speculated that defects or imbalance in the immune homeostatic/regulatory networks especially in the context of its response to bacteria could lead to tissue destruction as seen in many chronic inflammatory diseases including chronic inflammatory periodontal disease [16]. He further speculated that suppressor T cells have a regulatory role in periodontitis. This is evidenced by abrogation of suppressor cell activity by IL-2 and suppressor cells can in turn absorb the IL-2 which is required for IFN-γ production [17]. Moreover low amounts of IFN can selectively block the suppressor T Cell pathways [18]. Thus a delicate balance exists between IL-2, $PGE_{2,}$ and suppressor T cells. Disruption of this immuno-regulatory balance could lead to periodontal disease. In fact increased number of suppressor T cells in

periodontal disease [19, 20]; and the inability to detect IL-2 in some unstimulated cultures [21]; point towards that possibility [16].

Suppressor T cells have been rediscovered as T-regulatory cells (T_R) Cells in the mid-1990s and includes cells like T_R1 and Th3 cells [22]. Thus for an immuno- inflammatory response to occur it becomes necessary to overcome dominant negative regulation by these T_R Cells [23]. C. Pasare, and R. Medzhitov, in an elegant study showed that activation of Toll like receptor (TLR) signaling in dendritic cells (DCs) also acts in a cell extrinsic way to enhance T effector cell responses by overcoming $CD4^+CD25^+$ T_R cell suppression [24-26]. They also show that induction of co-stimulatory molecules alone is not sufficient to overcome suppression but requires the presence of DCs secreted IL-6 in the micro-environment [24-26].

Similar mechanisms may play a role in chronic periodontal disease pathogenesis and progression. Disruption of oral mucosal immune homeostasis has been implicated in pathogenesis of other oral mucosal inflammatory diseases like recurrent aphthous stomatitis [27] and oral lichen planus [11]. A link between pathogenesis of Behcet's disease and periodontitis has been proposed on the same premise [28-30].

Once the immune suppression by the T_R cells is overcome by cytokines secreted by antigen presenting cells like dendritic cells or Langerhans cells, the innate and adaptive immune cascade can proceed unhindered in the elimination of

the foreign antigen(s) that initiated the disruption of the tissue homeostatic networks. In this process host tissue suffers irreversible collateral damage.

In chronic periodontal disease the role of the different effector arms of T cell in the actual contribution towards tissue destruction has long been controversial and the interpretation of available data was dependent on the opinion of investigators [31]. A predominance of T_H2 cell type over T_H1 cells in periodontal lesions [32] and the development of plasma cell lesion in advanced periodontal disease [33-35] have led some investigators to the conclusion that a shift from cell mediated immunity to humoral immunity occurs during the development of periodontal disease [36].

On the other hand periodontal lesions have a predominance of T_H1 biasing cytokines in the milieu [37-39]. Although presence of T_H2 cytokines has been reported [40] it has not been consistently reported and moreover is found in lesser quantities [38, 39].

Furthermore Kawai et al demonstrated that T_H1 type T cells and not T_H2 type T cells could trigger periodontal bone resorption in a rat model of bone resorption [41]. Taubman et al found direct evidence for the engagement of OPG-L expressing T_H1 cells in periodontal destruction [37]. The anti-inflammatory cytokine IL-10 produced by both T_H1 and T_H2 cells [42] can induce B cell differentiation into plasma cells [43, 44] and this could explain the presence of plasma cells in the advanced periodontal lesion [37]. IL-10 is normally produced

as a feedback loop to prevent host-harmful T_H1 hyper-reactivity to infection [45, 46]. Taken together evidence points towards T_H1 type T cells as mediators of periodontal destruction in chronic periodontal disease.

Thus disruption of immune-homeostasis leads to freeing of the T_H1 arm for pathogenesis of oral inflammatory diseases including chronic periodontitis.

Langerhans cells an Enigmatic Antigen Presenting cell:

Antigen presenting cells like dendritic cells and Langerhans cells are important in the induction of immuno-stimulatory responses including determination between the types of adaptive immune response and induction of tolerance/anergy. In the following section the history, immuno-biology and the role of these cells, especially the role of Langerhans cells in chronic periodontitis and other oral inflammatory diseases will be reviewed.

A historical perspective:

Langerhans cells (LC) were first described in 1868 by Paul Langerhans while he was still a medical student. In a paper titled "on the nerves of human skin" (Langerhans 1868) he used a gold chloride labeling technique and a primitive light microscope to identify and describe a new type of epidermal cell [47]. This is considered a fortuitous identification since gold chloride technique is a very finicky technique and he might have been helped by the fact LC respond well to haptens in certain sensitive individuals. Initially they were thought to be nerve cells. The advent of electron microscopy dispelled that notion as they did not display any characteristic features of a nerve cell. In the 1950s, it was proposed that they were effete worn-out melanocytes that had lost the ability to produce melanin [48]. Birbeck, M.S. and colleagues in 1961 showed the presence of Langerhans cell in vitiligo, that is, epidermis lacking melanocytes [49]. This gave conclusive evidence against them being melanocytes. Birbeck also described the ultrastructural characteristics of the Langerhans cells [50]. He described the presence of rod and tennis racket shaped organelles in Langerhans cells which were later called Birbeck's Granules. These organelles had been previously described in histiocytic tumors or Histiocytosis X. These tumors are now called Langerhans cell Histiocytosis. These observations led to the conclusions that LC

were probably mesenchymal cells, histiocytes, or even macrophages. It was then shown the LC express adenosine triphosphatase (ATPase), a molecule known to be present in leukocytes [48, 51, 52]. In 1973 Inga Silberberg observed that LC accumulated in the dermis and were found in close contact (apposition) with lymphocytes in response to contact sensitizers. He went on to describe epidermal Langerhans cells as "the most peripheral outpost of the immune system" [53]. In the late 1970's many researchers in Scandinavia furthered the evidence that LC are genuine immune cells by demonstrating the presence of FC receptors, complement receptors and MHC II receptors [48]. In the 1980s evidence were presented of the antigen presentation capability of LC [48].

Ontogeny and Lineage of LC:

Perreault et al and Volc-Platzer et al confirmed the LC originates from bone marrow [54, 55]. The LC phenotype strongly suggests that it is of a myeloid origin [48]. For example, LC express typical myeloid antigens like CD11d and CD33 [56, 57]. In experimental situations LC can be generated from lymphoid origins [48]. Although, the fact that defects in lymphoid system do not affect

Langerhans cells in the epidermis and the role and important regulator of myeloid origin, the transcription factor PU.1 plays in driving CD34^{+} stem cells upon transduction argue for a myeloid origin of LC [48].

Antigen recognition and capture by LC:

LC can efficiently perform receptor mediated endocytosis and macropinocytosis [58]. They have been shown to internalize micro-organisms like *Saccharomyces cerevisiae, Staphylococcus aureus,* and yeast cell wall compound Zymosan [59]. LC can also internalize apoptotic bodies [60, 61]. LC express various PRRs on their surface. Although some reports suggest that LC do not express TLR-4 but express TLR-2 and TLR-6 [62], others have shown the presence of TLR4 in freshly isolated epidermal LC [63]. In vitro generated CD34+ LC also express TLR2 and TLR4 [64]. Dectin -1, a C-type lectin receptor which recognizes Zymosan, has been reported to be expressed in LC [65]. Expression of NOD1 and NOD2 in Langerhans cells but it's a potential pathways through which Langerhans cell could recognize molecules like peptidoglycan

[66]. Thus LC can recognize and efficiently process antigens derived from various micro-organisms.

Langerhans cells: mediators of immunity and tolerance:

Current paradigm suggests that the state of maturity of Langerhans cells determines whether migrating Langerhans cells signal immunity or tolerance. The state of maturity of a Langerhans cells is defined by the display of molecules that provoke immune responses i.e. histocompatibility molecules, co-stimulators, adhesion and homing receptors [67]. Lutz et al propose that DCs including epidermal LC induce tolerance or immunity based on the maturation stage. Immature and semi-mature DCs/LC promotes anergy or tolerance, while fully mature DCs are immunogenic. They further propose that the decisive immunogenic signal seems to be the release of proinflammatory cytokines from DCs/LC [68]. As explained in later sections IL-10 plays a crucial role in this decision as it can suppress pro-inflammatory cytokine secretion from DCs/LC. In fact Jonuleit et al propose the use of dendritic cells as a tool to induce anergic and

regulatory T cells. They specifically propose use of IL-10 towards this purpose [69]. Wang et al propose a model in which the epidermal cytokine milieu influences the emigration of LC. In this model epithelial homeostasis is achieved by a balance between pro-inflammatory cytokines like IL-1 and TNF-α and anti-inflammatory cytokines like IL-10. Disruption of this balance by antigens leads to activation and maturation of Langerhans cells and subsequent emigration into lymphatics and triggering the adaptive immune response. Subsequently when balance is restored by IL-10 secretion into the milieu epithelial homeostasis is re-established [70]. Such a homeostatic mechanism could exist in oral mucosa and gingiva.

Oral mucosal Langerhans cells:

Langerhans cells in the oral mucosa are organized as an antigen trapping network [71-75] and are usually found in the supra-basal layer of stratified epithelium. They can recognize, process and present antigen and proliferate T cells [74]. Ito et al studied the three dimensional appearance of Langerhans cells

in human gingival epithelium and found that the LC have their dendritic processes oriented towards the epithelial surface, ostensibly for sampling oral contents and mucosal bacteria [76]. A role of oral mucosal LC in initiation and development of gingival inflammation has been suggested by Seguier et al [77]. Difranco et al have shown increased number of LC in inflamed gingiva [78]. Oral mucosal Langerhans cells have been implicated in many oral inflammatory diseases like chronic periodontitis [77, 79-81], gingivitis [82, 83], oral lichen planus [84, 85], oral hairy leukoplakia [86], and oral squamous cell carcinoma [87]. Two groups have shown the deterioration of the LC network in the human gingival epithelium with age resulting in increased susceptibility for periodontal disease [79, 88]. The exact role of Langerhans cells in oral inflammatory diseases is poorly understood and needs to be explored in order to develop therapeutic strategies for these conditions based on modulating the immune responses of LC.

Role of cytokine micro-environment in the induction of T cell responses:

Induction of T cell responses are initiated by professional antigen presenting cells like dendritic cells, Langerhans cell, macrophages and to some

extent B cells [89] as discussed in the previous section. According to Guermonprez et al dendritic cells respond to two types of signals. DCs sense pathogens directly through PRRs (TLR, NODs and dectins [90-92]) or sense infection indirectly through inflammatory cytokines (TNF-α, IL-1β & PGE2 [93, 94]), internal cellular compounds and ongoing specific immune responses [95, 96]. Tissue specific environmental factors can also participate in the phenotypic differentiation of DCs and the resultant T cell response [97, 98].

APCs are traditionally described to produce three signals that are required for activation of antigen specific T cell responses [89, 99]. The first signal involves presentation of antigen on MHC class II molecules on APC surface to TCR (T cell receptor) on T cells. The second signal involves interaction of APC co-stimulatory molecules like CD80/CD86 with CD28 on T cells. The third signal is provided by the cytokine environment created by APCs by active secretion. This third signal is critical in the determination of the appropriate type of immune response [89, 99].

Cytokines like IL12 and IL-18 are key players in the development of T_H1 type of T cell response [100, 101]. While IL-4 has been implicated in the development of T_H2 response [89]. IL-10 conditioned and IL-10 secreting dendritic cells on the other hand produces anergic T cells or T_R1 type cells [69]. In fact the mucosal milieu containing IL-10 cells induce production of T_R1 type cells [10] as demonstrated by Akbari et al in pulmonary mucosa [102]. The

cytokine milieu also determines the maturation stage of DCs. Maturation stage in turn has implications in the development of T cell type response. Immature and semi-mature dendritic cells promote anergy and/or tolerance, whilst fully mature DCs for the most part elicit an immuno-inflammatory response in T cells [68].

Thus in chronic inflammatory disease like chronic periodontitis manipulation of the cytokine environment can be used to prevent the initiation progression of immuno-inflammatory destruction. In fact presence of anti-inflammatory cytokine IL-10 [103] and influx of T_R cells [37] into periodontal lesions indicate an attempt by the body to re-establish immune homeostasis. Augmenting this phenomenon may have some therapeutical potential.

Role of IL-10 in chronic periodontal disease:

IL-10 is an anti-inflammatory cytokine that plays a role in periodontal disease by inhibiting pro-inflammatory cytokines and stimulating protective antibody production [37, 104]. The immunobiology of IL-10 will be discussed in greater detail in a later section. Various authors have looked at the role of IL-10

and its signaling in the context of periodontal disease. Sumer et al report that polymorphisms in the IL-10 gene were significantly associated with generalized severe chronic periodontitis [104]. Garlet et al propose that expression of IL-10 and its downstream molecules, suppressor of cytokine signaling (SOCS) molecules, in periodontal disease could be a stop signal for disease progression. Lappin et al reported the presence of preponderance of IL-10 secreting cells in the periodontitis granulation tissue and suggest that these cells are involved in the down-regulation of inflammatory and immune response in periodontitis [103].

Sasaki et al reported that IL-10 is an important endogenous suppressor of infection –stimulated bone resorption and the mediate this effect by inhibiting IL-1α [105]. Our lab has demonstrated that IL-10 is resistant to repeated stimulation by LPS from periodontopathic bacteria *Porphyromonas gingivalis,* (PGLPS) and could play a role in the quiescence of inflammation in chronic periodontitis [106-108]. IL-10 has also been reported to play important roles in other chronic inflammatory diseases like inflammatory bowel disease [109].

IL-10 immunobiology:

History and molecular structure:

Interleukin-10 was first described as cytokine synthesis inhibitory factor (CSIF) produced in mouse T_H2 cells to inhibit activation and cytokine production by T_H1 cells [110]. Since then various studies have demonstrated that the human IL-10 (hIL-10) is not a typical T_H2 cell cytokine. It has been shown to be produced by T_H0 cells, T_H1 cells, T_R cells, B cells, monocytes/macrophages, keratinocytes, dendritic cells, and Langerhans cells [111, 112]. Human IL-10 protein consists of 160 amino acids and has a predicted molecular size of 18.5kDa [113]. It's found as a homo-dimer of 37kDa molecular size [113]. Recombinant hIL-10 and its viral counterpart viral IL-10 (vIL10) derived from Ebstein-Barr [113-115] Virus are 17-18kDa polypeptides that are not N-glycosylated unlike the mouse derived mIL-10 although glycosylation seems to have no influence on the biological activity [112].

Biological activity: Effects of IL-10 on Monocytes, and Macrophages:

IL-10 potently inhibits production of IL-1α, IL-1β, IL-6, IL-10 itself, IL-12, IL-18, GM-CSF, G-CSF, M-CSF, TNF, LIF, and platelet-activating factor (PAF) by activated Monocytes / macrophages [116, 117]. This inhibitory effect is crucial to its anti-inflammatory activities as these cytokines have synergistic activities on inflammatory pathways and processes. The inhibition also prevents amplification of these responses by secondary mediators like prostaglandins and PAF [112]. IL-10 also inhibits production of both CC and CXC chemokines by activated monocytes [118-120] and thus having ability to affect both T_H1 and T_H2 type responses. IL-10 inhibits expression of MHC class II antigens, CD54, CD80, and CD86 on monocytes even after the induction of these molecules by IL-4 of IFNγ [121-125]. It also inhibits antigen presentation by affecting the peptide loading of MHC II molecules to the plasma membrane [125]. Thus IL-10 inhibits cytokine, chemokine production, and antigen presentation in monocytes and macrophages.

Thus IL10 can play a role in periodontal diseases where there is an increased homing of monocytes and macrophages once the immune homeostasis is dysregulated

Biological activity: Effects of IL-10 on Neutrophils:

IL-10 inhibits cytokine and chemokine response of neutrophils 2 hours post-stimulation to LPS, LPS plus INFγ, or TNF, and this is dependent on inhibitory effects of IL-10 on endogenous IL-1β and TNF production. IL-10 inhibits neutrophil migration in-vivo by up-regulating IL-1RA. IL-10 has been proposed to indirectly affect the survival of neutrophil through inhibition of survival cytokines, but this role is controversial [112].

This effect of IL-10 while protecting periodontal tissue from destruction could potentially lead to persistence of already invaded pathogens.

Biological activity: Effects of IL-10 on B-cells and Immunoglobulin production:

IL-10 enhances survival of normal human B cells depending on their state of activation. IL-10 is a potent co-factor for proliferation of human B cells precursors or mature B Cells activated by anti-IgM or CD40 crosslinking. This effect is enhanced by IL-2 and IL-4. B cell-derived and exogenous IL-10 affected B cell differentiation and isotype switching. It also has roles in differentiation of B cells into plasma cells [112].

This effect of IL-10 has been observed in established and advanced periodontal lesions. Whether this is protective or destructive is controversial as discussed previously.

Biological activity: Effects of IL-10 on Dendritic Cells / Langerhans cells:

IL-10 inhibits production of IL-12 and expression of co-stimulatory molecules by various types of DCs [126-132]. This correlates with the ability of IL-10 to inhibit primary allo-antigen specific T cell responses [133, 134]. In fact IL-10 treatment of DCs can induce or contribute towards a state of anergy in allo-antigen or peptide antigen activated T cells [135-140]. LC isolated from epidermis when conditioned with IL-10 has also been shown to suppress T_H1 clones [141]. Thus IL-10 inhibits inflammatory T_H1 inflammatory response by its action on DCs and they achieve it either by inhibitory effect on inflammation inducing DCs or by induction of anti-inflammatory T cell population by IL-10 producing DCs [112].

Biological activity: Direct effect of IL-10 on T cells:

IL-10 can directly affect function of T cells and inhibit IL-2, TNF and IL-5 production depending on activation conditions [142, 143]. IL-10 stimulates $CD8^+$ cells and induces their recruitment, cytotoxic activity, and proliferation [144-147]. Activation of T cells in the presence of IL-10 can induce non-responsiveness/anergy. This cannot be reversed by IL-2 or stimulation with anti CD-3 and anti CD154 [135]. IL-10 can also induce T_R cells as discussed previously.

Differences between hIL-10 and vIL-10:

vIL10 shares many biological properties of mIL-10 and hIL-10 including cytokine synthesis inhibitory factor activity and down regulation of class II MHC molecules on antigen presenting cells. The key difference between vIL-10 and hIL-10 is that vIL-10 doesn't possess T cell co-stimulatory activity like hIL-10

[117, 148-150] and this occurs due to a difference in a single amino acid residue [150]. vIL-10 cannot enhance class II MHC molecules on B cells, and it cannot proliferate thymocytes and mast cells like cellular IL-10 [113, 151].

Summary of IL-10 function:

Thus IL-10 is pleiotropic cytokine that has a regulatory function on a various hemopoietic cells. IL-10's principal function is containment and eventual termination of inflammatory response. Thus it facilitates elimination of infectious organisms with minimal damage to host tissues. IL-10 also plays key roles in immune tolerance, development of T cells, dendritic cells including Langerhans cells and growth & differentiation of B Cells [112].

IL-10 as a therapeutic agent:

The function of IL-10 has made it an attractive candidate for the treatment of various acute and chronic inflammatory conditions, auto-immunity, allergies, cancer, and infectious disease. Phase I and Phase II clinical trials, investigating safety, tolerance, pharmacokinetics, pharmaco-dynamics, immunological and hematological effects of IL10 have been performed on healthy volunteers and specific patient populations under various settings [152-154]. These studies were done with intravenous or subcutaneous routes. IL-10 has been shown to be well tolerated without serious side effects at doses up to 25µg/Kg. Mild to moderate flu like symptoms were observed in a fraction of recipients at doses up to 100ug/kg. A detailed review of these studies has been done by Moore et al [112]. As discussed earlier Akbari et al reported tolerance induction in pulmonary DCs to respiratory antigens through IL-10 [102]. Recombinant human IL-10 is being tested in clinical trials for rheumatoid arthritis[155], inflammatory bowel disease[156], psoriasis[157], organ transplantation and chronic hepatitis C [158] and other diseases [159]. Dieu-Nosjean et al conclude that IL-10 through it actions on Langerhans cells contribute towards regulatory immune responses at the epithelial surfaces [160]. Use of IL-10 as therapeutic drug is not far off. Taken together we can conclude that there is a potential for the use of IL-10 in periodontal and other inflammatory disease treatment.

Rationale and Objectives of the dissertation:

As discussed in previous sections the role of Langerhans cells in oral inflammatory diseases including chronic periodontitis remains largely unexplored. Most studies of Langerhans cells have been in the oral mucosal context histomorphometrical analysis of healthy and inflamed tissues [79, 161]. In vitro studies on Langerhans cells have been limited due to the difficulty in isolating Langerhans cells from oral mucosa without changing the phenotype in the process of isolation [48]. Langerhans cells generated in-vivo have been mainly derived from monocytes (lymphoid origin) which as discussed before is not the true lineage of Langerhans cells in-vivo[48]. Caux et al have demonstrated that cord blood derived CD34+ hematopoietic stem cells grown under the aegis of GM-CSF and TNF-α result in a subset of cells that stain with antibody against Langerin/CD207 and possess Birbeck granules under electron microscopic observation [162]. This group and others have developed methods to enrich the Langerhans cell population in culture [163, 164]. These cells have a phenotype very similar to in-vivo Langerhans cells [48] and are considered in-vitro representatives of human Langerhans cells [165]. These LC have been studied in the context of dermatological disorders. Few studies have taken advantage of

these LC to study the immunological mechanisms involved in the pathogenesis of oral inflammatory diseases.

Hence we wanted to analyze the immunological functions of Langerhans cells to oral MAMPs. Specifically we wanted to study the response of Langerhans cells to LPS of *Porphyromonas gingivalis*, peptidoglycan of gram positive bacteria, and zymosan of yeasts cells. The potent MAMP; LPS of *Escherichia coli* was used as a positive control to study the immunobiology of these LPS.

We have been able to successfully generate a highly enriched population of Langerhans cells from umbilical cord blood derived CD34+ progenitor cells by using a proprietary RPMI based medium, DC-MM medium (MatTek, Ashland, MA). This medium contained among other things cytokines like GMCSF, TNF-α, and transforming growth factor-β which help in the maintenance of the Langerhans cell phenotype. These cells have been confirmed to be bonafide Langerhans cells with Birbeck granules by examination under an electron microscopy [166]. We have been able to further enrich this population by Langerin antibody mediated cell sorting to obtain a population of 100% pure Langerin+ Langerhans cells by employing stringent gating conditions.

After establishing the immunological profile of these Langerhans cells we wanted to modulate the response with help of recombinant IL-10. It is well known that IL-10 has a very short half life in vitro and in vivo. The effects of IL-10 on immune cells are reversible. Thus loss of anti-inflammatory effects of IL-10 can

be lost much quicker than desired. A continuous secretion of IL-10 from transduced epithelia could potentially overcome this obstacle and sustain anti-inflammatory and immunomodulatory responses in oral mucosal inflammatory diseases. Thus we wanted to construct a genetically modified conditioning epithelium which continuously secrete high quantities of IL-10. Since vIL-10 is superior to hIL-10 since it doesn't have any immuno-stimulatory effects on other immune cells we decide to transduce the vIL-10 gene with a help of retro-viral vector into the epithelial keratinocytes to achieve this. Once we had established different cell lines which secreted different levels of vIL10 based on multiplicity of infections we screened and selected, through ELISA of culture supernatants, an epithelial cell line which secreted 20ng of vIL-10 into the supernatant per million cells. Once we had done that we wanted to establish the immunomodulatory effects of not only the supernatant but also the epithelial cells on the Langerhans cells immuno-stimulatory capacity.

Thus the overall objective of the dissertation is to determine the specific role that human LC play in activation and suppression of immune/inflammatory responses towards microbes and their antigens that are encountered by the oral mucosa. The overall goal of the dissertation is to determine how to stably suppress inflammation mediated by LC response to microbial ligands, while preserving innate immune functions of LC. These results can be applied to development of an immunocompetent organotypic model of oral mucosa for

better establishing the mechanisms of oral disease pathogenesis and of new therapies for stable dampening of inflammation.

In order to achieve the objectives and goals stated above we formulated the three specific aims. The specific aims and the hypotheses formulated to address them have been described in the following section.

Specific Aims:

Aim 1:

To determine the innate response and immuno-stimulatory function of human LCs in response to MAMPs in vitro

The following hypotheses were formulated to address Aim 1:

Hypothesis 1:

Human LC express a repertoire of pattern recognition receptors (PRRs) that can respond to MAMP activation.

Hypothesis 2:

The immuno-stimulatory capacity of LC is mediated by MAMP- induced activation/maturation of signals necessary for cell activation (MHC-II, co-stimulatory molecules and cytokines).

Aim 2:

To establish the ability to modulate innate response and immuno-stimulatory functions of human LCs in vitro through IL10 conditioning

The following hypotheses were formulated to address Aim 2:

Hypothesis 1:

Expression of activation markers, antigen presenting molecules by LCs in response to microbial ligand can be modulated by cytokine conditioning.

Hypothesis 2:

The immuno-stimulatory capacity of LCs towards CD4+ T cells is dependent on microbial ligand induced activation/maturation, and can also be thus modulated by cytokine conditioning.

Aim 3:

To develop a genetically engineered conditioning epithelial cell micro-environmental model to study and modulate Langerhans cell responses in vitro

Hypothesis:

Expression of activation markers, antigen presenting molecules by LCs in response to microbial ligand can be modulated by genetically engineered conditioning epithelial cell micro-environment.

Chapter II

Materials and methods

Cell Isolation and culture:

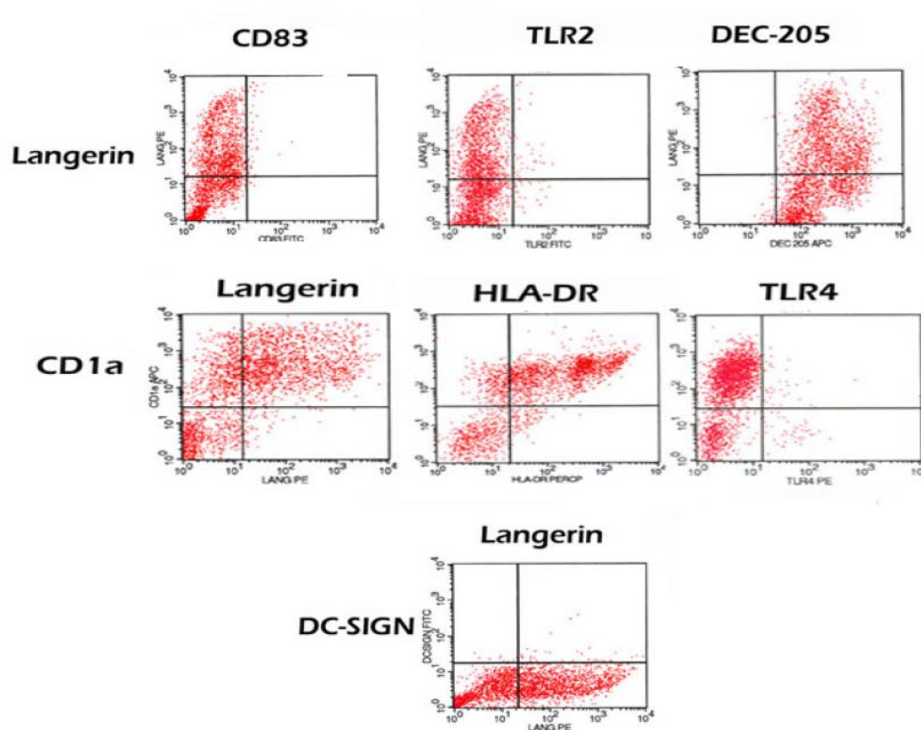
Langerhans cells:

In vitro generation of CD34$^+$-derived LC/DC from human embryonic cord blood:

CD34$^+$ DC progenitors were isolated from 60 ml umbilical cord blood using magnetic associated cell sorting (dynabeads^{TM}, (Miltenyi Biotech, Gladbach, Germany). These cells were then incubated for up to 48 days in growth medium supplemented with a cocktail of cytokines containing GM-CSF, TNF-α and TGF-β (DC-100-MM, MatTek Inc, Ashland, MA) [167, 168]. These conditions yielded an LC/DC expansion of up to approximately 250-fold. This was evidenced by an increase from 1.76 x 10^6 CD34+ precursors to 4.43 x 10^6 LC/DC. Analysis of DC phenotype by FACS shows 38% CD123+/CD11c- plasmacytoid DCs and 21% CD123-CD11c+ myeloid DCs.

Phenotypic characterization of LC/DC population:

Figure II-1: Phenotype of LC/DC population characterized by flow cytometry



LC/DC thus generated where characterized with help of antibody mediated flow cytometric characterization as described in subsequent sections. These LC/DC populations expressed large amounts of Langerin molecules on their surface. When a double staining was done along with Langerin the following phenotype was found. Langerin+ LCs expressed almost no CD83 on their surface.

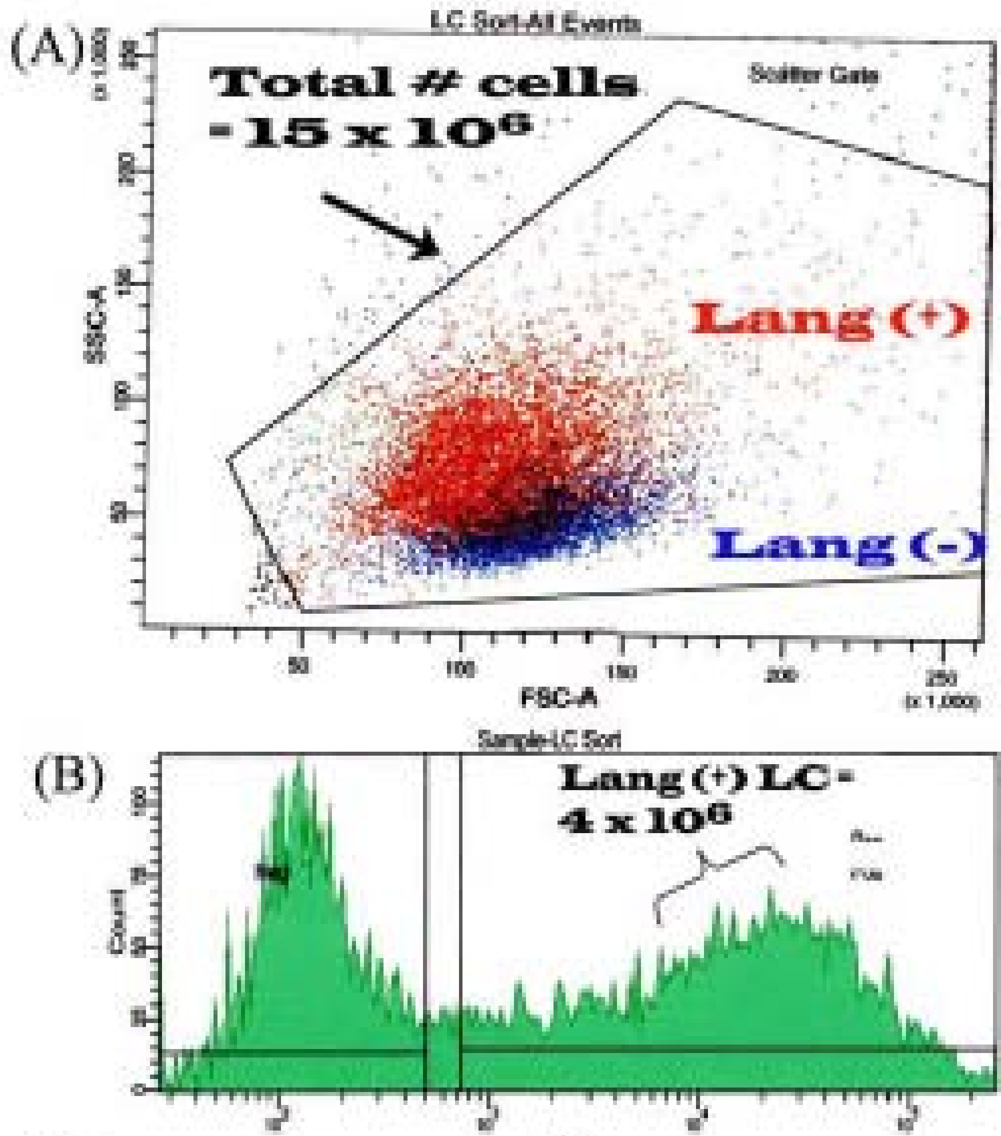
Low levels of TLR2 staining were observed in these cells. These cells also stained positive for DEC205 (Fig 1). CD1a was also abundantly expressed by LC/DC. Double stained reveal most of CD1a+LC/DC express Langerin, HLADR. These cells are negative for TLR2 and DCSIGN (Figure 1). These cells also express E-cadherin, CLA etc (data not shown).

Isolation of LCs from CD34$^+$-derived LC/DC:

Langerin is a definitive marker for Langerhans cells [169], hence we decided to isolate LC from the above generated CD34$^+$- derived LC/DC mixed population of cells. LC/DC are then stained under sterile conditions with pure PE-conjugated anti-langerin antibodies –Clone #DCGM4, (Beckman coulter, Fullerton, CA.) as per protocol described by the manufacturers. The concentration of antibody used was 100μl of antibody for 1 million cells suspended in 1ml of 0.5% PBSA. The cell suspension is then sorted by FACSVantage SE cell sorter (Becton Dickinson, Franklin lakes, NJ) under sterile conditions based on PE stain. The sorting is done under strict gating into three populations of highly positive,

intermediate and highly negative. Only highly negative and highly positive cells are collected with the intermediate cells being discarded. Thus the sorting then typically yields about 4-5 X 10^6 Langerin positive Langerhans cells when starting with about 1 x 10^7 CD34+-derived LC/DC (figure 2). These cells are then maintained in a cytokine rich DC maintenance medium (DC-100-MM, MatTek Inc. Ashland. MA).

Figure II-2: Langerhans cell isolation through fluorescence associated cell sorting



Keratinocytes and Fibroblasts

Isolation and culture of keratinocytes

Primary keratinocytes were obtained from human newborn foreskin and grown in submerged cultures in the presence of a feeder layer of irradiated NIH 3T3 cells [170], using keratinocyte medium described by Wu et al [171]. The keratinocyte medium consisted of a mixture of 3:1 Ham's F12 medium and Dulbecco's Modified Eagle Medium (DMEM), supplemented with 5 % fetal calf serum, *5* µg/ml insulin (Sigma, St Louis, MO), 0.5 µg/ml hydrocortisone (Sigma, St Louis, MO, USA) , 0.1nM cholera toxin (ICN Biochemicals, Cleveland, OH), 100 ng/ml epidermal growth factor (Chiron, Emeryville, CA), 10 µg/ml penicillin, and 10 µg/ml streptomycin (Sigma, St Louis, MO, USA) .

Isolation and culture of fibroblasts:

Human dermal fibroblasts were obtained from outgrowth of skin biopsies and are maintained and cultured in Dulbecco's Modified eagle medium (DMEM) supplemented with 10% fetal bovine serum, 10 μg/ml penicillin, and 10μg/ml streptomycin (Sigma, St Louis, MO, USA) .

T Lymphocytes

Autologous and Allogeneic T cell isolation

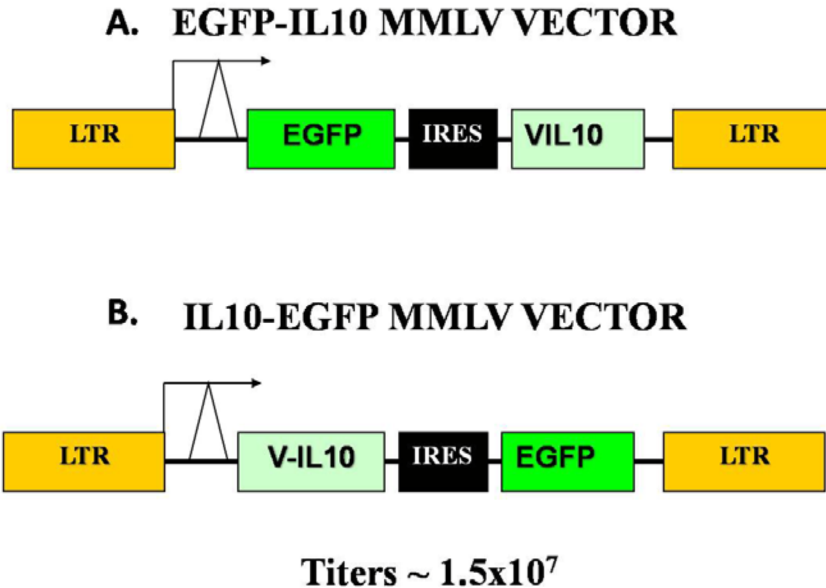
Autologous $CD4^+$ cells were isolated from the peripheral blood derived mononuclear component of the same donor that was used for CD34+ cells isolation whichever subsequently used for generation and isolation of Langerhans cells. Allogeneic T Cells were isolated from peripheral blood derived mononuclear component obtained from buffy coats purchased from Long Island

Blood Bank, (Central Islip, NY). The mononuclear component was separated from the buffy coats with help of Ficoll gradient separation as per manufactures instructions. Cells bearing CD4 surface marker were isolated from the mononuclear fraction through negative selection with a cocktail of monoclonal antibodies conjugated with micro beads (Miltenyi Biotech, Gladbach, Germany) [172]. Isolation of naïve $CD4^{+}$ by negative selection with Mini-MACS separation columns ((Miltenyi Biotech, Gladbach, Germany) as described by the manufacturer will eliminate any possibility of activation of the CD4+ helper T lymphocytes during the purification procedure. The isolated cells were checked for purity by staining them with FITC conjugated anti-CD4 or APC conjugated anti-CD4 monoclonal antibody and analyzing the cells by flow cytometry. The purity of the CD4+ T cells were typically 95-99% compared to isotype matched (negative) antibody staining.

Recombinant retrovirus:

Production and transduction:

Figure II-3: Design of retroviral vectors



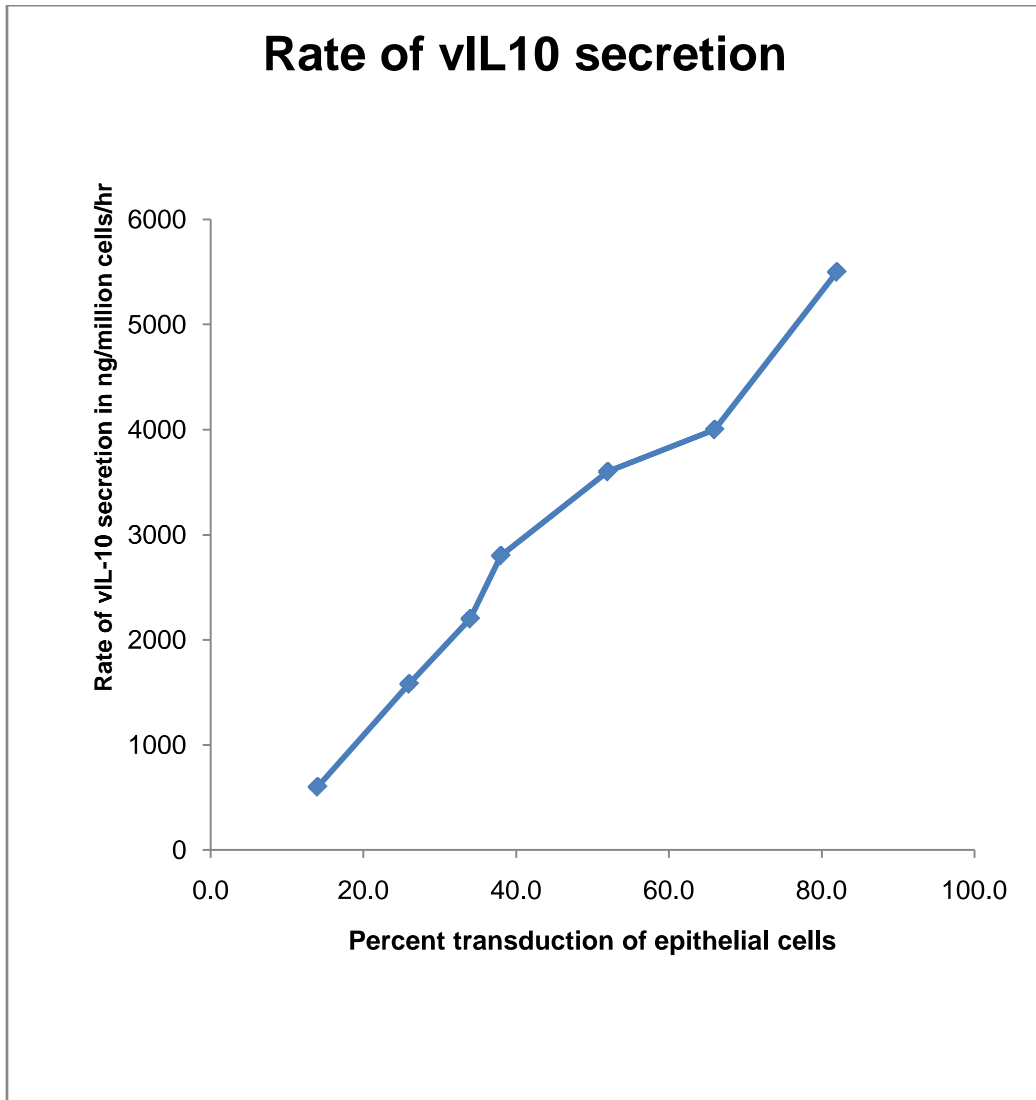
Two bi-cistronic retroviral vector constructs as described in figure 3 were used to stably transduce the Keratinocytes and fibroblasts to make them secrete vIL10. The retroviral constructs were made from murine moloney leukemia viral backbone (a gift from the Taichman lab, Stony Brook, NY). vIL-10 gene sequence in combination with the reporter gene eGFP were inserted into this backbone using recombinant DNA techniques. The promoters from which the genes are expressed have an influence on the efficiency of the gene output. Hence two constructs were made to so as to screen determine the more effective vector in terms of secreting vIL-10. The vector depicted in figure 3a expresses eGFP of the viral long terminal repeat (LTR) promoter and vIL-10 is expressed from the

IRES element. In the vector depicted in figure 3b eGFP is expressed from the IRES and vIL-10 is expressed of the LTR promoters. The recombinant viral vectors were then used for transfecting 293 GP cells, retroviral packaging cells, using the calcium phosphate co-precipitation protocol. After 2 days the cells were 'passaged' into a medium containing puromycin (1µg/ml) (Sigma, St Louis, MO, USA) and are maintained in this selective medium. Resistant producer cells were then transiently transfected with pHCMVG DNA encoding vesicular stomatitis virus-glycoprotein G (gift from Taichman Lab, Stony Brook, NY) [173, 174]. 48 hours after transfection, the producer cells lyse and release the replication incompetent retroviruses into the supernatant [173, 174]. The supernatants were filtered through 0 .45-µm pore size filter (Gelman science, Ann Arbor, MI, USA) and were either stored as is at -70°C or concentrated further by ultra-centrifuging using a protocol described elsewhere [173, 174]. Retroviral transduction was achieved by adding known volumes of viral supernatant to a predetermined number of oral mucosal keratinocytes (or fibroblasts) in six well plates in the presence of 8 µg/ml poly-brene (Sigma, St Louis, MO, USA) at 37°C [173, 174]. After 2–3 h, fresh medium is added and 2 days after transduction, cells were then either passaged or harvested for FACS analysis. The percentage of cells positive for GFP was then used to calculate viral titers. Viral titers ranged from 1.5x 10^7 – 8x10^7 infective units. Once viral titers were determined the viruses are then used to achieved different multiplicities of infection (MOI) ranging from 0.3 – 1.

Thus transduced cells are then analyzed FACS for the presence of GFP which indirectly reflects the percentage of transduction.

The different percentage transduced cells are then plated, passaged and harvested and stored in -70^{0}C. The supernatants are then analyzed for secretion of vIL10 using ELISA. Figure 4 depicts the rate of vIL-10 secretion by epithelial cells as a function of percent of transduction. 30% transduced epithelial cells were used for conditioning Langerhans cells as they produced approximately 20ng of vIL10 per million cells per hour.

Figure II-4: vIL-10 secretion by epithelial cells as a function of percentage of cells expressing eGFP



Microbe Associated Molecular Patterns:

LPS isolation and determination of the purity

LPS was isolated from either Pg 381 or *E. coli* (type ATCC strain 25299) by hot phenol-water extraction, followed by isopycnic density gradient centrifugation and was further purified of contaminating nucleic acids, proteins, lipoproteins, as previously described [175]. In addition, some of the LPS preparations (purified identically) were provided courtesy of T.E. Van Dyke, Boston University Goldman School of Dental Medicine.

The purity of the LPS was determined by running 50 μg of LPS through two Polyacrylamide gels under reducing conditions. One of the gels was silver stained and the other stained with Coomassie blue. The purity of LPS was confirmed by detecting visible LPS staining during silver staining and not through Coomassie blue staining.

Stock solutions of 10mg/ml were prepared in PBS which was then serially diluted as per experimental requirements. The LPS solutions were sonicated for 2 minutes before experimental use to ensure equal distribution in the solution.

Zymosan Preparation:

Zymosan was commercially purchased (ZYMOSAN A from *Saccharomyces cerevisiae*; Sigma, St Louis, MO, USA) and it was re-suspended in 0.5% PBSA as per manufacturer’s instruction. Zymosan is not soluble in water but can be uniformly suspended in saline. 1mg/ml suspension was prepared as stock solution and was serially diluted as per experimental requirements. Care was taken to ensure uniform suspension by vortexing each time before use.

Peptidoglycan Preparation:

Peptidoglycan was commercially purchased (Sigma, St Louis, MO, USA) and stock solution of 1mg/ml was prepared in 0.5%PBSA. The stock solution and was then serially diluted as per experimental requirements. Care was taken to ensure uniform suspension before use by sonicating it in ultrasonic water bath for total of 5 minutes. The tube containing the suspension was removed every other minute so as to avoid any temperature elevation of the suspension vortexing each time before use.

Conditioning agents:

Recombinant Human IL10 (rhIL10)

Recombinant human rhIL10 was commercially purchased from BD biosciences (San Jose, CA). The lyophilized powder is reconstituted in 1%PBSA to make stock solutions of 1mg/ml and stored at -75°C. The stock solution was thawed and working concentrations were made from it, based on the individual experimental protocols as listed in later sections.

Recombinant CD40 Ligand (CD40L)

Recombinant human CD40L was commercially purchased from BD biosciences (San Jose, CA). The lyophilized powder is reconstituted in 1%PBSA to make stock solutions of 1mg/ml and stored at -75°C. The stock solution was thawed and working concentrations were made from it based on the individual experimental protocols as listed in later sections.

Recombinant human IL1β (IL1β)

Recombinant human IL1β was commercially purchased from BD biosciences (San Jose, CA). The lyophilized powder is reconstituted in 1%PBSA to make stock solutions of 1mg/ml and stored at -75°C. The stock solution was thawed and working concentrations were made from it based on the individual experimental protocols as listed in later sections.

Recombinant human TNFα (TNFα)

Recombinant human TNFα was commercially purchased from BD biosciences (San Jose, CA). The lyophilized powder is reconstituted in 1%PBSA to make stock solutions of 1mg/ml and stored at -75°C. The stock solution was thawed and working concentrations were made from it based on the individual experimental protocols as listed in later sections.

SB202190

SB202190 was commercially purchased from Sigma (Sigma, St Louis, MO, USA) was used in concentration of 50 μM [176, 177].

Pyrrolidine dithiocarbamate (PDTC)

PDTC was commercially purchased from Sigma (Sigma, St Louis, MO, USA) was used in concentration of 10 μM [176, 178, 179].

Biochemical and molecular Assays

Multi-Parametric Flow cytometry analysis:

Flow cytometric analysis was done on the various cell types used in this study to both characterize the phenotypes of the cells as well as to analyze their functional responses to the various conditioning and challenges (Cytokines, Microbe associated molecular patterns etc.,). The various conditioning and challenges and effects obtained will be discussed in detail in the later sections.

Flow cytometric analysis was done based on the following protocol. The cells were incubated with monoclonal antibodies (mAB), or their respective isotype matched controls, to analyze the expression of the surface proteins. Cells were incubated for 30 minutes at 4°C, and then washed with 1%PBSA. Cells were then fixed in 1% paraformaldehyde and protected from direct light until analysis through flow cytometry. Analysis was performed with FACScalibur^{TM} (Becton Dickinson, Franklin lakes, NJ). The expression of the surface proteins were analyzed as the percentage of positive cells in the relevant population defined by forward scatter and side scatter characteristics. Expression levels were evaluated by assessing mean fluorescence intensity (MFI) indices calculated by relating MFI noted with the relevant mAb to that with the isotype control monoclonal antibodies for samples labeled in parallel and acquired using the same setting. Unstained cells were also run as further controls. An attempt to use directly conjugated antibodies where possible was employed as it simplifies the methodology of staining. Table I provides the list of antibodies, their sources and the respective clones employed for these studies.

Table I : List of Antibodies

Antibody	Clone	Isotype	Source
Langerin	DCGM4	Ms IgG1, K	Beckman Coulter, Fullerton, CA
TLR2	TL2.1	Ms IgG2a, k	eBioscience
TLR4	HTA125	Ms IgG2a, k	eBioscience
CD1a	HI149	Ms IgG1, k,	BD Biosciences, San Jose, CA
DEC205	MG38	Ms IgG_{2b}, k	BD Biosciences
HLADR	L243	Ms IgG2a, k	BD Biosciences
DCSIGN	DCN46	Ms IgG_{2b}, k	BD Biosciences
CD83	HB15e	Ms IgG1, k	BD Biosciences
CD80	L307.4	Ms IgG1, k,	BD Biosciences
CD86	FUN-1	Ms IgG1, k	BD Biosciences
CD4	RPA-T4	Ms IgG1, k	BD Biosciences

Flow cytometry based Cytokine analysis

The various culture supernatants were collected and the inflammatory cytokines (TNF-α, IL-6, IL-8, IL1β, and IL-10) were analyzed by flow cytometry using cytometric bead array (BD™ Cytometric Bead Array (CBA) Human Inflammation Kit, BD Biosciences. San Jose, CA). The protocol for staining and analysis were done as per manufacturer's instructions. Based on a standard curve for each cytokine, the CBA software calculates levels in pg/ml.

PCR analysis

RNA extraction & cDNA synthesis

Cells were placed in RNAlater RNA stabilizing reagent (Qiagen) and frozen at -80C until the desired sample size was obtained. Frozen Cells were re-suspended in lysis buffer and total RNA was extracted using Qiagen RNeasy mini kits according to manufacturer's instructions. Avian RT first strand kits (Sigma, St Louis, MO, USA) were used to synthesize cDNA from total RNA. The concentration of total RNA was determined at OD260 and small discrepancies in the number of cells which were used as starting material was corrected by loading the same concentration of RNA for cDNA synthesis. The purity of cDNA was determined by analysis of the OD260 / OD280 ratio.

Primers for PCR

Nucleotide sequences were determined from NCBI website (Pubmed search engine) and the primers were custom designed using primer3 software. Table II lists the primer sequences and the amplification product sizes used for real time PCR.

Table II: Primers for real time RT PCR

Gene ID	Left Primer sequences (5' to 3')	Right Primer sequences (5' to 3')	Product size (bp)
TLR 2	GGAGGCTGCATATTCCAAGG	GCCAGGCATCCTCACAGG	216
TLR 4	CTTGACCTTCCTGGACCTCTC	ACTTGGAAAATGCTGTAGTTCC	217
TLR 6	TGCCTCCATTATCCTCATGC	CATTTGGGAAAGCAGAGTGG	203
DEC 1	GGCAACTGGGCTCTAATCTC	GATGGGTTTTCTTGGGTAGC	205
NOD 1	TCCAAGTTCGTGCTGTGCTA	GATGGTCTCACCCTGCTCAT	174
NOD 2	GAGGCTTTTCAGGCACAGAG	CCTTATTCCAGACGGTGTCC	193
beta-Act	ACTCTTCCAGCCTTCCTTCC	GTTGGCGTACAGGTCTTTGC	204

Real time RT-PCR quantification

Real-time 5'-nuclease fluorogenic RT-PCR analysis was performed on ICycler iQ Real-Time PCR (Bio-Rad, Hercules, CA with SYBR green kits (Bio-Rad, Hercules, CA) and mRNA quantitation was done as described [180]. Levels of B-actin mRNA will serve as an internal control to normalize samples for variations in sample volume loading, presence of inhibitors, and nucleic acid recovery during extraction and cDNA synthesis procedures. The normalized expression is calculated according to the equation:

$$NE = \frac{(E_{target})^{CT-Target(Control-treated)}}{(E_{reference})^{CT-Reference(Control-treated)}}$$

In the formulae, NE stands for Normalized Expression, E_{target} stands for efficiency of amplification for the target gene, CT_{target} stands for thresh-hold cycle of amplification for target gene, $E_{(ref)}$ stands for efficiency of amplification for the reference gene (B-Actin) and CT_{ref} stands for thresh-hold of cycle of amplification for reference gene . Thus the normalized gene expression is directly proportional to the amount of RNA of a certain target sequence (i.e., the target gene) relative to the amount of RNA of the reference gene and can be used to compare levels in different experimental groups. All analyses were performed in triplicate.

T cell Proliferation assay

Intracellular staining of T-cells with CFSE [181]:

CFSE (carboxyfluorescein diacetate succinimidyl ester) is an intracellular dye which labels the proteins in the cytoplasm of a cell. The dye forms adducts with intracellular protein. Thus formed label is inherited by daughter cells after either cell division or cell fusion, and is not transferred to adjacent cells in a population [182-184].

CFSE staining of T Cells is done as per manufacturer's instructions (see reference).Briefly the CFSE cell trace is prepared by mixing the 2 components from the CellTrace™ CFSE Cell Proliferation Kit (Molecular Probes, Carlsbad, CA). Component A contains the Dye and Component B contains DMSO This stock solution is further diluted 1:5 in DMSO so as to give the appropriate signal to noise ratio as determined through a set of dilution experiments to optimize the experimental conditions. The T cells are re-suspended in PBS to get 10^6 /ml cells suspensions. To this suspension appropriate amount of diluted dye is added (2 µl

per million cells). Dye and cells are incubated at 37 ^{0}C for 10 minutes. Then the cells are quenched by adding 5 times of ice cold culture media to the cell suspension and incubated on ice for 5 minutes. Cells are washed twice in fresh media and incubated at 37^{0}C for 2 days before use. Incubating for 2 days stabilizes the cytoplasmic staining so that further distribution of the stain in the proliferating cells is reflective of the actual proliferation.

The stained T cells are co-cultured with LCs as described in the following section and are followed up to determine the rate of proliferation with the help of a flow cytometry using a flow-cytometry instrument equipped with a 488 nm excitation source.

Autologous and allogeneic naïve CD4 T cell stimulation by LCs

Autologous and allogeneic naïve CD4^{+} cells were purified from PBMC as previously described. Pre-characterized LCs were treated with microbial agonists and / or conditioning agents as per experimental requirements as discussed in the later sections. Pulsed DCs were seeded in graded doses (1000, 500 and 300

cells/well) and were co-cultured with fixed number of CFSE (Molecular Probes, Carlsbad, Ca) stained, 5000 cells / well, autologous or allogeneic naïve $CD4^{+ve}$ T cells. The cells were incubated at 37^0 C for 5 days in enriched T cell medium. $CD4^+CFSE^+$ T cells were then analyzed by FACS analysis using Cellquest software. Based on level of expression of intracellular CFSE (1/2 CFSE is lost with each generation), proliferating $CD4^+$ T cells were be determined.

Chapter III

Aim - I

Effect of oral MAMPs on Langerhans cells

Introduction:

Background & rationale:

The isolation of Langerhans cells from the epidermis and mucosa in a naïve form i.e., without activating the Langerhans cell is difficult and challenging. The very process of extracting Langerhans cells with help of enzymes, chemo-attractants etc., leads to the activation and maturation of Langerhans cells.

This results in a compromise in the quality of the Langerhans cells that can be used to understand the in-vitro innate and immuno-stimulatory response of these cells. Even if successful, such methods typically have a poor quantitative yield required extensive amounts of starting material (skin or mucosa).

Thus most studies of Langerhans cells are done with help of in-vitro generated Langerhans cells. Typically Langerhans cells were derived from monocytes or from $CD34^+$ cells under special culturing conditions containing various cocktails of cytokines. Generally most studies use a combination of markers to isolate and identify Langerhans cells. This typically includes CD1A and/or HLADR etc.

Langerin and Birbeck granules are two of the most conclusive identifiers of Langerhans cells. Birbeck granules are typically visualized under electronic

microscope. Langerin on the other hand can be easily stained with conjugated antibodies. We were able to successfully generate and isolate Langerhans cells from CD34+ve embryonic cord blood cells in high yields as described in previous chapters.

Very few studies have looked at Langerhans cells isolated with the help of Langerin antibodies. Furthermore these studies were derived from monocytes. To our knowledge no study exist which look into in vitro response of CD34+ve embryonic cord blood cells Langerhans cells to MAMPs specifically oral MAMPS.

Hence we decided to explore the same, and towards that, the following objective was formulated and pursued.

Aim - I:

To determine the innate response and immuno-stimulatory function of human LCs in response to MAMPs in vitro

Specific Hypotheses

To explore the stated aim we formulated the following hypotheses.

Hypothesis 1:

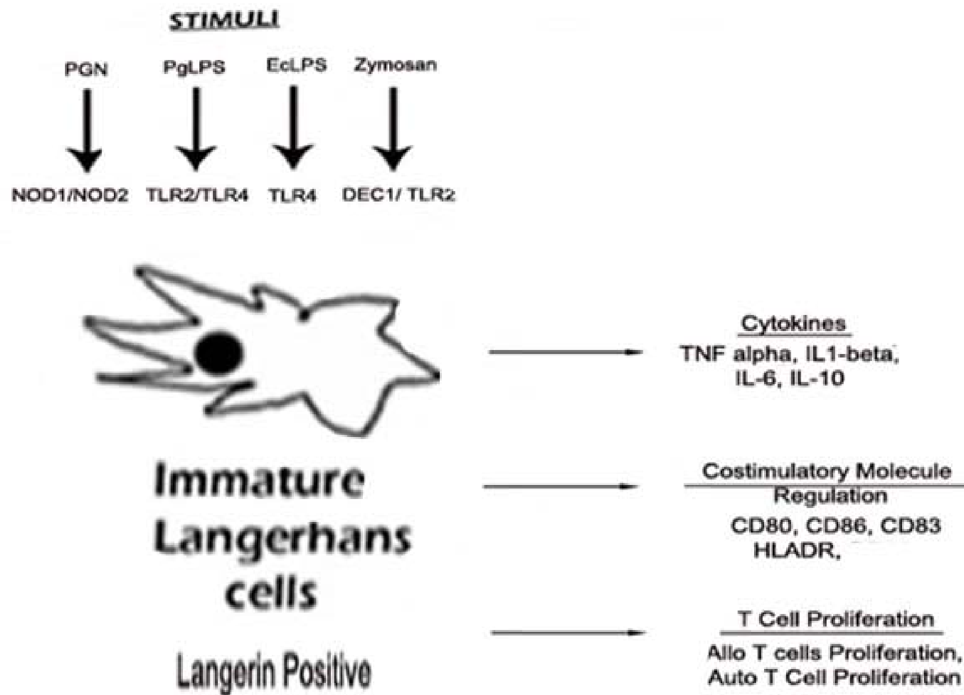
CD34^{+ve} derived Langerin^{+} LC express a unique repertoire of pattern recognition receptors (PRRs). These PRRs determine the level of response to microbial ligands, (MAMPs) and which in turn would determine their homeostatic function and innate immune function.

Hypothesis 2:

The immuno-stimulatory capacity of LC is dependent on MAMP-induced activation and maturation.

Experimental Design

Figure III-1: Experimental design for Aim-I



Challenge Protocol:

Isolated immature Langerhans cells are challenged with different doses of four types of MAMPs, i.e., 1000pg, and 10000pg. The cells were incubated with these MAMPS for 24 hours at 37^0C in a 5% CO_2 incubator. The different types of MAMPs are listed and described below.

Peptidoglycan has been well established as a ligand for NOD1/NOD2 receptors. Hence we chose to use it to evaluate presence and extent of the innate and

immunostimulatory response of Langerhans cells through the NOD1/NOD2 receptor pathway activation.

Similarly PGLPS is a ligand for the signal transduction and subsequent activation for TLR2/TLR4 signaling in dendritic cells and Langerhans cell. ECLPS is a ligand for TLR4 signaling. Thus we chose PGLPS and ECLPS to see the effects of TLR2 and TLR4 signaling in Langerhans cells.

Zymosan is a beta-glucan which activates the DEC-1 scavenger receptor in dendritic cells and Langerhans cells and was chosen to evaluate the Langerhans cell response.

Experimental Readouts:

The Innate immune response and the immunostimulatory capacity of Langerhans cells were evaluated as follows.

Co-stimulatory molecules:

The activation and maturation of Langerhans cells can be assessed by measuring the regulation of co-stimulatory molecules expressed on the surface of

Langerhans cells in response to various environment agents including cytokines, MAMPS etc., We decided to use flow-cytometry based analysis and quantification of the change in expression of CD83, CD86, CD80 and HLADR to assess the response of Langerhans cells. Thus the cells which were incubated with MAMPs for 24 hours were spun down, stained with fluorescence conjugated antibodies, and then fixed. These cells were analyzed with help of flow-cytometry as described in the materials and methods chapter.

Cytokines:

It is well known that activated Langerhans cells express cytokines and the measure of these cytokines not only enables us to quantitatively assess the Langerhans cell response but also sheds some light on the qualitative aspect of the Langerhans cell response.

Thus we decided to assess the change in levels of the following cytokines secreted by LCs in response to MAMPs challenge, namely TNFα, IL-1β, IL-6, and IL-10 with the help of a flow-cytometry based cytokine assay.

Towards this the supernatants of MAMPs stimulated Langerhans cells culture supernatants were pipetted out of the tubes containing the centrifuged cells and were stored under -75^{0}C until further analysis. The supernatants are then

quickly thawed and stained with help of Cytokine assay kit and then analyzed through flow-cytometry as described in the materials and methods chapter.

T cell Proliferation:

The immunostimulatory capacity of an activated antigen presenting cells like the dendritic cells or Langerhans cells have traditionally been studied with the help of T cell proliferation assays.

Thus we decided to use a CFSE based T cell proliferation assay to evaluate this. The assay has been described in detail in the materials and methods chapter. The Langerhans cells were first stimulated with different MAMPs and then incubated for 24 hours. Following which each of the thus stimulated Langerhans cells were spun down and co-cultured with the T cells as described previously for 4-6 days before being analyzed with the help of flow-cytometry.

Receptor Regulation:

LC expresses low levels of some PRRs constitutively. LC expresses very low levels of TLR2, and TLR4 as determined by flow cytometric analysis.

Fluorescent conjugated antibodies against these PRRs and PRRs like NOD1 and NOD2 have been reported to be of poor quality especially in terms of cells which have low constitutive expression. Since we wanted to analyze how MAMP challenge affects the surface expression of PRRs we instead decided to use real time PCR based analysis for the transcripts of these PRRs.

Towards this the cells which were incubated with MAMPs for 24 hours were spun down and were stored in RNAlater. Messenger RNA was extracted from these cells and converted to cDNA and used for real time PCR analysis as described in the material methods section.

Results:

Co-stimulatory Molecules

As described in the Experimental design the expression of CD83, CD86, CD80, HLADR on Langerhans cells upon stimulation, with the four different MAMPs, was analyzed with flow cytometry). The statistical significance was determined with help of Kolmogrov-Smirnov statistics. The treated Langerhans cells if significant had a ‘p’ value less than or equal to 0.001 when compared to untreated Langerhans cells.

CD83:

EFFECT OF DIFFERENT DOSES OF FOUR DIFFERENT MAMPS:

Langerhans cells express very low constitutive levels to almost no CD83 on their surface as compared to matched isotype controls indicating their immature status (Figure 2 &3

Figure III-2: Effect of 1000pg of four different MAMPs on CD83 expression on Langerhans Cells

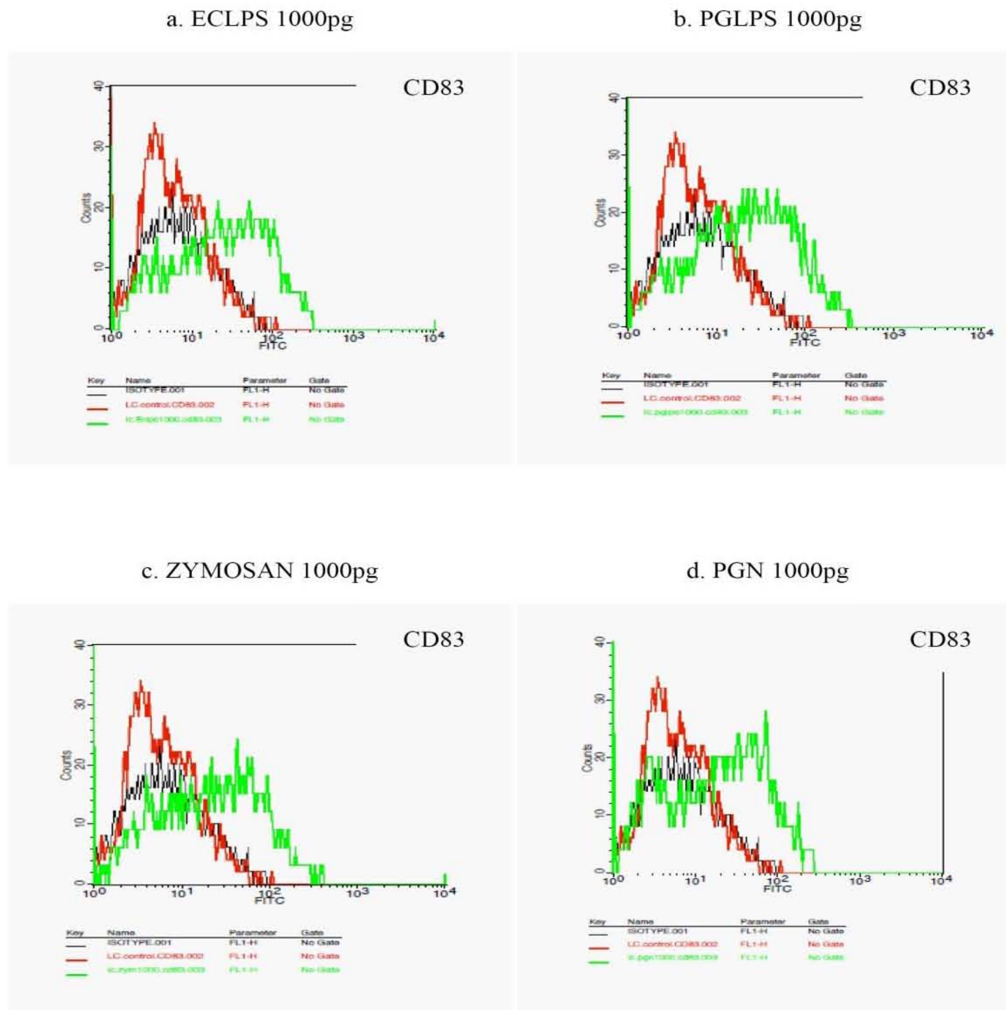


Figure Legend

Isotype

Untreated LC

LC + MAMP

PGLPS 1000pg:

Langerhans cells stimulated with 1000pg dose of PGLPS increased surface expression of CD83 substantially (Figure 2b) in a statistically significant manner.

ECLPS 1000pg:

Langerhans cells stimulated with 1000pg dose of ECLPS increased surface expression of CD83 substantially (Figure 2a) in a statistically significant manner.

Zymosan 1000pg:

Langerhans cells stimulated with 1000pg dose of Zymosan increased surface expression of CD83 substantially (Figure 2c) in a statistically significant manner.

Peptidoglycan (PGN) 1000pg:

Langerhans cells stimulated with 1000pg dose of PGN increased surface expression of CD83 substantially (Figure 2d) in a statistically significant manner.

Figure III-3: Effect of 10000pg of different MAMPs on CD83 expression on Langerhans Cells.

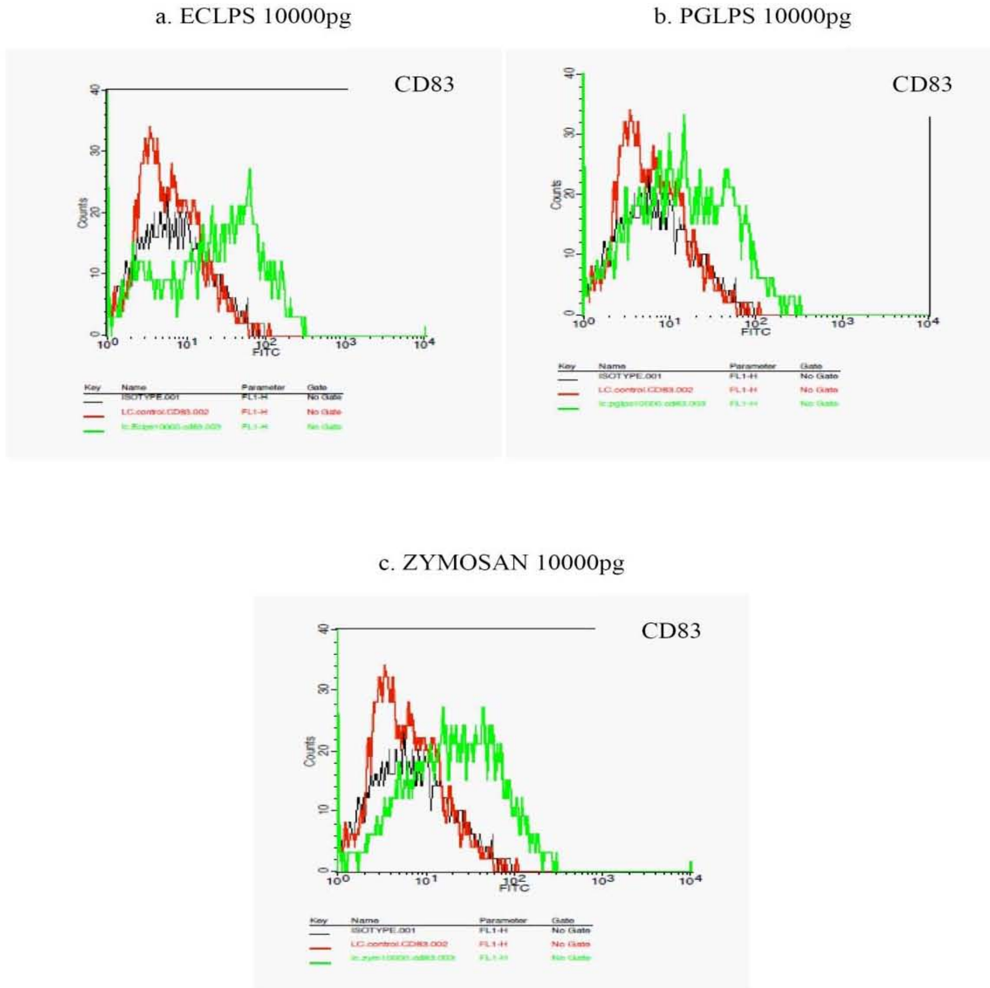


Figure Legend

Isotype

Untreated LC

LC + MAMP

PGLPS 10000pg:

Langerhans cells stimulated with 10000pg dose of PGLPS also increased surface expression of CD83 substantially (Figure 3b) in a statistically significant manner.

ECLPS 10000pg:

Langerhans cells stimulated with 10000pg dose of ECLPS increased surface expression of CD83 substantially (Figure 3a) in a statistically significant manner.

Zymosan 10000pg:

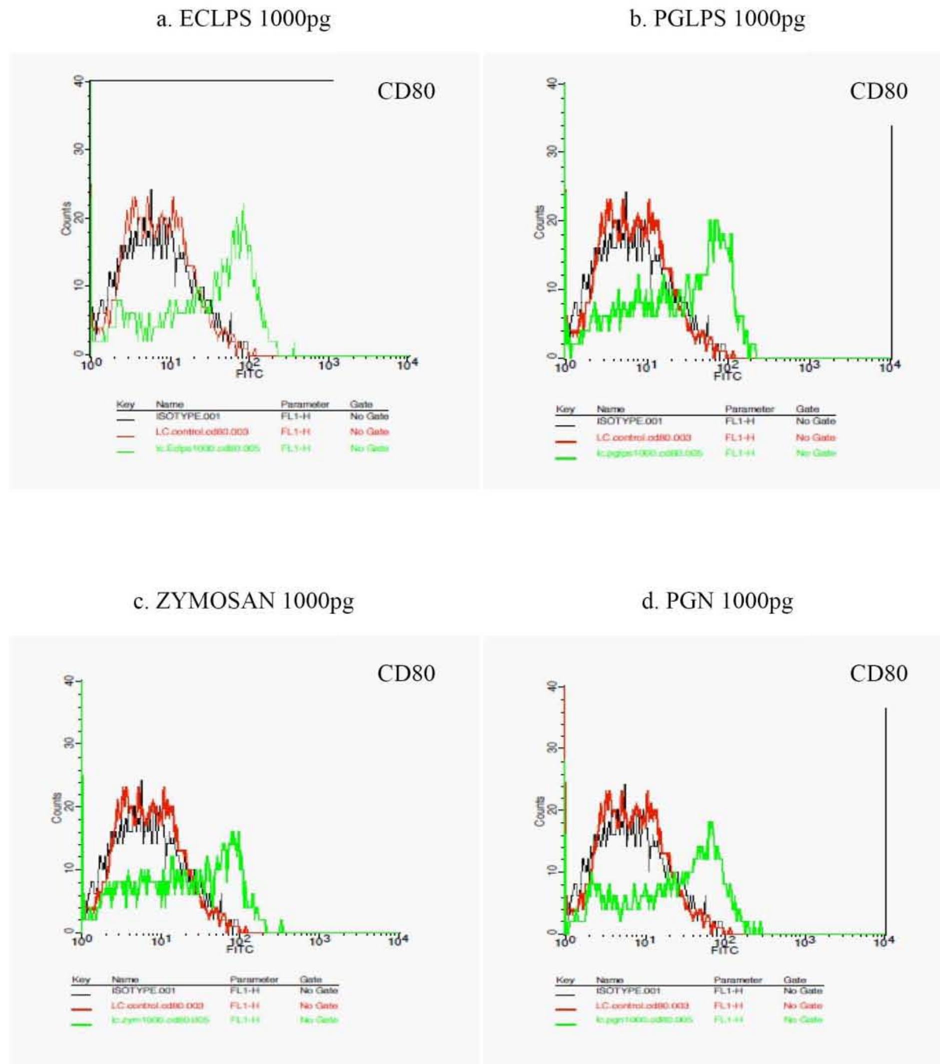
Langerhans cells stimulated with 10000pg dose of Zymosan increased surface expression of CD83 substantially (Figure 3c) in a statistically significant manner.

CD80

EFFECT OF DIFFERENT DOSES OF FOUR DIFFERENT MAMPS:

Langerhans cells express very low constitutive levels to almost no CD80 on their surface as compared to matched isotype controls indicating their lact of activation or rest status (Figure 4 &5).

Figure III-4: Effect of 1000pg of four different MAMPs on CD80 expression on Langerhans Cells



PGLPS 1000pg:

Langerhans cells stimulated with 1000pg dose of PGLPS increased surface expression of CD80 substantially (Figure 4b) in a statistically significant manner.

ECLPS 1000pg:

Langerhans cells stimulated with 1000pg dose of ECLPS increased surface expression of CD80 substantially (Figure 4a) in a statistically significant manner.

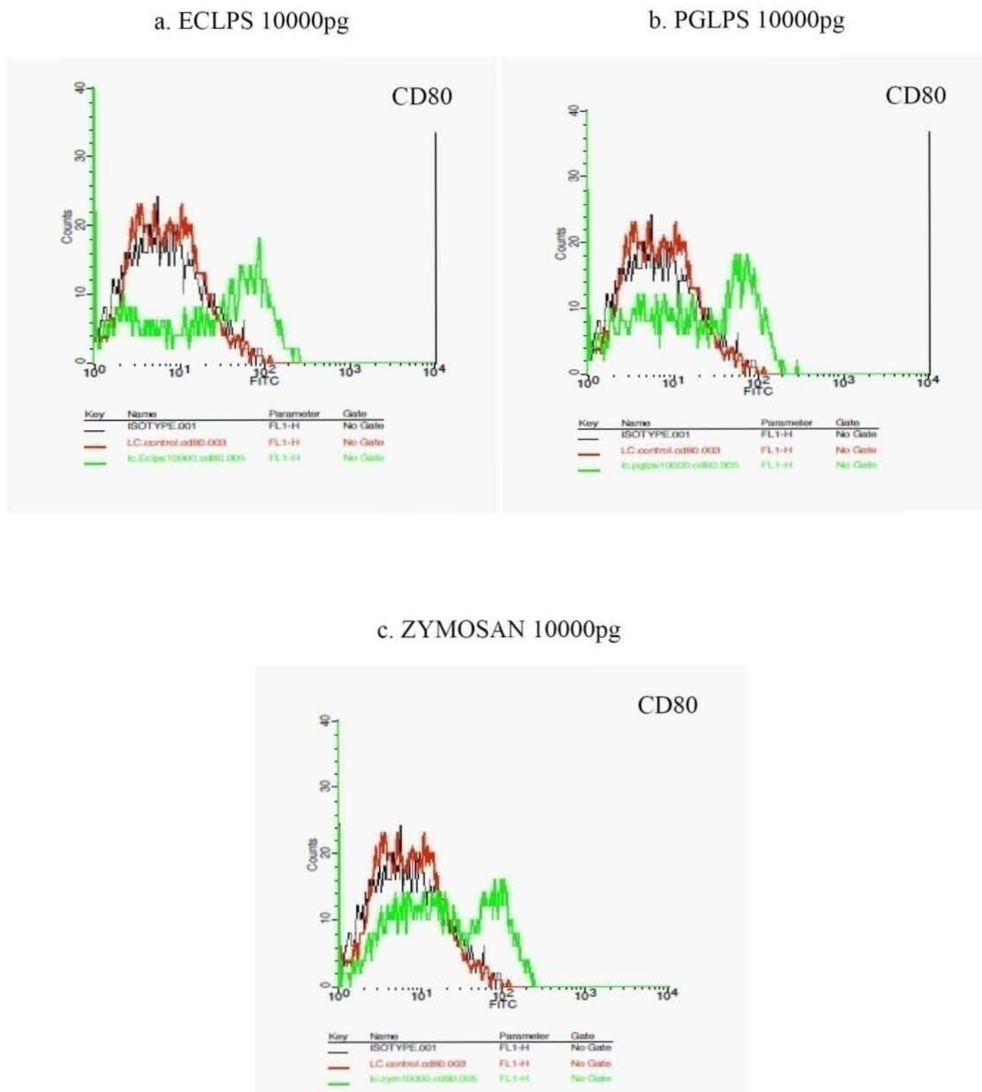
Zymosan 1000pg:

Langerhans cells stimulated with 1000pg dose of Zymosan increased surface expression of CD80 substantially (Figure 4c) in a statistically significant manner.

Peptidoglycan (PGN) 1000pg:

Langerhans cells stimulated with 1000pg dose of PGN increased surface expression of CD80 substantially (Figure 4d) in a statistically significant manner.

Figure III-5: Effect of 10000pg of different MAMPs on CD80 expression on Langerhans Cells.



PGLPS 10000pg:

Langerhans cells stimulated with 10000pg dose of PGLPS increased surface expression of CD80 substantially (Figure 5b) in a statistically significant manner.

ECLPS 10000pg:

Langerhans cells stimulated with 10000pg dose of ECLPS increased surface expression of CD80 substantially (Figure 5a) in a statistically significant manner.

Zymosan 10000pg:

As described in the Experimental design the expression of CD80 on Langerhans cells upon stimulation, with Zymosan, was analyzed with flow cytometry. CD80 was expressed constitutively in Langerhans cells (Figure 5). Langerhans cells stimulated with 10000pg dose of Zymosan increased surface expression of CD80 substantially (Figure 5c). This inducible expression was statistically significant.

CD86:

EFFECT OF DIFFERENT DOSES OF FOUR DIFFERENT MAMPS:

Langerhans cells express low constitutive levels to almost no CD86 on their surface as compared to matched isotype controls indicating their inactive state or rest status (Figure 6 & 7).

Figure III-6: Effect of 1000pg of four different MAMPs on CD86 expression on Langerhans Cells.

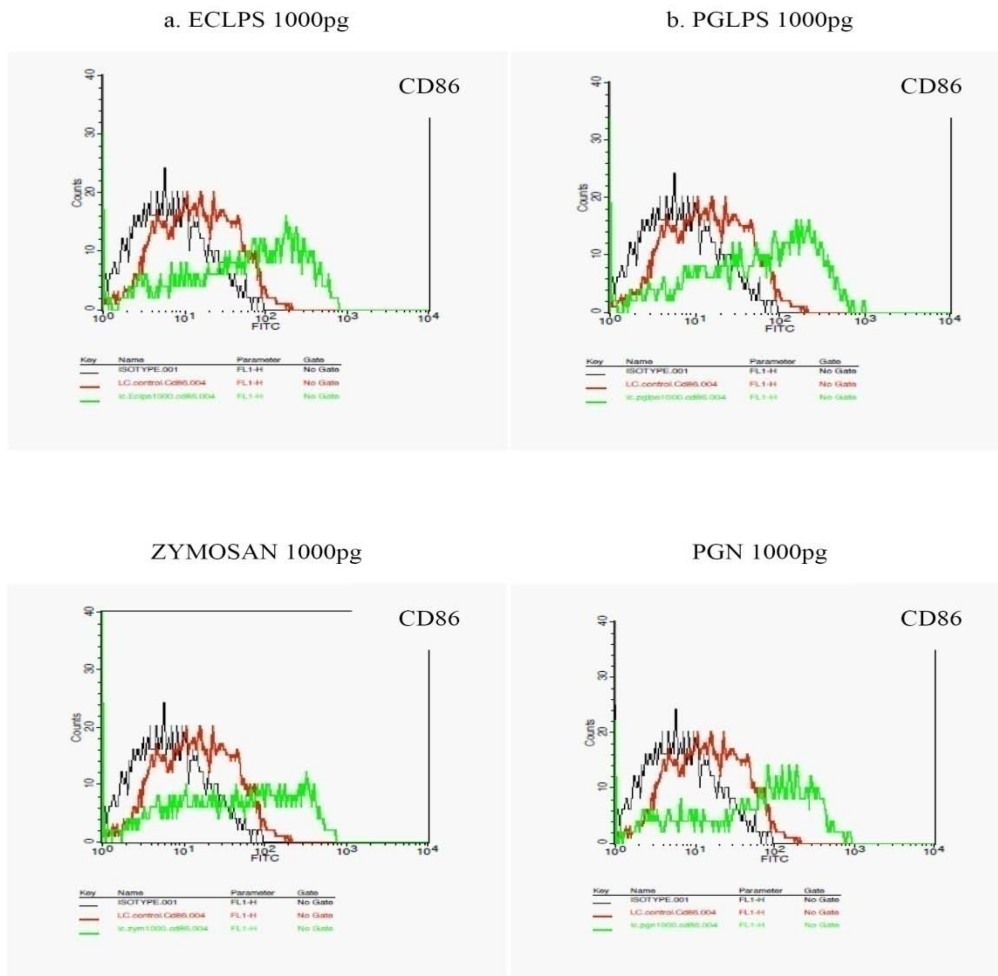


Figure Legend

Isotype

Untreated LC

LC + MAMP

PGLPS 1000pg:

Langerhans cells stimulated with 1000pg dose of PGLPS increased surface expression of CD86 substantially (Figure 6b). This inducible expression was statistically significant.

ECLPS 1000pg:

Langerhans cells stimulated with 1000pg dose of ECLPS increased surface expression of CD86 substantially (Figure 6a). This inducible expression was statistically significant.

Zymosan 1000pg:

Langerhans cells stimulated with 1000pg dose of Zymosan increased surface expression of CD86 substantially (Figure 6c). This inducible expression was statistically significant.

Peptidoglycan (PGN) 1000pg:

Langerhans cells stimulated with 1000pg dose of PGN increased surface expression of CD86 substantially (Figure 6d). This inducible expression was statistically significant.

Figure III-7: Effect of 10000pg of different MAMPs on CD86 expression on Langerhans Cells.

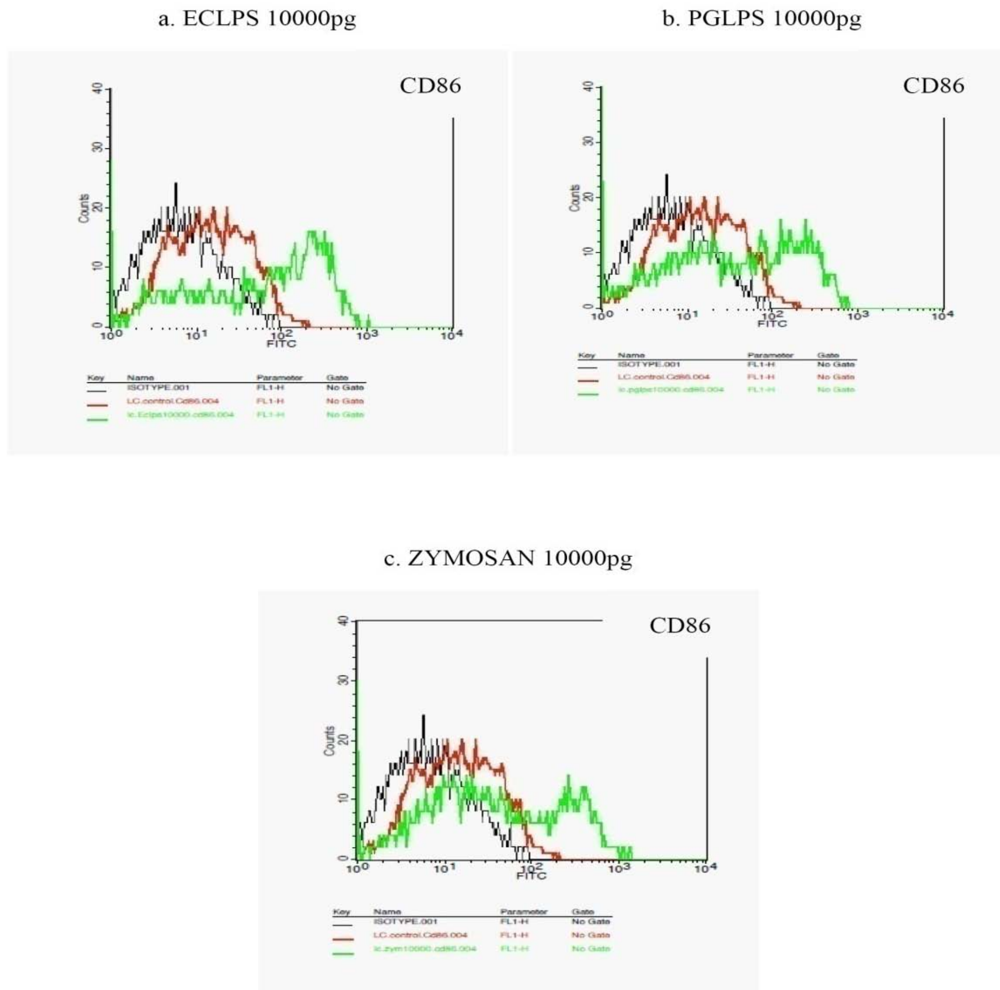


Figure Legend

Isotype

Untreated LC

LC + MAMP

PGLPS 10000pg:

Langerhans cells stimulated with 10000pg dose of PGLPS increased surface expression of CD86 substantially (Figure 7b). This inducible expression was statistically significant.

ECLPS 10000pg:

Langerhans cells stimulated with 10000pg dose of ECLPS increased surface expression of CD86 substantially (Figure 7a). This inducible expression was statistically significant.

Zymosan 10000pg:

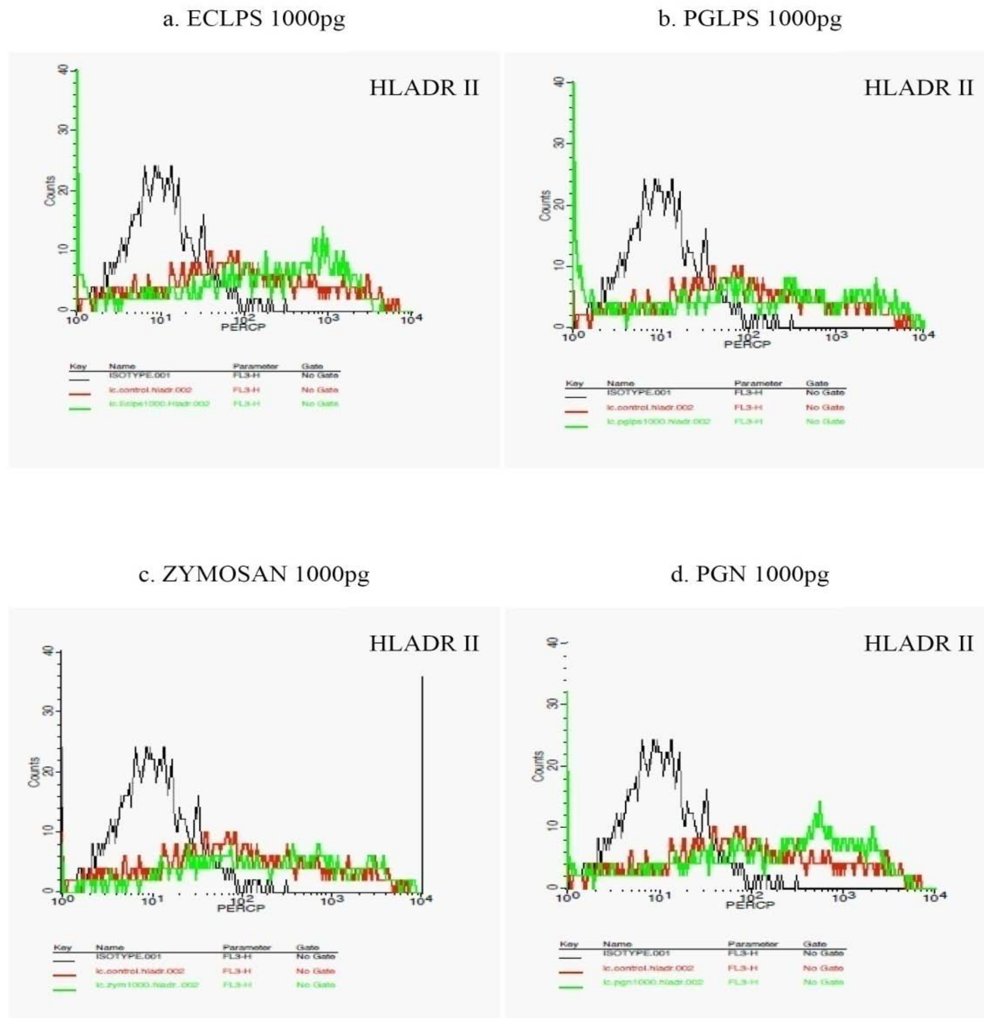
Langerhans cells stimulated with 10000pg dose of Zymosan increased surface expression of CD86 substantially (Figure 7c). This inducible expression was statistically significant.

HLADR II:

EFFECT OF DIFFERENT DOSES OF FOUR DIFFERENT MAMPS:

Langerhans cells express high constitutive levels of HLADRII on their surface as compared to matched isotype controls (Figure 8 & 9). Previous studies have HLADRII used as a marker for Langerhans cells in combination with CD1A.

Figure III-8: Effect of 1000pg of four different MAMPs on HLADR II expression on Langerhans Cells.



PGLPS 1000pg:

Langerhans cells stimulated with 1000pg dose of PGLPS did not increase the surface expression of HLADR II in a statistically significant manner (Figure 8a).

ECLPS 1000pg:

Although the Langerhans cells stimulated with 1000pg dose of ECLPS did not increase the surface expression of HLADR II in a statistically significant manner (Figure 8b), the amount of high staining HLADR II molecules were increased substantially in these cells.

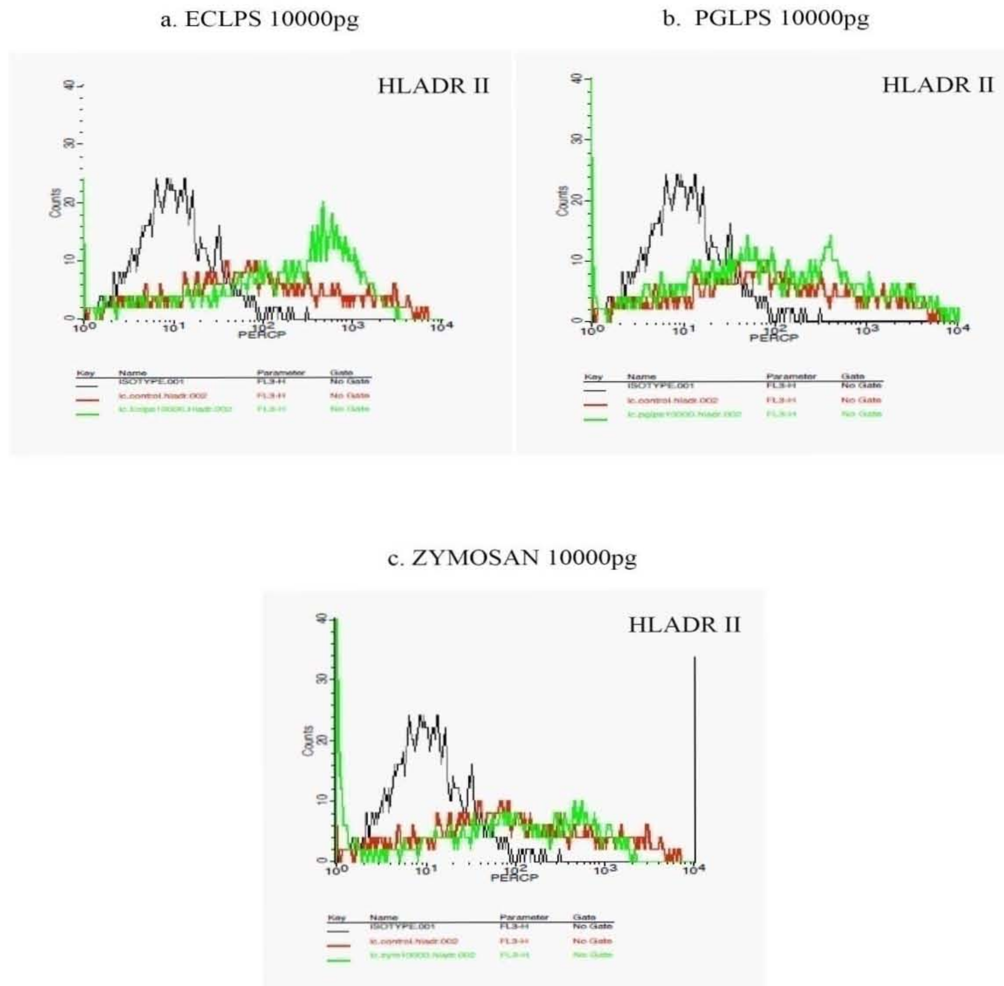
Zymosan 1000pg:

HLADR II was expressed constitutively in Langerhans cells (Figure 8). Langerhans cells stimulated with 1000pg dose of Zymosan did not increase the surface expression of HLADR II in a statistically significant manner (Figure 8c.

Peptidoglycan (PGN) 1000pg:

Similar to ECLPS stimulated Langerhans cells, Langerhans cells stimulated with 1000pg dose of PGN, did not increase the surface expression of HLADR II in a statistically significant manner (Figure 8d), but the amount of high staining HLADR II molecules were increased substantially in these cells.

Figure III-9: Effect of 10000pg of different MAMPs on HLADR II expression on Langerhans Cells.



PGLPS 10000pg:

Although the Langerhans cells stimulated with 10000pg dose of PGLPS did not increase the surface expression of HLADR II in a statistically significant manner (Figure 8b), the amount of high staining HLADR II molecules were increased substantially in these cells. Interestingly this was not noted in the 1000pg PGLPS treated Langerhans cells.

ECLPS 10000pg:

The Langerhans cells stimulated with 10000pg dose of ECLPS also did not increase the surface expression of HLADR II in a statistically significant manner (Figure 8b), but the amount of high staining HLADR II molecules were again increased substantially in these cells.

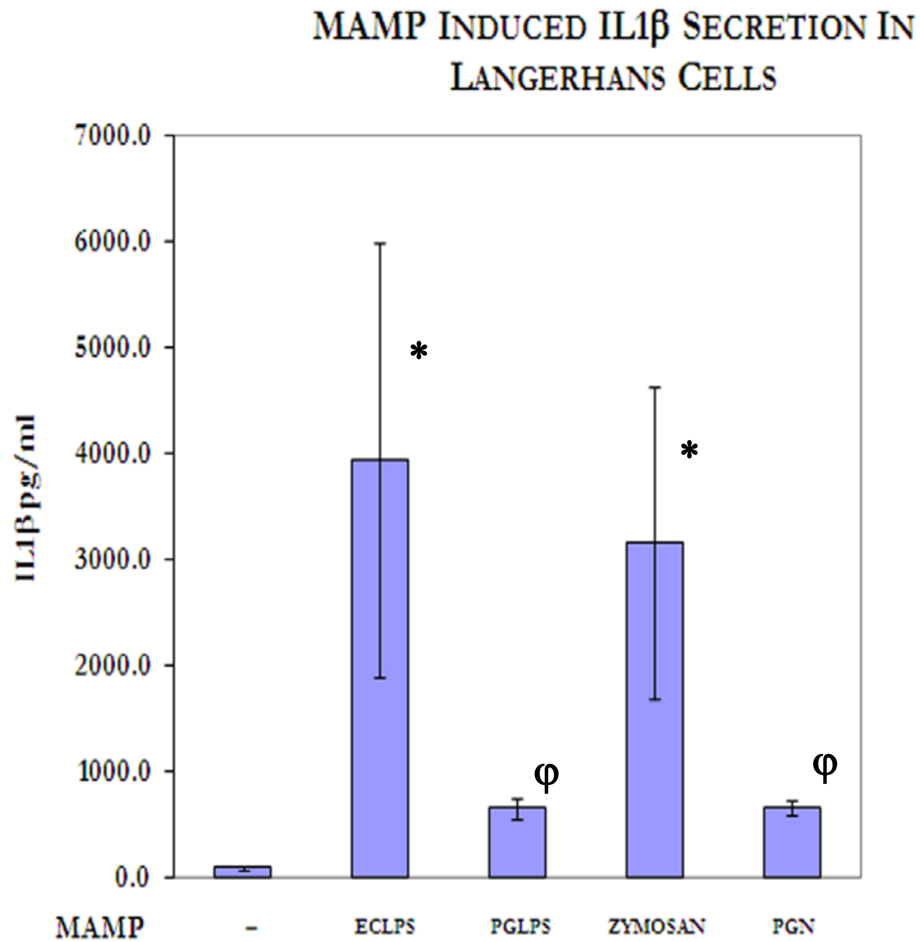
Zymosan 10000pg:

Langerhans cells stimulated with 10000pg dose of Zymosan did not increase the surface expression of HLADR II in a statistically significant manner (Figure 8d), In fact the HLADR II staining of cells shifted to the left signifying a possible down-regulation of HLADRII molecules with zymosan.

Cytokines

IL-1β

Figure III-10: IL-1β secretion of Langerhans cells in response to 1000pg of different MAMPs:



∗–Statistical significance with a P value of $\leq$ 0.05 as compared to untreated LCs; φ – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated LCs.

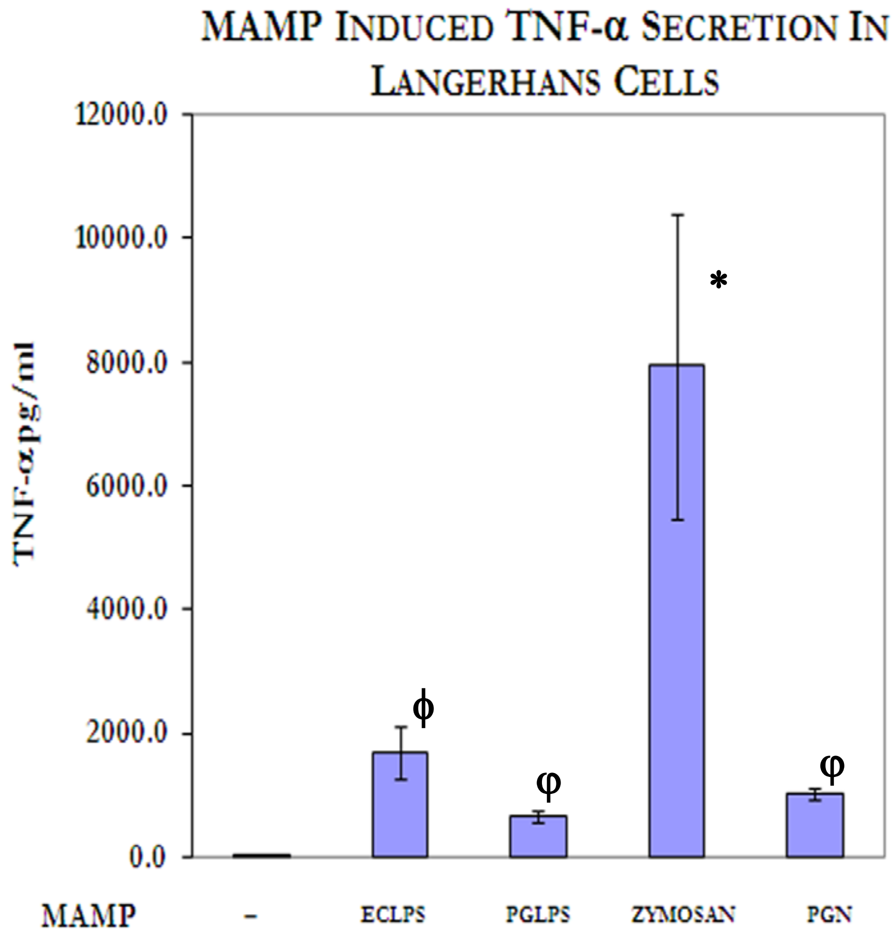
As described in the experimental design section Langerhans cells were challenged with different MAMPs at a uniform dosage of 1000pg. The IL1β response to these MAMP challenges were measured with help of a flow-cytometry based bead assay.

The results of the assay are plotted in Figure 10 in which the IL1β levels in picograms are plotted against Langerhans stimulated with different MAMPs. The results (Figure 10) show that Langerhans cells constitutively express low levels of IL1β.

We show here that when Langerhans cells are challenged with 1000pg of different MAMPs, they secrete elevated amounts of IL1β. ECLPS and Zymosan challenge elicits the highest amounts of IL1β production from these Langerhans cells. PGLPS and PGN produce similar amounts of IL1β. and produce almost 1000 fold more than the constitutive expression. ECLPS and Zymosan produce approximately 5 fold more the PGLPS and PGN.

TNF-α

Figure III-11: TNF−α secretion of Langerhans cells in response to 1000pg of different MAMPs:



∗−Statistical significance with a P value of $\leq$ 0.05 as compared to untreated LCs; ϕ − Statistical significance with a P value of $\leq$ 0.05 as compared to untreated LCs; φ − Statistical significance with a P value of $\leq$ 0.05 as compared to untreated LCs;

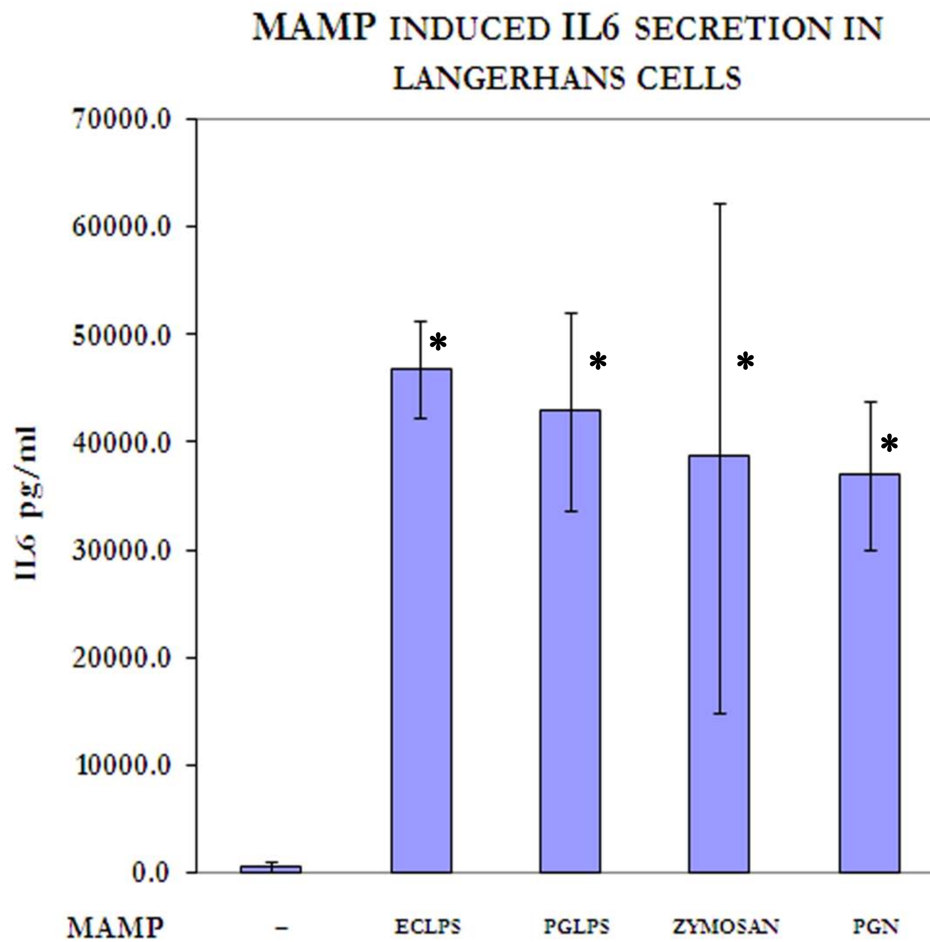
As described in the experimental design section Langerhans cells were challenged with different MAMPs at a uniform dosage of 1000pg. The TNFα response to these MAMP challenges were measured with help of a flow-cytometry based bead assay.

The results of the assay are plotted in Figure 11 in which the TNFα levels in picograms are plotted against Langerhans stimulated with different MAMPs. The results (Figure 11) show that Langerhans cells constitutively express low levels of TNFα.

When these Langerhans cells are challenged with 1000pg of different MAMPs, they secrete elevated amounts of TNFα. PGLPS and PGN produce similar amounts of TNFα which is 1000-1500 folds more than the constitutive expression. ECLPS secrete 2 fold TNFα more the PGLPS and PGN. Zymosan challenge elicits the highest amounts of TNFα production from these Langerhans cells. Zymosan challenged Langerhans cells produced almost 10 fold more the PGLPS challenged Langerhans cells.

IL-6:

Figure III-12: IL-6 secretion of Langerhans cells in response to 1000pg of different MAMPs:



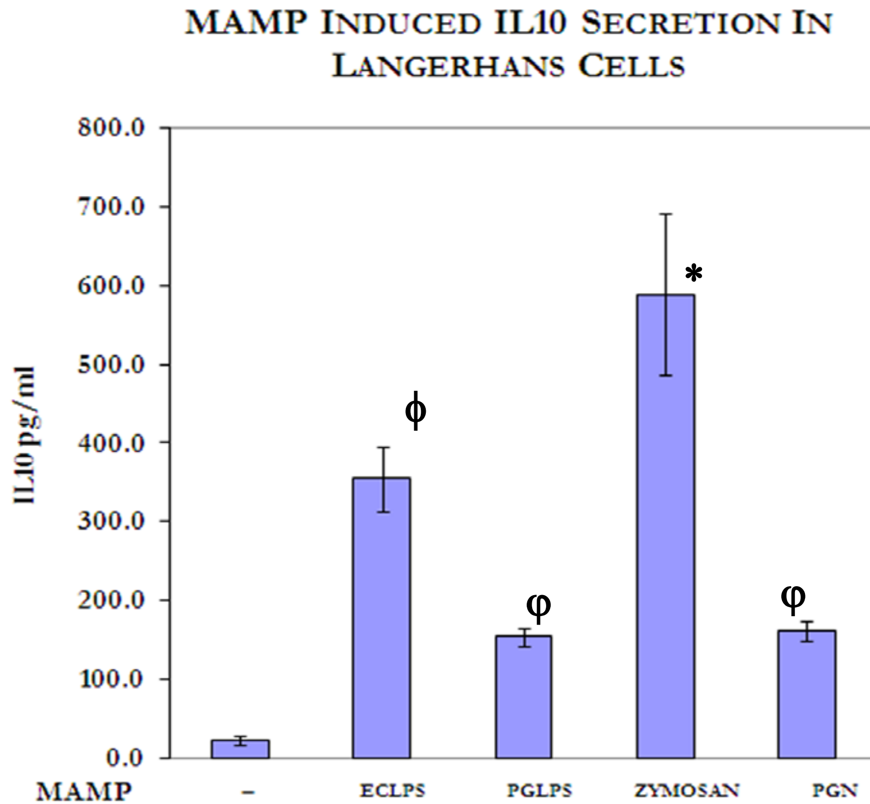
∗−Statistical significance with a P value of $\leq$ 0.05 as compared to LCs;

As described in the experimental design section Langerhans cells were challenged with different MAMPs at a uniform dosage of 1000pg. The IL-6 response to these MAMP challenges were measured with help of a flow-cytometry based bead assay.

The results of the assay are plotted in Figure 12 in which the IL-6 levels in picograms are plotted against Langerhans stimulated with different MAMPs. The results (Figure 12) show that Langerhans cells constitutively express low levels of IL-6. When these Langerhans cells are challenged with 1000pg of different MAMPs, they secrete elevated amounts of IL-6. All four MAMPs secrete approximately similar amounts of IL-6. IL-6 was secreted almost 40000 folds more than the constitutive levels secreted by Langerhans cell upon challenge with the four different MAMPs.

IL-10

Figure III-13: IL-10 secretion of Langerhans cells in response to 1000pg of different MAMPs:



∗–Statistical significance with a P value of ≤ 0.05 as compared to untreated LCs

ϕ – Statistical significance with a P value of ≤ 0.05 as compared to untreated LCs; φ – Statistical significance with a P value of ≤ 0.05 as compared to untreated LCs;

As described in the experimental design section Langerhans cells were challenged with different MAMPs at a uniform dosage of 1000pg. The IL-10 response to these MAMP challenges were measured with help of a flow-cytometry based bead assay.

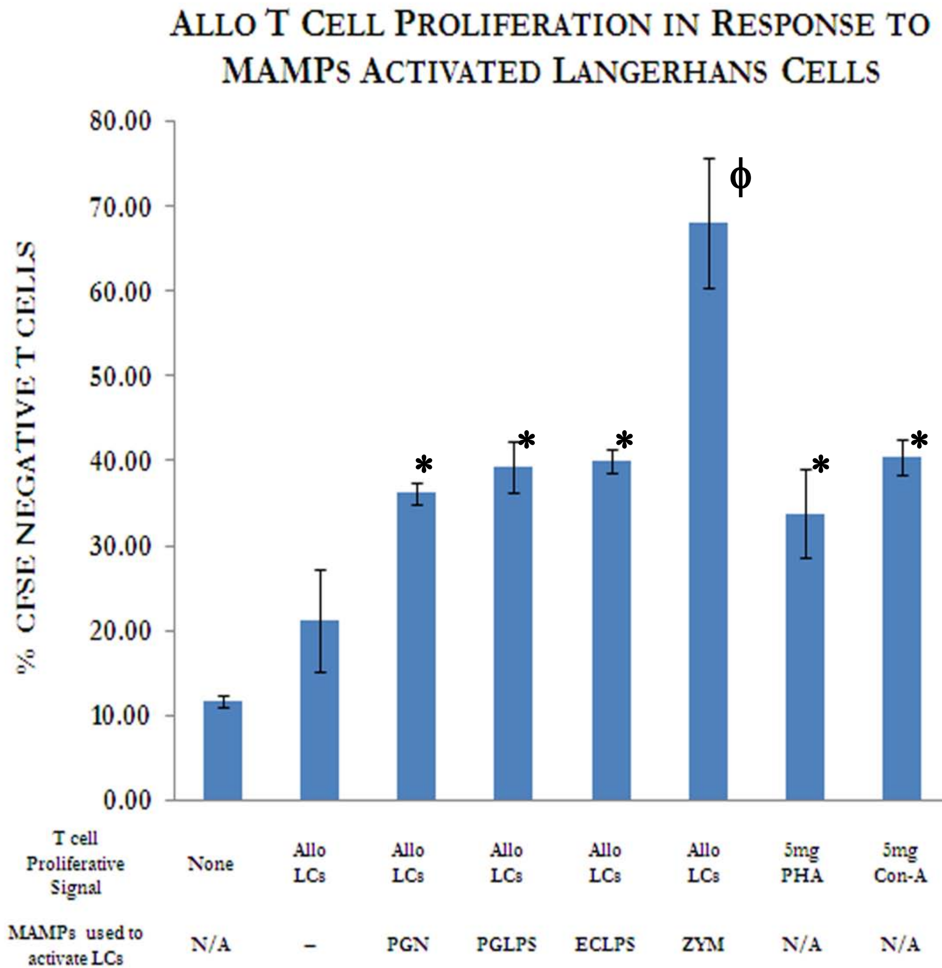
The results of the assay are plotted in Figure 13 in which the IL-10 levels in picograms are plotted against Langerhans stimulated with different MAMPs. The results (Figure 13) show that Langerhans cells constitutively express low levels of IL-10.

When these Langerhans cells are challenged with 1000pg of different MAMPs, they secrete elevated amounts of IL-10. ECLPS and Zymosan challenge elicits the highest amounts of IL-10 production from these Langerhans cells.
PGLPS and PGN produce similar amounts of IL-10 and produce almost 200 fold more than the constitutive expression. ECLPS and Zymosan produce approximately 2 - 3 fold IL-10 more the PGLPS and PGN.

Induction of T cell response:

Allo T cell response

Figure III-14: Allo T cell response of Different MAMP matured Langerhans cells:



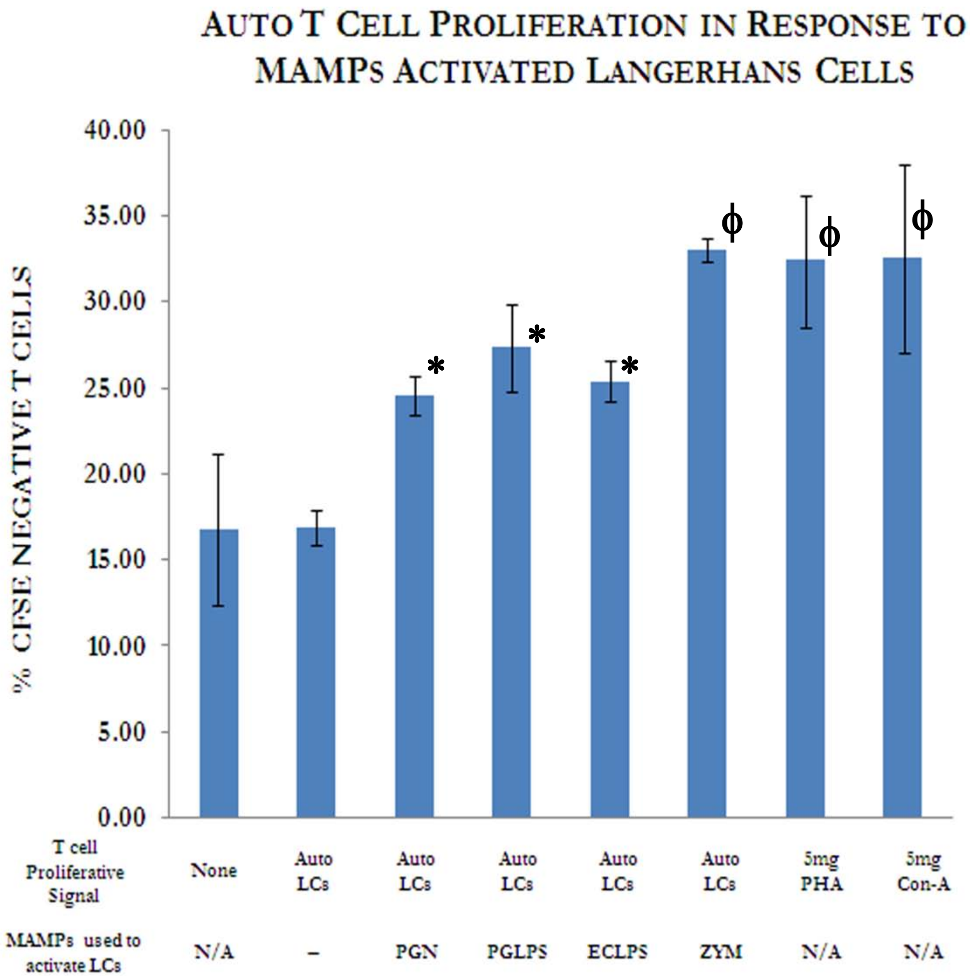
∗–Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls; ϕ – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls;

As described in the experimental design section, Langerhans cells were activated with MAMPs and then co-cultured with Allo-T cells stained with CFSE. The proliferating cells lose the CFSE stain due to division of the cytoplasm among the daughter cells. Thus the percentage of CFSE negative T cells would be directly indicative of the amount of T cell proliferation induced by the Langerhans cell. Figure 14 shows the increase in percentage of CFSE negative Allo T cells after co-culturing these cells with the differentially activated Langerhans cells. The X axis displays the MAMPs used to activate the Langerhans cells which were then co-cultured with T cells. 5μg PHA and 5μg concavalin A was used as positive controls for proliferation. T cells alone or T cells with untreated Langerhans cells were used as negative controls.

The results show that Zymosan activated Langerhans cells produces a profound allo-T cell proliferative response. The other MAMPs activated Langerhans cells produce a modest allo-T cell proliferative response which is comparable to the concavalin A and PHA treated T Cells.

Auto T cell response

Figure III-15: Auto T cell response of Different MAMP matured Langerhans cells.



∗–Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls; ϕ – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls;

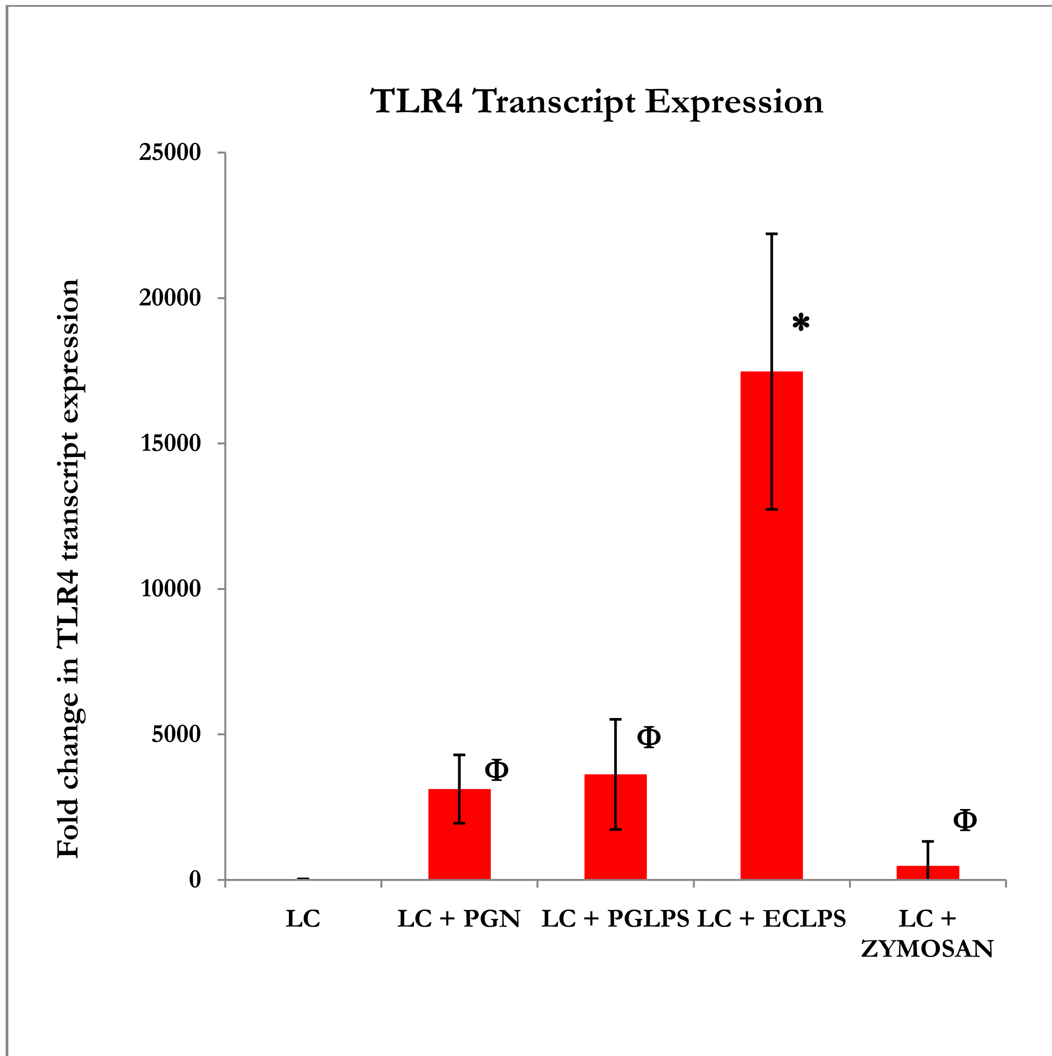
As described in the experimental design section, Langerhans cells were activated with MAMPs and then co-cultured with Auto-T cells stained with CFSE. The proliferating cells lose the CFSE stain due to division of the cytoplasm among the daughter cells. Thus the percentage of CFSE negative T cells would be directly indicative of the amount of T cell proliferation induced by the Langerhans cell. Figure 15 shows the increase in percentage of CFSE negative Auto T cells after co-culturing these cells with the differentially activated Langerhans cells. The X axis displays the MAMPs used to activate the Langerhans cells which were then co-cultured with T cells. 5μg PHA and 5μg concavalin A was used as positive controls for proliferation. T cells alone or T cells with untreated Langerhans cells were used as negative controls.

The results show that MAMPs activated Langerhans cells produce a modest auto-T cell proliferative response which is similar irrespective of the MAMP activation. Zymosan appears to induce proliferation of the T cells more than the other MAMPs and is comparable to the concavalin A and PHA treated T Cells.

Pattern Recognition Receptor regulation:

TLR4:

Figure III-16: Regulation of TLR4 in MAMPs challenged LCs.

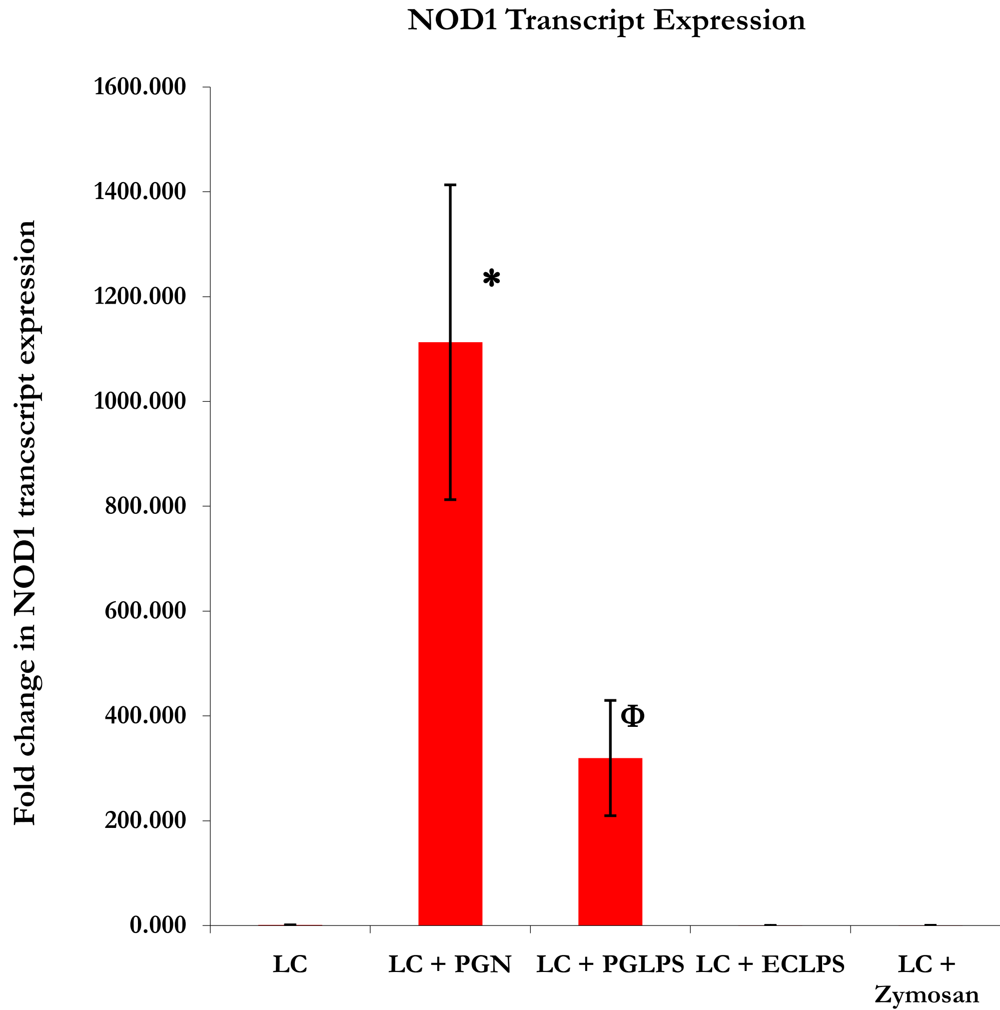


∗–Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls; ϕ – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls;

The expression of TLR-4 on LCs in response to different MAMPs as determined by real time PCR is shown in figure 16. LC expresses constitutively low levels of transcripts for TLR-4. When challenged with 1000pg of PGN LCs up-regulate transcripts for TLR-4 in a significant manner (300 fold). PGLPS (1000pg) challenged LCs express approximately 300 fold more TLR4 transcripts as compared to unstimulated LCs. 1000pg challenge of ECLPS induces a robust up regulation of TLR-4 transcripts in LCs (1000 fold change). 1000pg of Zymosan challenged again induces a modest up regulation of TLR-4 transcripts in the LCs (30 fold).

NOD1:

Figure III-17: Regulation of NOD1 in MAMPs challenged LCs.

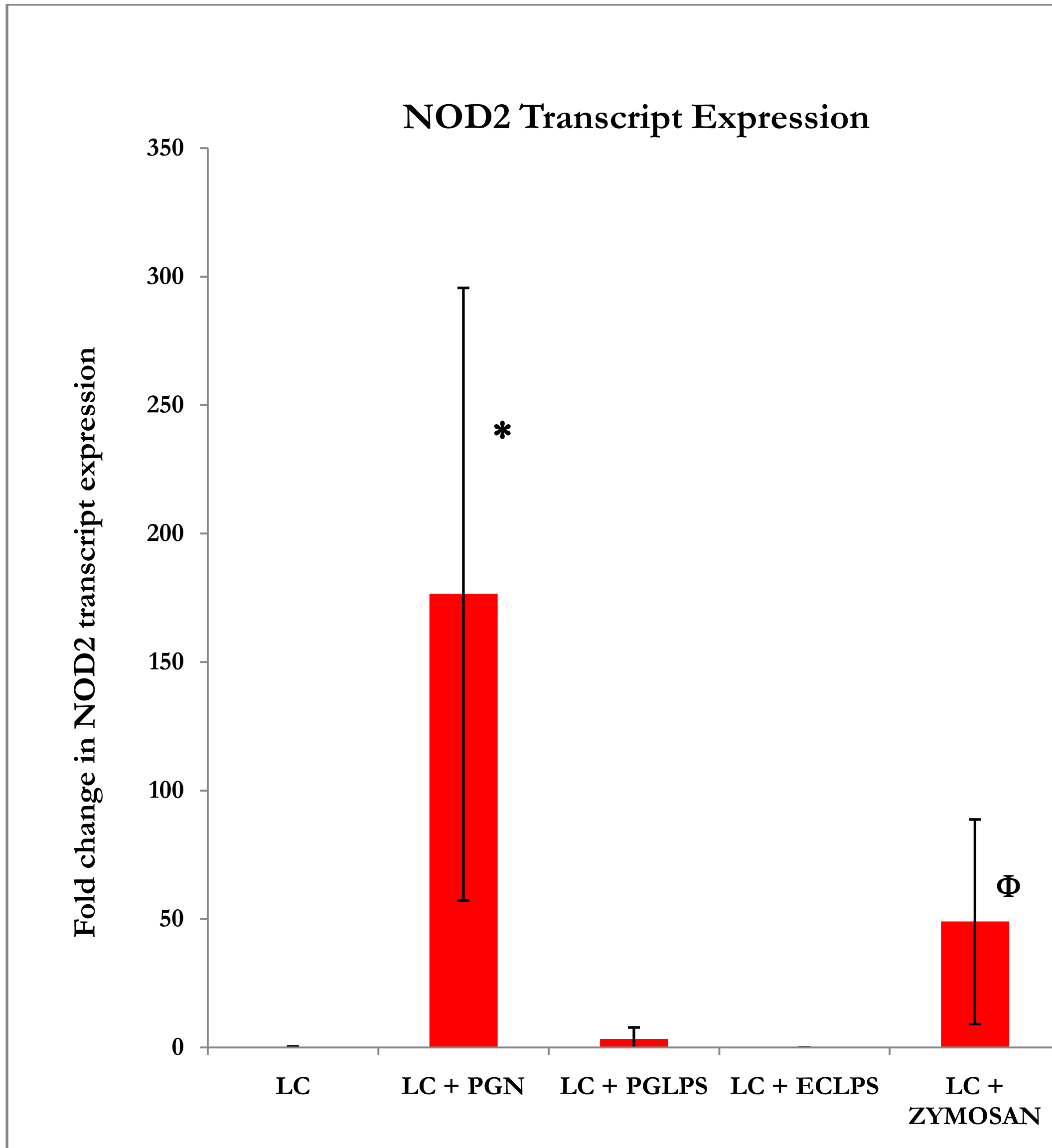


∗–Statistical significance with a P value of ≤ 0.05 as compared to untreated controls; ϕ – Statistical significance with a P value of ≤ 0.05 as compared to untreated controls;

The expression of NOD1 on LCs in response to different MAMPs as determined by real time PCR is shown in figure 17. LC expresses very low constitutive levels of transcripts for NOD1. When challenged with 1000pg of PGN LCs up-regulate transcripts for NOD1 in a robust manner (1100 fold). PGLPS (1000pg) challenged LCs express approximately 300 fold more NOD1 transcripts as compared to unstimulated LCs. NOD1 transcripts were undetectable in LCs challenged with 1000pg ECLPS, as well as in LCs challenged with 1000pg of Zymosan.

NOD2:

Figure III-18: Regulation of NOD2 in MAMPs challenged LCs.



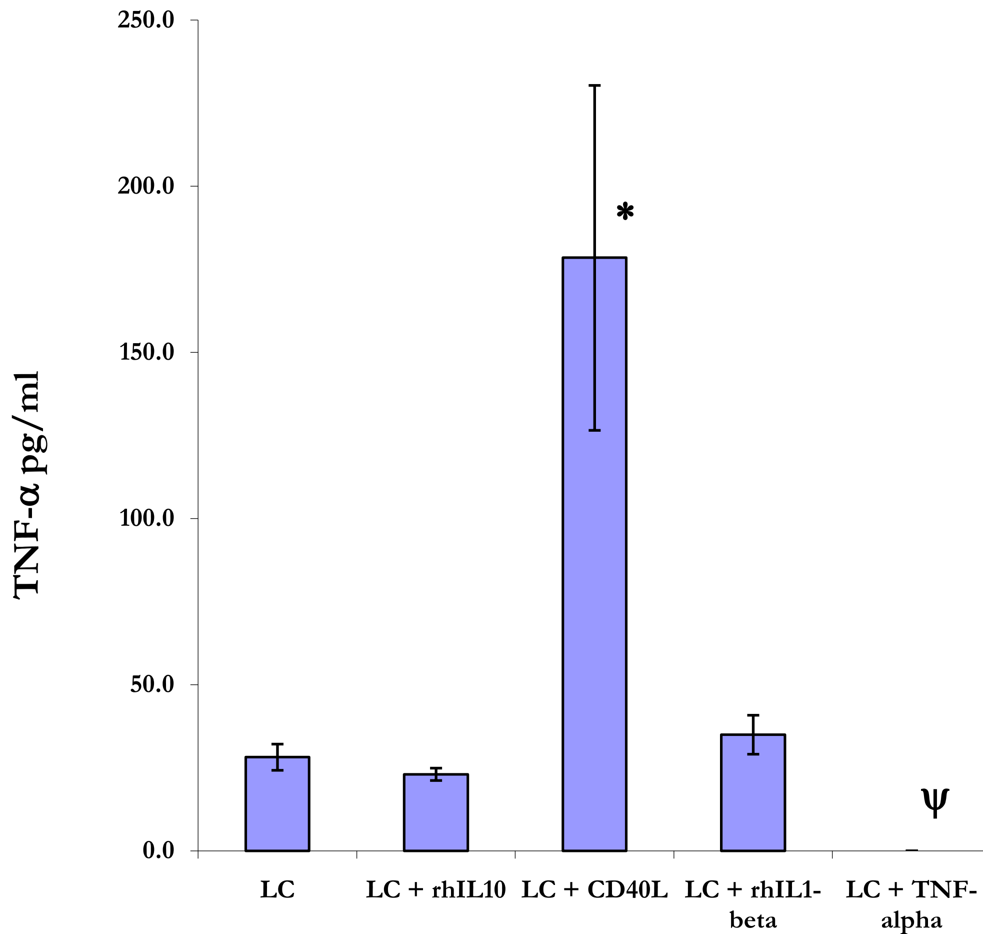
$*$−Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls; ϕ − Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls;

The expression of NOD2 on LCs in response to different MAMPs as determined by real time PCR is shown in figure 18. LC expresses very low constitutive levels of transcripts for NOD2. When challenged with 1000pg of PGN LCs up-regulate transcripts for NOD2 in a robust manner (1000 fold). There is modest but statistically insignificant up regulation of NOD2 transcripts in PGLPS challenged LCs (17 fold as compared to unstimulated LCs). NOD2 transcripts were undetectable in LCs challenged with 1000pg ECLPS. On the other hand Zymosan challenged LCs up regulate NOD2 in a statistically significant manner (250 fold as compared to unstimulated LCs).

Effect of conditioning on the cytokine response of LC:

To determine the modulation of LC immune response by the chosen conditioning agents described in the next chapter LCs were treated with different conditioning agents. The concentrations and protocol for conditioning agents are described in the materials and methods chapter. The following sections described the results of the modulation of cytokine response when LCs are conditioned. The modulation of co-stimulatory molecules and activation markers are described in the next chapter. Cytokine analysis was done with help of flow cytometry based cytometric bead assay also described in the materials and methods section. The cytokines analyzed were TNF-α, IL-10, IL-6, and IL1β and are depicted in figures 19-22

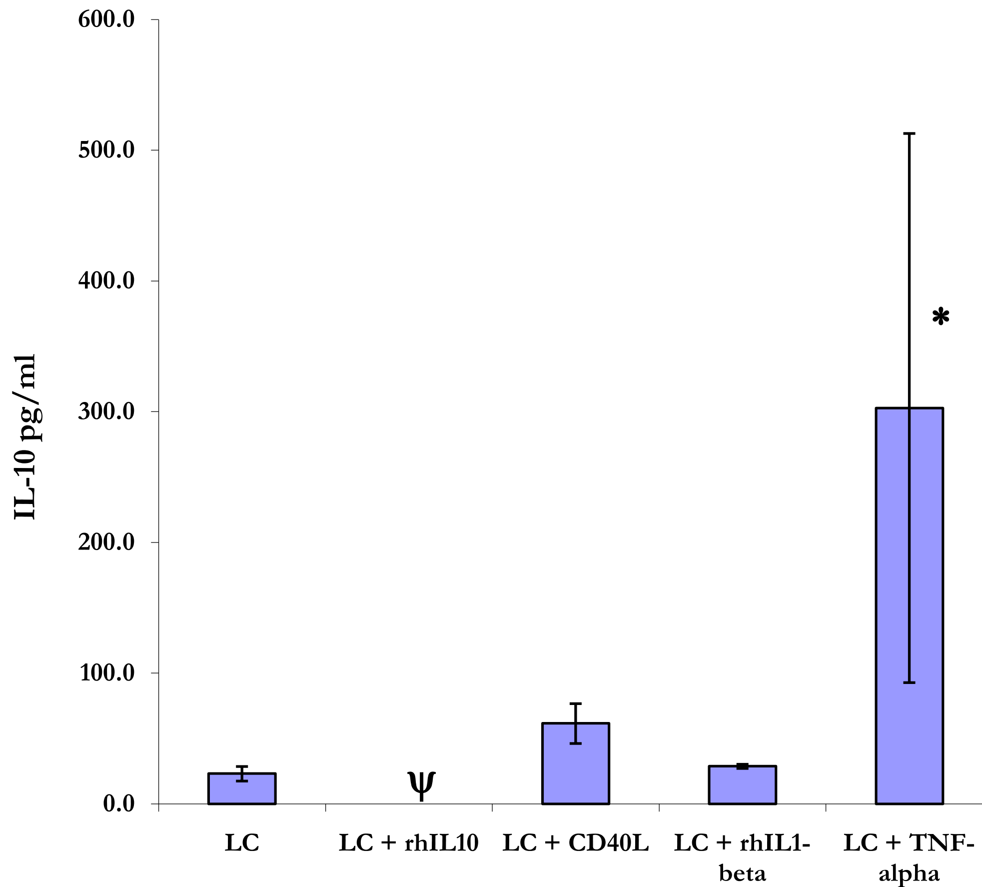
Figure III-19: Effect of conditioning on TNF-α Secretion from LC



*–Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls; ψ – exogenously added TNF-α;

LCs secrete constitutively low levels of TNFα. When conditioned with rhIL-10 this expression becomes slightly reduced. CD40L induces a 10 fold increase in TNFα secretion. LCs conditioned with rhIL1β induces very slight up regulation of TNFα secretion (1.5 fold).

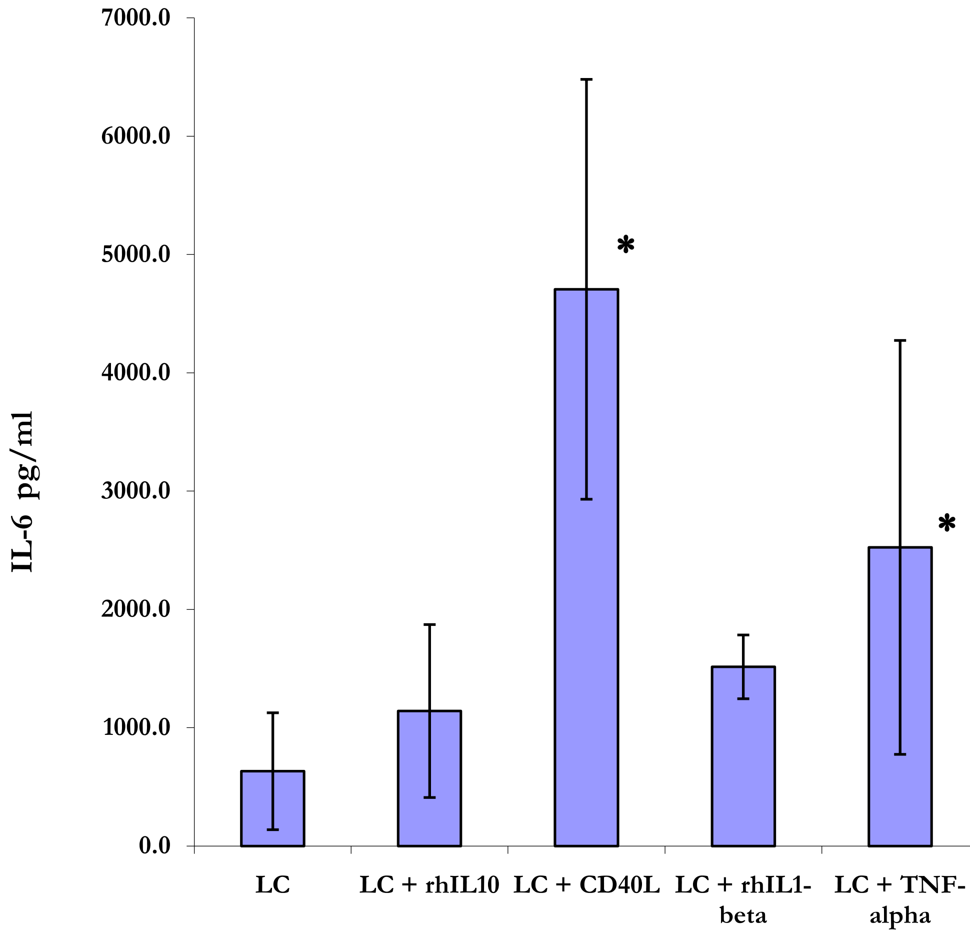
Figure III-20: Effect of conditioning on IL-10 secretion from LC



∗−Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls; ψ− exogenously added rhIL-10

LCs secrete constitutively low levels of IL-10. CD40L induces a 3 fold increase in IL-10 secretion. LCs conditioned with rhIL1β doesn't increase IL-10 secretion. TNFα increases IL-10 secretion in LCs by 15 fold.

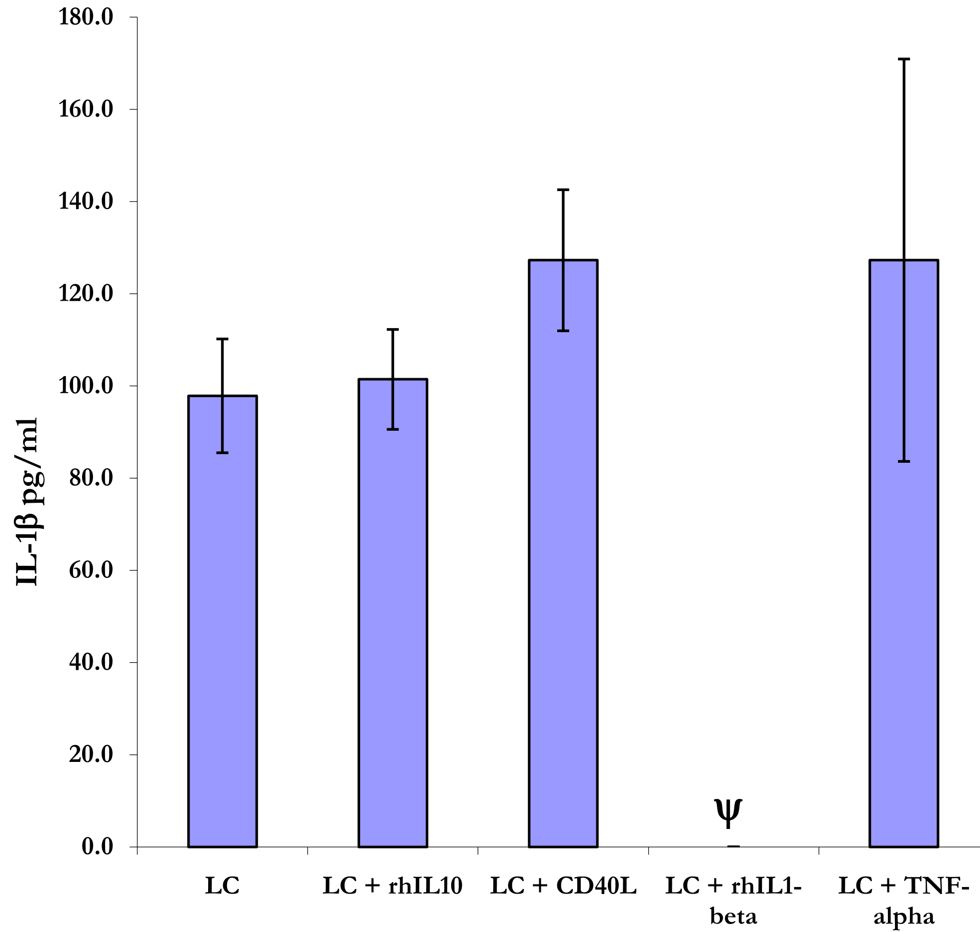
Figure III-21: Effect of conditioning on IL-6 secretion from LC



*–Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls

LCs secrete constitutively low levels of IL-6. When conditioned with rhIL-10 LC secrete 2 fold more IL-6. CD40L induces an 8 fold increase in IL-6 secretion. LCs conditioned with rhIL1β induces 2.5 fold up regulation of IL-6 secretion. TNFα induces a 4 fold up regulation if IL-6.

Figure III-22: Effect of conditioning on IL-1β secretion from LC



ψ- exogenously added rhIL-1β.

LCs secrete constitutively low levels of IL1β. None of the four conditioning agents changed IL1β secretion in LCs.

Summary of Results I:

Surface markers of LC activation:

The results indicate that Langerhans cells respond in a dose dependent manner to all the MAMPS by up regulation of CD83 (Figures 2 &3), CD80 (Figures 4 &5) and CD86 (Figures 6 & 7). However, HLA-DR, which was constitutively expressed to a high level on resting LCs, was not up regulated by PGLPS, ECLPS or zymosan at 1000 pg (Figure 8), but was increased at10,000 pg over constitutive level (Figure 9).

Cytokine secretion

The cytokine responses of LC stimulated with different MAMPs are summarized below:

1. LC stimulated with ECLPS or Zymosan secreted approximately 4 fold more IL-1β as compared to LC stimulated with PGLPS or PGN (Figure 10).

2. Zymosan induced a profound TNF-α secretory response when used to stimulate the LCs. Zymosan induced an almost 4 fold increase in LC secreted TNF-α , while ECLPS induced a 2 fold increase in TNF−α as compared to LC stimulated with either PGLPS or PGN (Figure 11).

3. IL-6 secretion increased dramatically from constitutive levels after stimulation with four different MAMPs. The increase in levels of IL-6 was approximately the same irrespective of the MAMP used (Figure 12).

4. LC stimulated with Zymosan secreted approximately 4 fold more IL-10 as compared to LC stimulated with PGLPS or PGN. LC stimulated with ECLPS on the other hand secreted approximately 3 fold more IL-10 as compared to LC stimulated with PGLPS and PGN (Figure 13).

Allo T cell Proliferation:

Our results indicate that LC activated by PGN or ECLPS or PGLPS induce modest allo T cell proliferation. Allo T cell proliferation by these MAMP activated LC is similar to Concavalin A or PHA mediated T cell proliferation as

shown in figure 14. Zymosan activated LC induces a strong Allo T cell proliferative response even higher than PHA or Concavalin A (figure 14).

Auto T cell Proliferation:

In terms of auto T cell proliferative ability of LC activated by MAMPS out results show that LC activated by PGN or ECLPS or PGLPS induce modest auto T cell proliferation. Auto T cell proliferation by these MAMP activated LC is less than Concavalin A or PHA mediated T cell proliferation as shown in figure 15. Zymosan activated LC induces a strong Auto T cell proliferative response similar to T Cell proliferation induced by PHA or Concavalin A (figure 15).

Summary of PRR Regulation:

1) ECLPS stimulation induces a profound up regulation of TLR4 message in LCs almost 6 times more than PGLPS or PGN stimulated LC and almost 1000 fold more constitutive expression. Zymosan induced only a modest increase in TLR4 message levels as shown in figure 16.

2) Only PGN and PGLPS challenged LC increase NOD1 message significantly with PGN inducing a fold increase as compared to PGLPS as seen in figure 17.

3) PGN increases NOD2 expression a 1000 fold more than constitutive in LC followed by zymosan (250 fold) and PGLPS (17 fold) as seen in figure 18.

Summary of effect of conditioning on LC cytokine secretion:

1) CD40L conditioning induces the highest amount of TNF-α, IL-6 as compared to other conditioning agents (figure 19-22).

2) TNF-α conditioning induces a high IL-6 response and very high IL-10 response (figure 19-22).

3) IL-1β conditioning fails to induce a significant cytokine response in LC as shown in figure 19-22.

4) None of the conditioning agent up regulated IL-1β in LC except for CD40L (figure 19-22).

Chapter IV

Aim - II

Effect of IL-10 conditioning on immune responses of Langerhans cells to Oral MAMPS

Introduction:

Background & rationale:

The results for AIM I indicated that the CD34$^+$ derived Langerin$^+$ LC express a unique repertoire of pattern recognition receptors (PRRs)and these receptors determine the level of response to microbial ligands, (MAMPs). The CD34$^+$ derived Langerin$^+$ LC respond to the different MAMPs robustly albeit with qualitative differences especially in the amounts of cytokines they secrete. The LCs become activated and primed for antigen presentation. These activated LCs are also capable of causing T cell proliferation. As discussed in the introduction section the inhibition of co-stimulatory molecules in an antigen presenting cell leads to anergy [68]. Furthermore it has also been shown that microbial activation of TLR pathway leads to breaking of tolerance [25]. Taking all this together we wanted to explore the possibility of inhibiting the activation of Langerhans cells with help of IL-10. Towards this the following objective was formulated and pursued.

Aim II:

To establish the ability to modulate innate response and immuno-stimulatory functions of human LCs in vitro through IL-10 conditioning

Specific Hypotheses:

To explore the stated aim we formulated the following hypotheses.

Hypothesis 1:

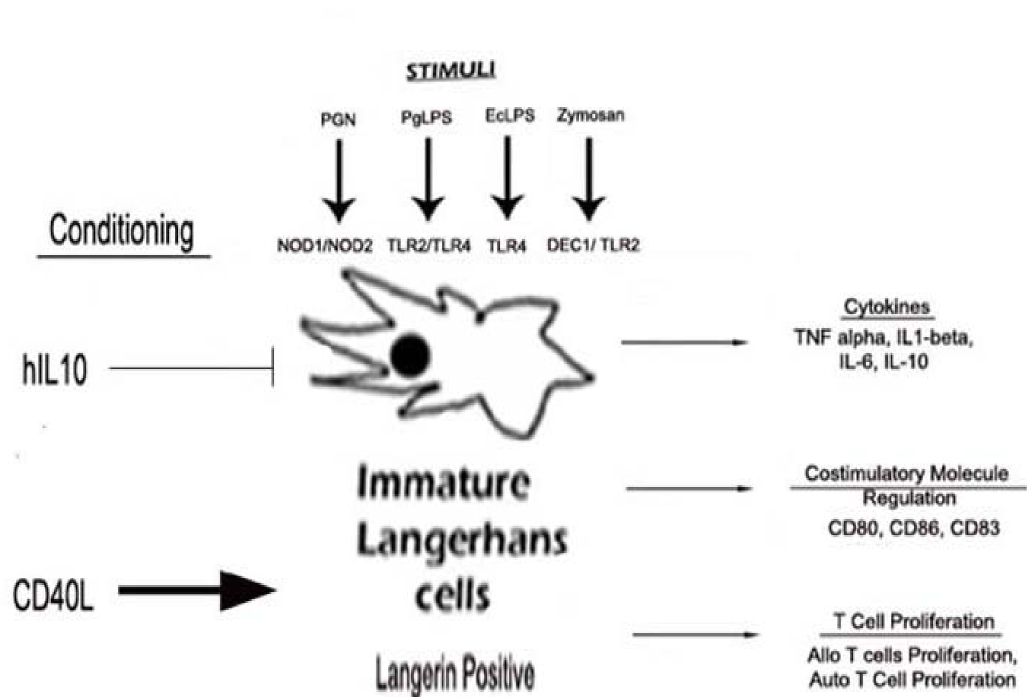
The innate immune function of $CD34^{+}$ derived $Langerin^{+}$ LCs against MAMPs can be modulated by conditioning the LCs through a conditioning cytokine micro-environment.

Hypothesis 2:

The immuno-stimulatory capacity of MAMPs activated $CD34^{+}$ derived $Langerin^{+}$ LCs towards CD4+ T cells can be modulated by conditioning the LCs in a conditioning cytokine micro-environment.

Experimental Design

Figure IV-1: Experimental Design for AIM II



Conditioning Protocol:

Isolated immature Langerhans cells are conditioned with either 50ng/µl of recombinant human IL-10 (rhIL-10) or 1µg/µl of recombinant human CD40L (rhCD40L). The Langerhans cells are incubated with either of the cytokines for 24 hrs in a 5% CO_2 incubator at 37^0C. These cells were then stimulated with one of the four different MAMPs as described below.

Challenge Protocol:

The conditioned Langerhans cells were the further challenged with 1000pg of one of the four types of MAMPs i.e., the cells were incubated with these MAMPS for 24 hours at 37^0C in a 5% CO_2 incubator as seen in chapter III.

Experimental Readouts:

The modulation of innate immune response and the immunostimulatory capacity of Langerhans cells activated/matured by four different MAMPs by conditioning them with two different cytokines, i.e., were evaluated as follows:

Co-stimulatory molecules:

We hypothesized that conditioning of Langerhans cells will change the response of the Langerhans cells to stimulation by MAMPs. Thus we expected that the Langerhans cells will become unresponsive to MAMPs in the presence of rhIL-10. In contrast in the presence of CD40L we expected the response to MAMPs will be enhanced at the least in an additive manner. This down-modulation or up-regulation of activation / maturation of Langerhans cells can be

assessed by measuring the regulation of co-stimulatory molecules expressed on the surface of Langerhans cells. We decided to use flow-cytometry based analysis and quantification of the change in expression of CD83, CD86, and CD80 to assess the response of Langerhans cells. Thus, the cells were incubated with MAMPs for 24 hours after conditioning with the cytokines and were then spun down, stained with fluorescence conjugated antibodies, and then fixed.

Appropriate negative controls were included. These cells were then analyzed with help of flow-cytometry as described in the materials and methods chapter.

Cytokines:

It is well known that activated Langerhans cells secrete cytokines and the measure of these cytokines not only enables us to quantitatively assess the Langerhans cell response but also sheds some light on the qualitative aspect of the Langerhans cell response. rhIL-10 conditioned Langerhans cells are expected to be incapable or have reduced inflammatory cytokine response to MAMPs. On the other hand CD40L conditioned Langerhans cells are expected to show either enhanced inflammatory cytokine response or expression of a different set of cytokines.

Towards this we decided to assess the change in levels of the following cytokines secreted by LCs in response to MAMPs challenge, namely TNFα, IL-1β, IL-6, and IL-10 with the help of a flow-cytometry based cytokine assay.

Towards this the supernatants of 24 hour conditioned and subsequently MAMPs stimulated Langerhans cells culture supernatants were pipetted out of the tubes containing the centrifuged cells and were stored under -75^{0}C until further analysis. The supernatants are then quickly thawed and stained with help of cytokine assay kit and then analyzed through flow-cytometry as described in the materials and methods chapter.

T cell Proliferation:

The immunostimulatory capacity of an activated antigen presenting cells like the dendritic cells or Langerhans cells have traditionally been studied with the help of T cell proliferation assays. We expected the rhIL-10 conditioned, MAMPs stimulated Langerhans cells to be less immunostimulatory than either MAMPs activated Langerhans cells or CD40L conditioned MAMPs activated Langerhans cells.

We decided to use a CFSE based T cell proliferation assay to evaluate this. The assay has been described in detail in the materials and methods chapter. The Langerhans cells were first conditioned with rhIL-10 or rhCD40L for 24 hours and then subsequently stimulated with different MAMPs and then incubated for another 24 hours. Following which each of the thus stimulated Langerhans cells

were spun down and co-cultured with the T cells as described previously for 4-6 days before being analyzed with the help of flow-cytometry.

Results:

Co-stimulatory Molecules:

CD83:

Modulation of CD83 expression on Langerhans cells through cytokine conditioning:

Figure IV-2: Effect of cytokine conditioning on CD83 expression on Langerhans Cells

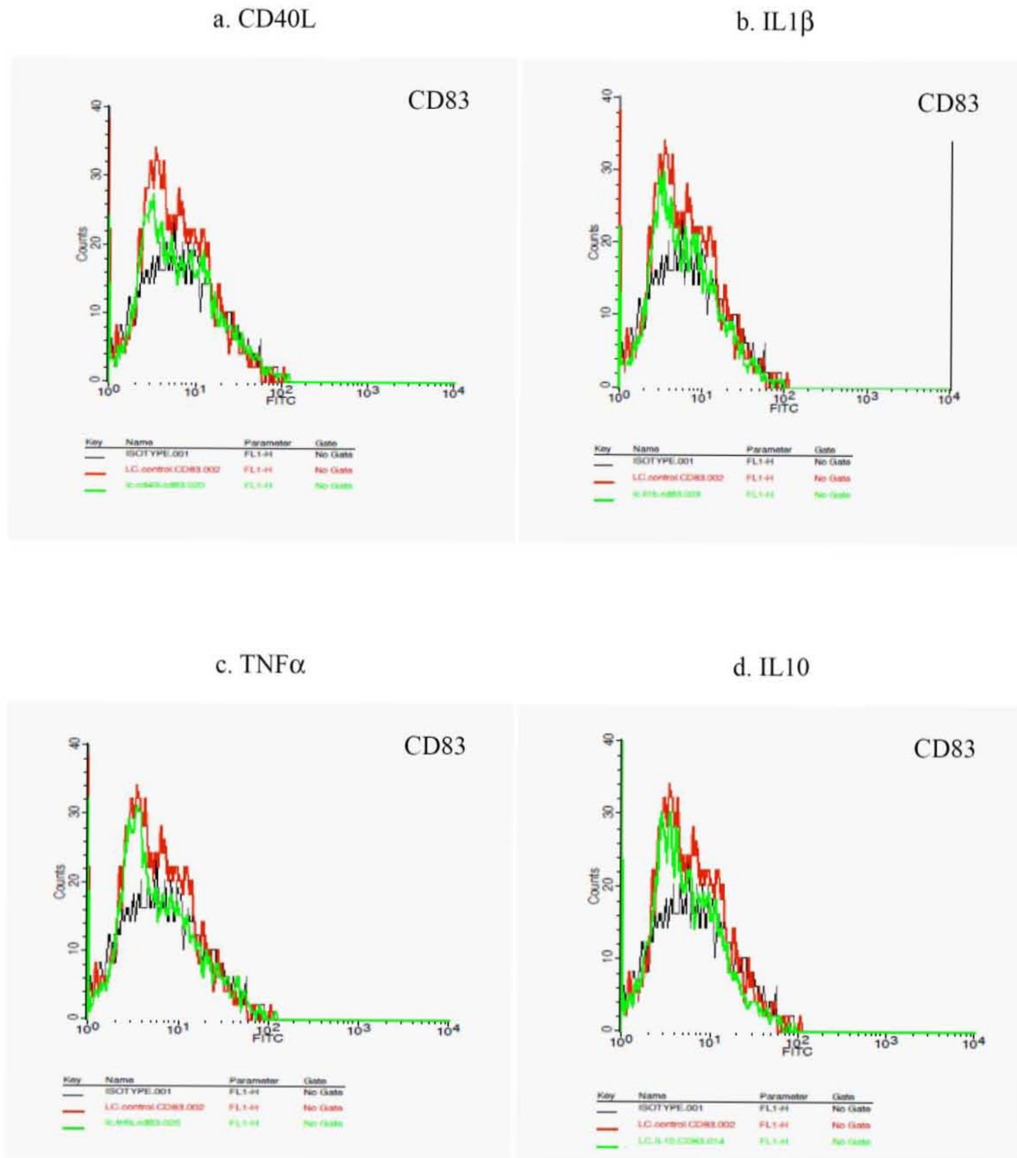


Figure Legend

Isotype

Untreated LC

LC + Conditioning agent

As described in the Experimental design the expression of CD83 on Langerhans cells upon condtioning with four different cytokines, was analyzed with flow cytometry. The results are shown in Figure 2. The statistical significance was determined with help of Kolmogrov-Smirnov statistic.

CD40L:

Langerhans cells conditioned with 1µg/µl of CD40L did not show any increase in the surface expression of CD83 (Figure 2a). The statistical significance was determined with help of Kolmogrov-Smirnov statistic.

IL-1β:

Langerhans cells conditioned with 50ng/µl of IL-1β did not show any increase in the surface expression of CD83 (Figure 2b). The statistical significance was determined with help of Kolmogrov-Smirnov statistic.

TNFα:

Langerhans cells conditioned with 50ng/µl of TNFα did not show any increase in the surface expression of CD83 (Figure 2c). The statistical significance was determined with help of Kolmogrov-Smirnov statistic.

IL-10:

Langerhans cells conditioned with 50ng/µl of IL-10 did not show any increase in the surface expression of CD83 (Figure 2d). The statistical significance was determined with help of Kolmogrov-Smirnov statistic.

Figure IV-3: Effect of rhIL-10-conditioning on CD83 expression on Langerhans cell response to MAMPs

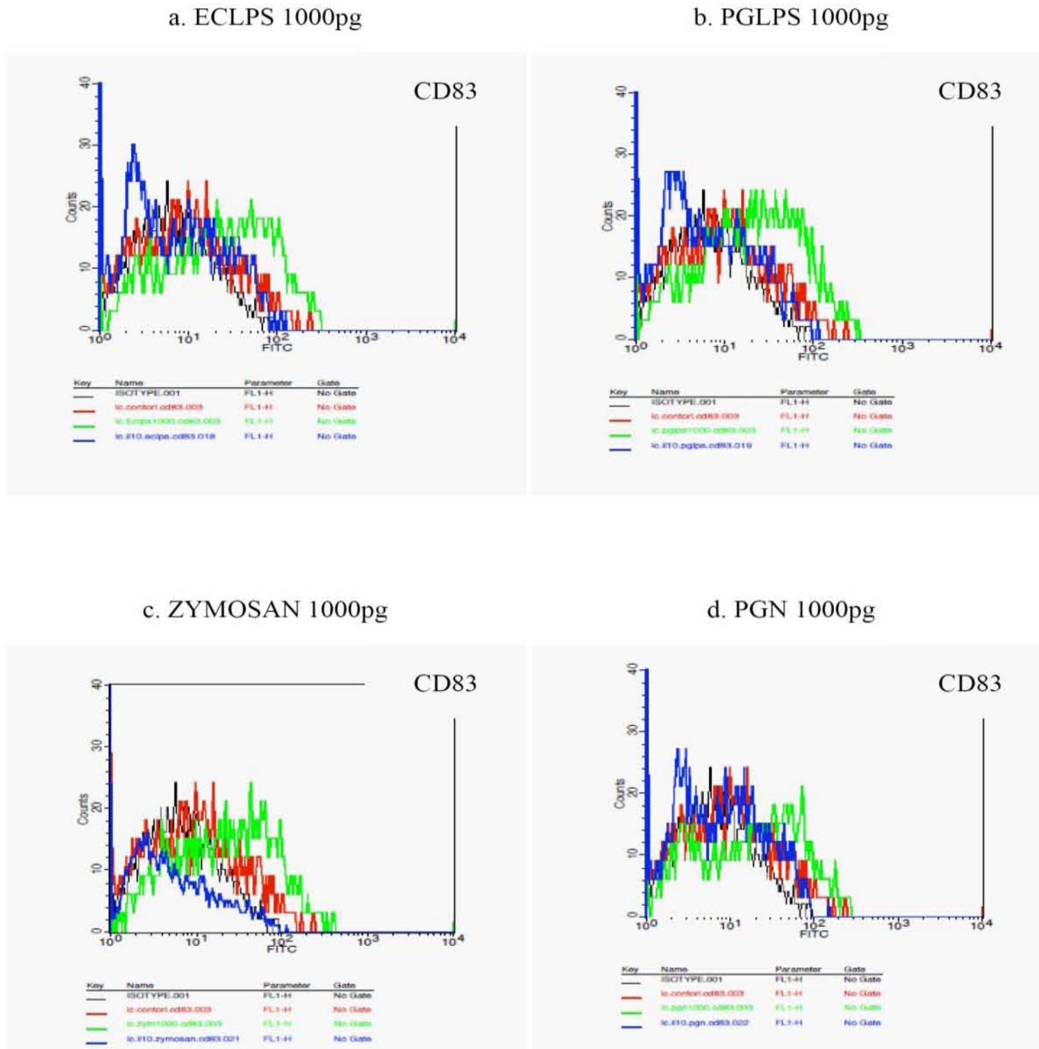


Figure Legend

- Isotype
- Untreated LC
- LC + MAMP
- IL-10 conditioned LC + MAMP

As described in the experimental design the expression of CD83, on rhIL-10 conditioned Langerhans cells, upon stimulation with the four different MAMPs, was analyzed with flow cytometry (Figure 3). CD83 was expressed constitutively in Langerhans cells (Figure 3).

PGLPS 1000pg:

Langerhans cells stimulated with 1000pg dose of PGLPS increased surface expression of CD83 substantially (Figure 3b). This inducible expression was statistically significant. When the Langerhans cells were conditioned with rhIL-10, and subsequently challenged with 1000pg of PGLPS, they failed to up-regulate the surface expression of CD83 (Figure 3b).

ECLPS 1000pg:

Langerhans cells stimulated with 1000pg dose of ECLPS increased surface expression of CD83 substantially (Figure 3a). This inducible expression was statistically significant. When the Langerhans cells were conditioned with rhIL-10, and subsequently challenged with 1000pg of ECLPS, they failed to up-regulate the surface expression of CD83 (Figure 3a).

Zymosan 1000pg:

Langerhans cells stimulated with 1000pg dose of zymosan increased surface expression of CD83 substantially (Figure 3c). This inducible expression was statistically significant. When the Langerhans cells were conditioned with rhIL-

10, and subsequently challenged with 1000pg of zymosan, they failed to up-regulate the surface expression of CD83 (Figure 3c).

Peptidoglycan (PGN) 1000pg:

Langerhans cells stimulated with 1000pg dose of PGN increased surface expression of CD83 in a statistically significant manner (Figure 3d). When the Langerhans cells were conditioned with rhIL-10, and subsequently challenged with 1000pg of PGN, they failed to up-regulate the surface expression of CD83 (Figure 3d).

Figure IV-4: Effect of CD40L conditioning on CD83 expression on Langerhans cell response to MAMPs

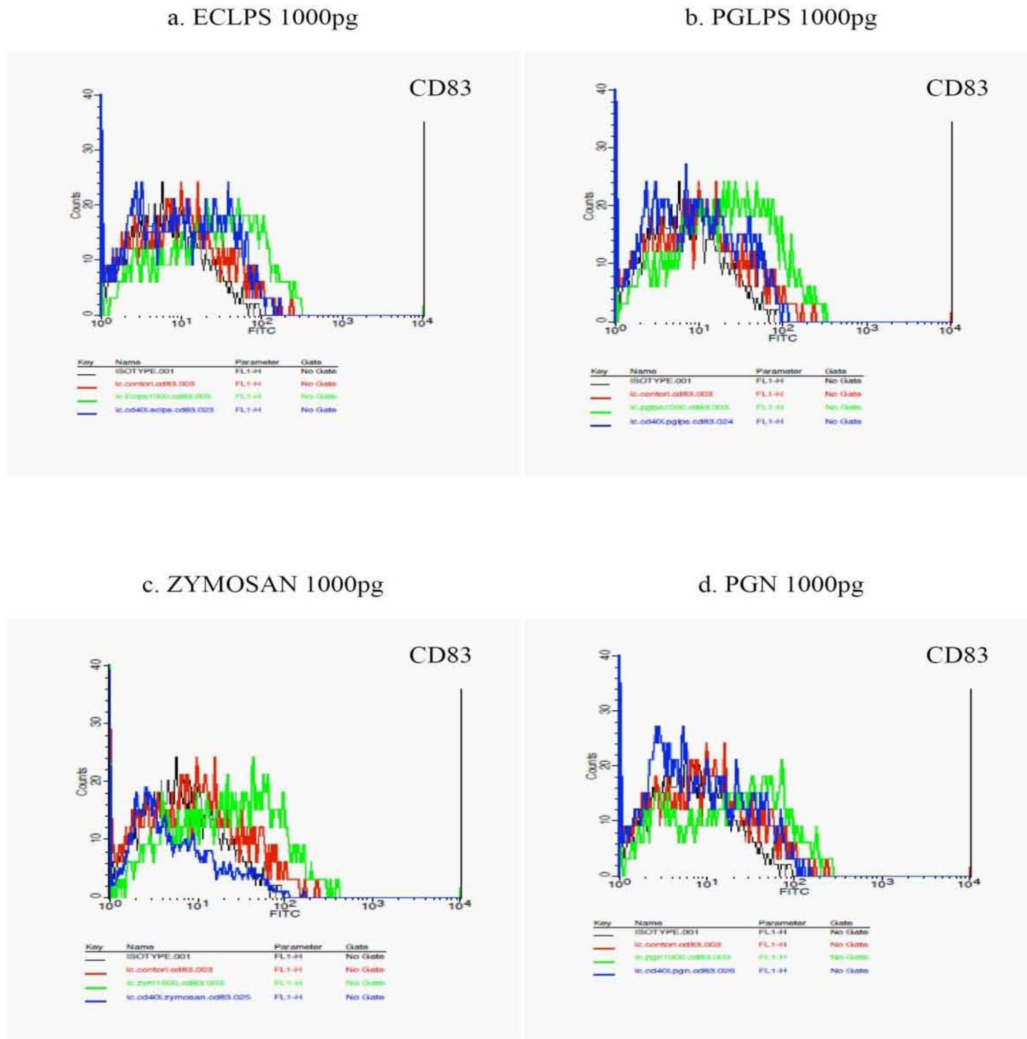


Figure Legend

- Isotype
- Untreated LC
- LC + MAMP
- CD40L conditioned LC + MAMP

As described in the experimental design the expression of CD83, on CD40L treated Langerhans cells, upon stimulation with the four different MAMPs, was analyzed with flow cytometry (Figure 4). CD83 was expressed constitutively in Langerhans cells (Figure 4).

PGLPS 1000pg:

Langerhans cells stimulated with 1000pg dose of PGLPS increased surface expression of CD83 substantially (Figure 4b). This inducible expression was statistically significant. Interestingly, when Langerhans cells were conditioned with CD40L, and subsequently challenged with 1000pg of PGLPS, they failed to up-regulate the surface expression of CD83 (Figure 4b).

ECLPS 1000pg:

Langerhans cells stimulated with 1000pg dose of ECLPS increased surface expression of CD83 substantially (Figure 4a). This inducible expression was statistically significant. Similar to PGLPS, Langerhans cells when conditioned with CD40L, and subsequently challenged with 1000pg of ECLPS, failed to up-regulate the surface expression of CD83 (Figure 4a).

Zymosan 1000pg:

Langerhans cells stimulated with 1000pg dose of zymosan increased surface expression of CD83 substantially (Figure 4c). This inducible expression was statistically significant. Langerhans cells that were conditioned with CD40L,

and subsequently challenged with 1000pg of zymosan, also failed to up-regulate the surface expression of CD83 (Figure 4c).

Peptidoglycan (PGN) 1000pg:

Langerhans cells stimulated with 1000pg dose of PGN increased surface expression of CD83 in a statistically significant manner (Figure 4d). Interestingly, as with the other MAMPS, Langerhans cells that were conditioned with CD40L, and subsequently challenged with 1000pg of PGN, failed to up-regulate the surface expression of CD83 (Figure 4d).

CD86:

Figure IV-5: Effect of cytokine conditioning on CD86 expression on Langerhans Cells

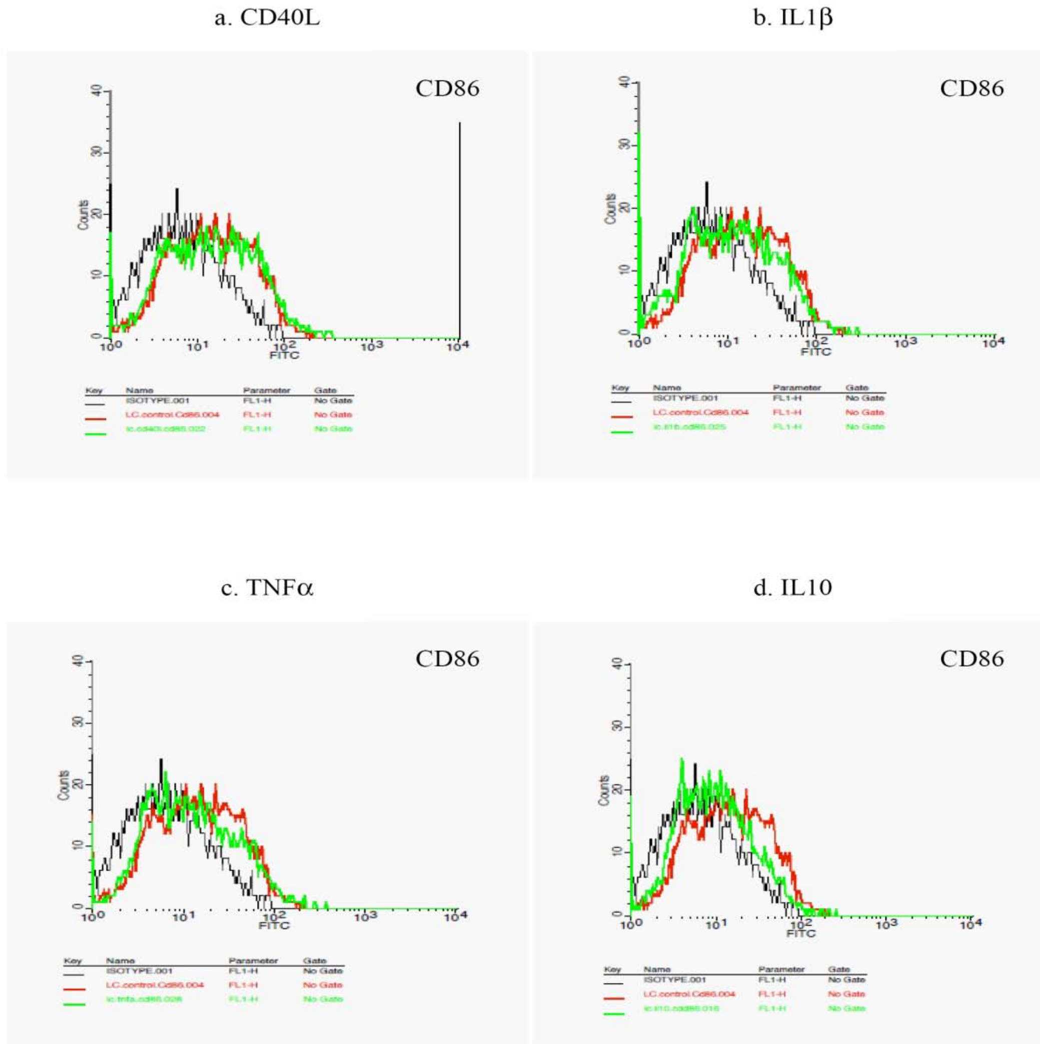


Figure Legend

Isotype

Untreated LC

LC + Conditioning agent

The effect of cytokine conditioning on CD86 expression on Langerhans cells was analyzed with flow cytometry for four different cytokines. The results are shown in Figure 5.

CD40L:

Langerhans cells conditioned with 1µg/µl of CD40L did not show any increase in the surface expression of CD86 (Figure 5a).

IL-1β:

Langerhans cells conditioned with 50ng/µl of IL-1β did not show any increase in the surface expression of CD86 (Figure 5b).

TNFα:

Langerhans cells conditioned with 50ng/µl of TNFα did not show any increase in the surface expression of CD86 (Figure 5c).

IL-10:

Langerhans cells conditioned with 50ng/µl of IL-10 showed as significant decrease in the surface expression of CD86 (Figure 5d).

Figure IV-6: Effect of rhIL-10 conditioning on CD86 expression on Langerhans cell response to MAMPs

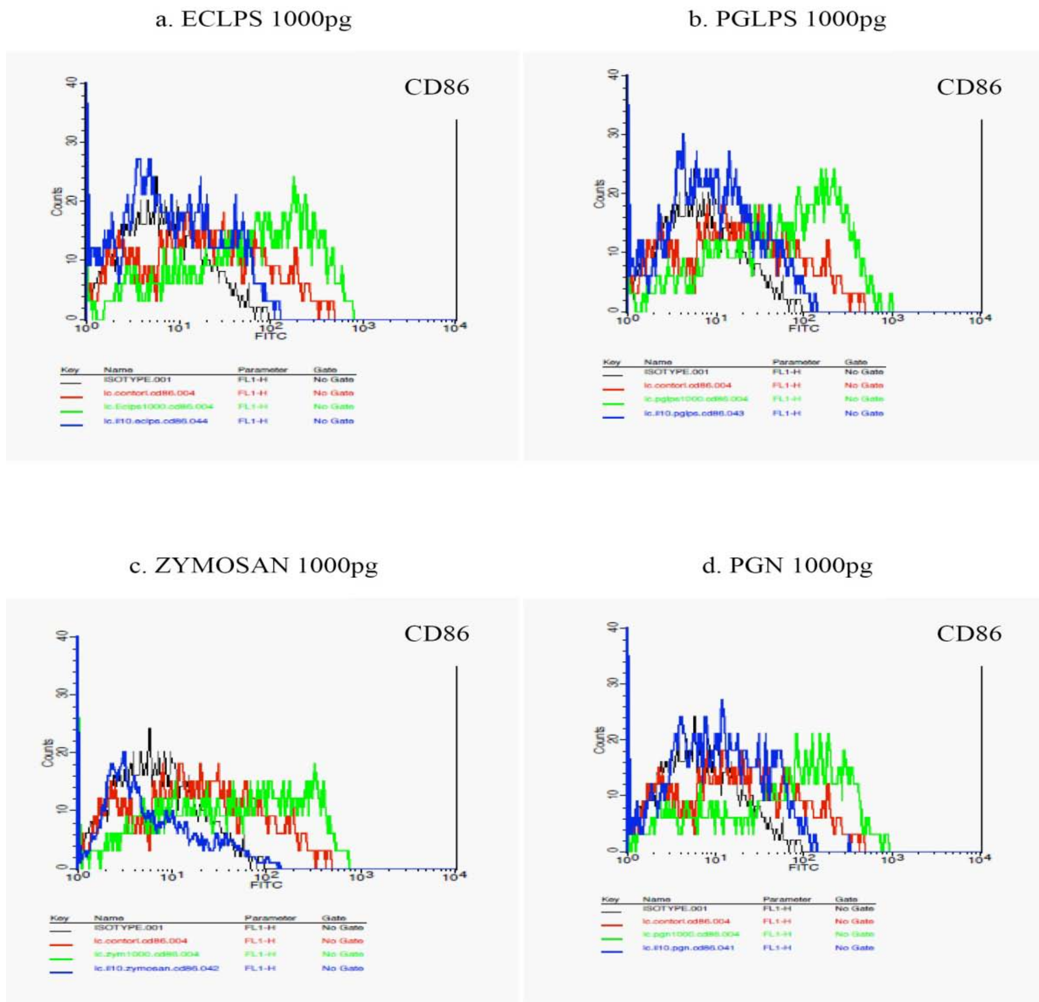


Figure Legend

- Isotype
- Untreated LC
- LC + MAMP
- IL-10 conditioned LC + MAMP

The expression of CD86, on rhIL-10 conditioned Langerhans cells, upon stimulation with the four different MAMPs, was analyzed with flow cytometry (Figure 6). CD86 was found to be expressed constitutively in Langerhans cells (Figure 6).

PGLPS 1000pg:

Langerhans cells stimulated with 1000pg dose of PGLPS increased surface expression of CD86 substantially (Figure 6b). This inducible expression was statistically significant. Langerhans cells that are conditioned with rhIL-10, and subsequently challenged with 1000pg of PGLPS, failed to up-regulate the surface expression of CD86 (Figure 6b).

ECLPS 1000pg:

Langerhans cells stimulated with 1000pg dose of ECLPS increased surface expression of CD86 substantially (Figure 6a). This inducible expression was statistically significant. When the Langerhans cells were conditioned with rhIL-10, and subsequently challenged with 1000pg of ECLPS, they failed to up-regulate the surface expression of CD86 (Figure 6a).

Zymosan 1000pg:

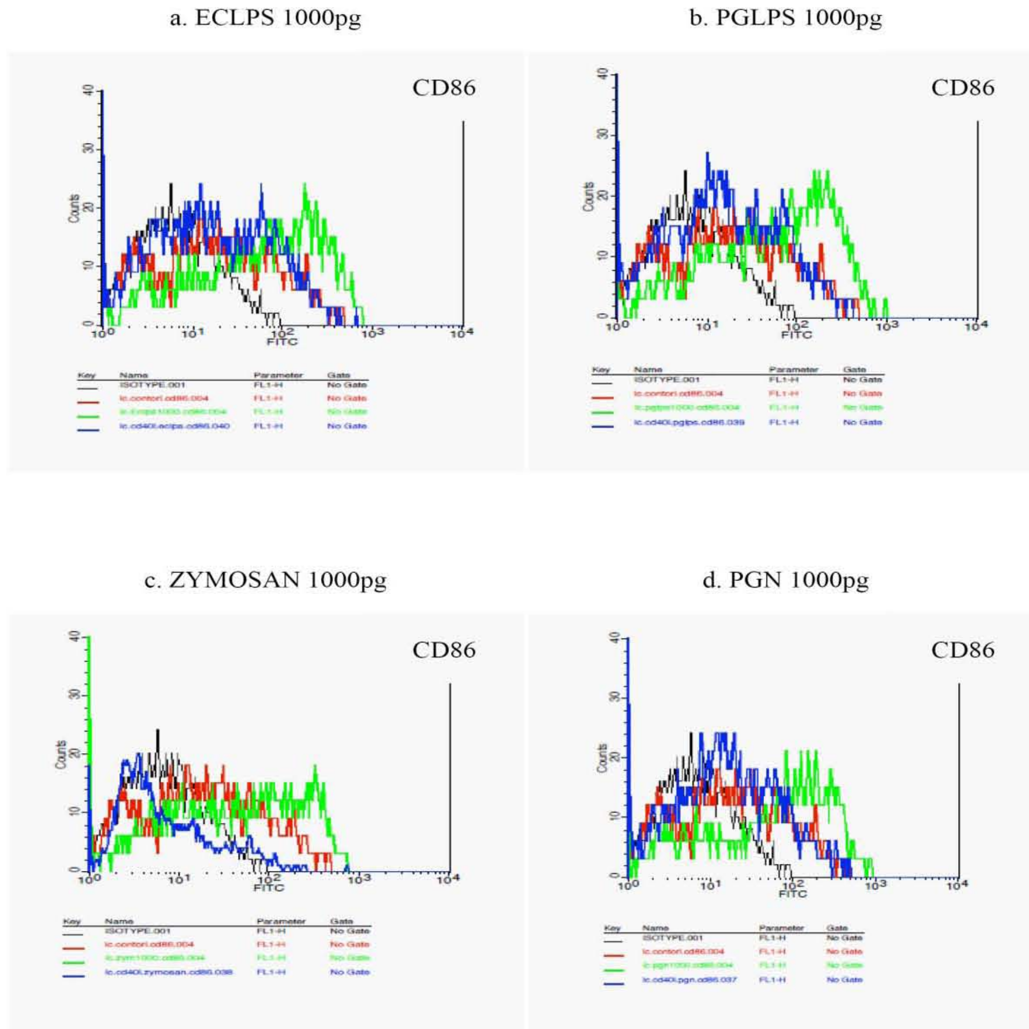
Like PGLPS and ECLPS stimulated LC, Langerhans cells stimulated with 1000pg dose of zymosan increased surface expression of CD86 substantially (Figure 6c). This inducible expression was statistically significant. Furthermore

when the Langerhans cells were conditioned with rhIL-10, and subsequently challenged with 1000pg of zymosan, they also failed to up-regulate the surface expression of CD86 (Figure 6c).

Peptidoglycan (PGN) 1000pg:

Langerhans cells stimulated with 1000pg dose of PGN increased surface expression of CD86 substantially (Figure 6d). This inducible expression was statistically significant. When the Langerhans cells were conditioned with rhIL-10, and subsequently challenged with 1000pg of PGN, they failed to up-regulate the surface expression of CD86 (Figure 6d).

Figure IV-7: Effect of CD40L conditioning on CD86 expression on Langerhans cell response to MAMPs



Expression of CD86, on CD40L treated Langerhans cells, upon stimulation with the four different MAMPs, was analyzed with flow cytometry (Figure 7). CD86 was expressed constitutively in Langerhans cells (Figure 7).

PGLPS 1000pg:

Langerhans cells stimulated with 1000pg dose of PGLPS increased surface expression of CD86 substantially and in a statistically significant manner (Figure 7b). Whereas Langerhans cells were conditioned with CD40L, and subsequently challenged with 1000pg of PGLPS, they failed to up-regulate the surface expression of CD86 (Figure 7b) beyond constitutive levels of expression.

ECLPS 1000pg:

A statistically significant increase in expression of CD86 was found on Langerhans cells stimulated with 1000pg dose of ECLPS (Figure 7a). Interestingly, when these Langerhans cells were conditioned with CD40L, and subsequently challenged with 1000pg of ECLPS, they failed to show similar up-regulation of the surface expression of CD86 (Figure 7a).

Zymosan 1000pg:

Langerhans cells stimulated with 1000pg dose of zymosan increased surface expression of CD86 substantially (Figure 7c). This inducible expression was statistically significant. Interestingly, when the Langerhans cells were conditioned with CD40L, and subsequently challenged with 1000pg of zymosan, they failed to up-regulate the surface expression of CD86 (Figure 7c).

Peptidoglycan (PGN) 1000pg:

Langerhans cells stimulated with 1000pg dose of PGN increased surface expression of CD86 substantially (Figure 7d). This inducible expression was statistically significant. Interestingly, when the Langerhans cells were conditioned with CD40L, and subsequently challenged with 1000pg of PGN, they failed to up-regulate the surface expression of CD86 (Figure 7d).

CD80

Figure IV-8: Effect of cytokine conditioning on CD80 expression on Langerhans Cells

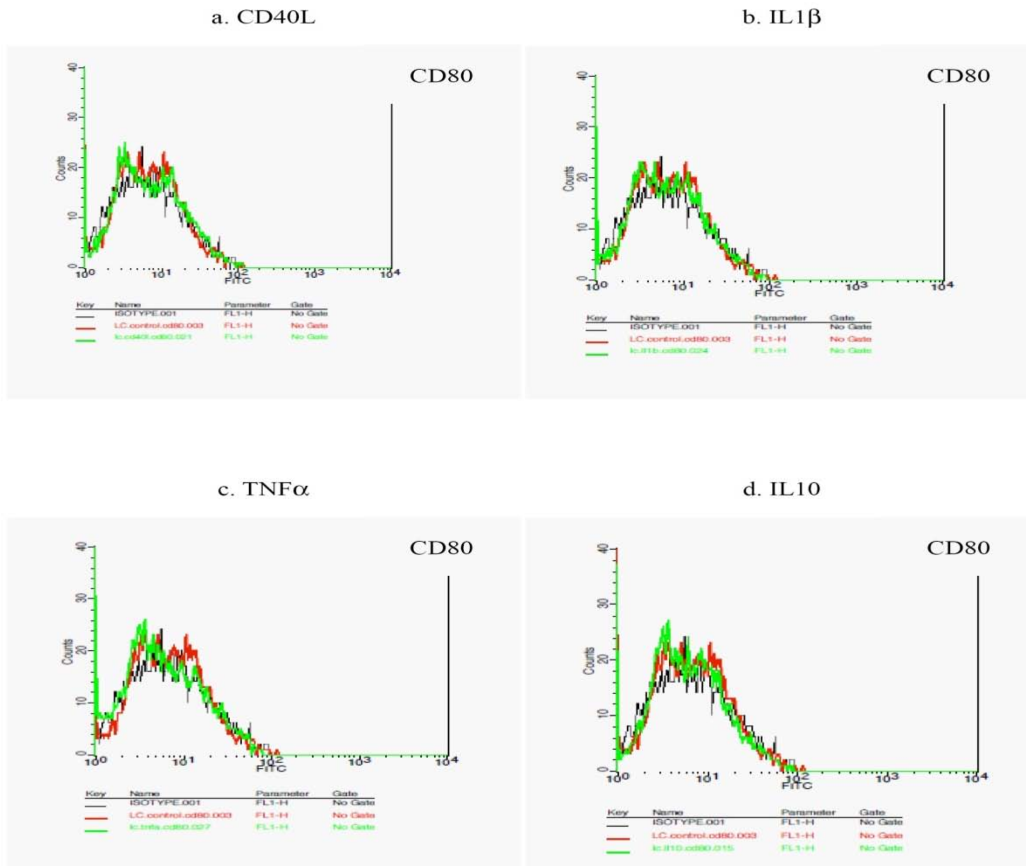


Figure Legend

Isotype

Untreated LC

LC + Conditioning agent

As described in the Experimental design the expression of CD80 on Langerhans cells upon conditioning with four different cytokines, was analyzed with flow cytometry. The results are shown in Figure 8.

CD40L:

Langerhans cells conditioned with 1μg/μl of CD40L did not show any increase in the surface expression of CD80 (Figure 8a).

IL-1β:

Langerhans cells conditioned with 50ng/μl of IL-1β did not show any increase in the surface expression of CD80 (Figure 8b).

TNFα:

Langerhans cells conditioned with 50ng/μl of TNFα did not show any increase in the surface expression of CD80 (Figure 8c).

IL-10:

Langerhans cells conditioned with 50ng/μl of IL-10 did not show any increase in the surface expression of CD80 (Figure 8d).

Figure IV-9: Effect of rhIL-10 conditioning on CD80 expression on Langerhans cell response to MAMPs

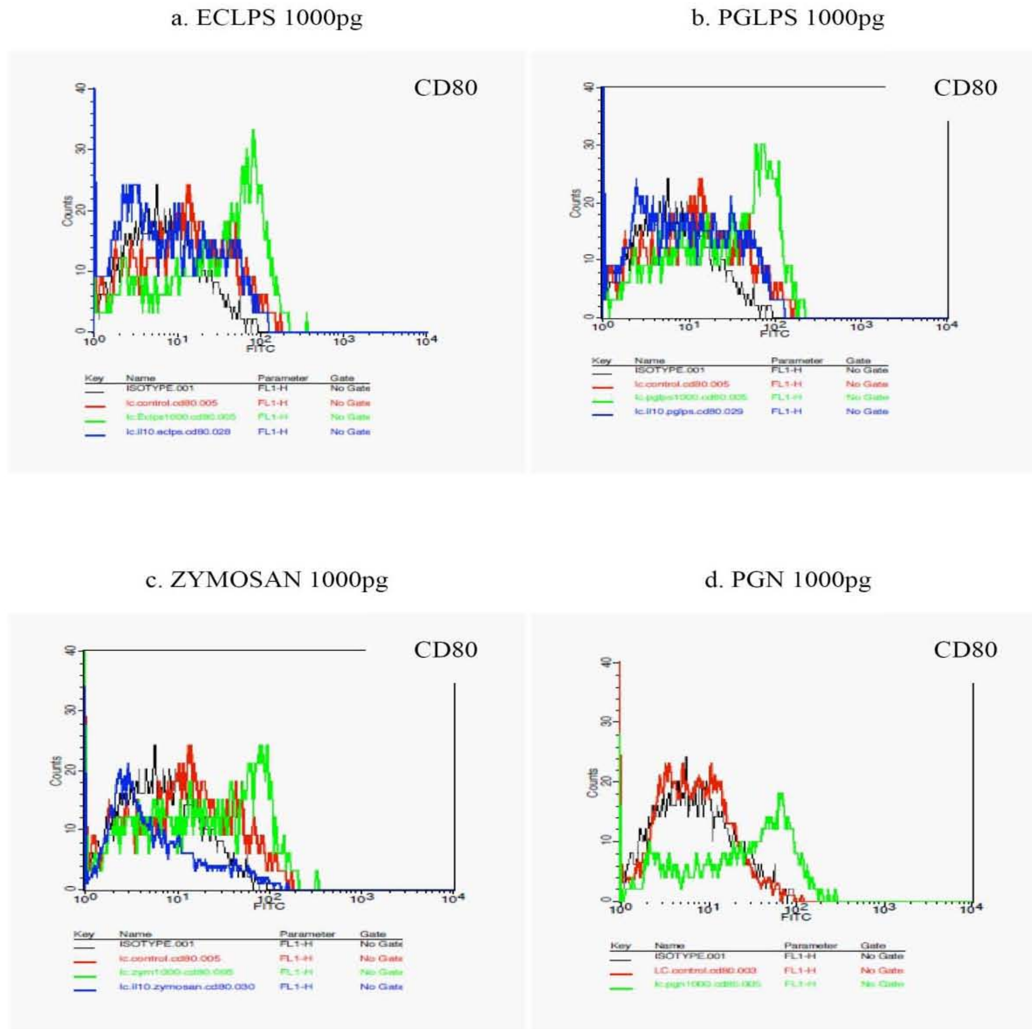


Figure Legend

Isotype

Untreated LC

LC + MAMP

IL-10 conditioned LC + MAMP

As described in the experimental design the expression of CD80, on rhIL-10 conditioned Langerhans cells, upon stimulation with the four different MAMPs, was analyzed with flow cytometry (Figure 9). CD80 was expressed constitutively in Langerhans cells (Figure 9).

PGLPS 1000pg:

Langerhans cells stimulated with 1000pg dose of PGLPS increased surface expression of CD80 substantially (Figure 9b). This inducible expression was statistically significant. When the Langerhans cells were conditioned with rhIL-10, and subsequently challenged with 1000pg of PGLPS, they failed to up-regulate the surface expression of CD80 (Figure 9b).

ECLPS 1000pg:

Langerhans cells stimulated with 1000pg dose of ECLPS increased surface expression of CD80 substantially (Figure 9a). This inducible expression was statistically significant. When the Langerhans cells were conditioned with rhIL-10, and subsequently challenged with 1000pg of ECLPS, they failed to up-regulate the surface expression of CD80 (Figure 9a).

Zymosan 1000pg:

Langerhans cells stimulated with 1000pg dose of zymosan increased surface expression of CD80 substantially (Figure 9c). This inducible expression was statistically significant. When the Langerhans cells were conditioned with

rhIL-10, and subsequently challenged with 1000pg of zymosan, they failed to up-regulate the surface expression of CD80 (Figure 9c).

Peptidoglycan (PGN) 1000pg:

Langerhans cells stimulated with 1000pg dose of PGN increased surface expression of CD80 substantially (Figure 9d). This inducible expression was statistically significant.

When the Langerhans cells were conditioned with rhIL-10, and subsequently challenged with 1000pg of PGN, they failed to up-regulate the surface expression of CD80 (Figure 9d).

Figure IV-10: Effect of CD40L conditioning on CD80 expression on Langerhans cell response to MAMPs

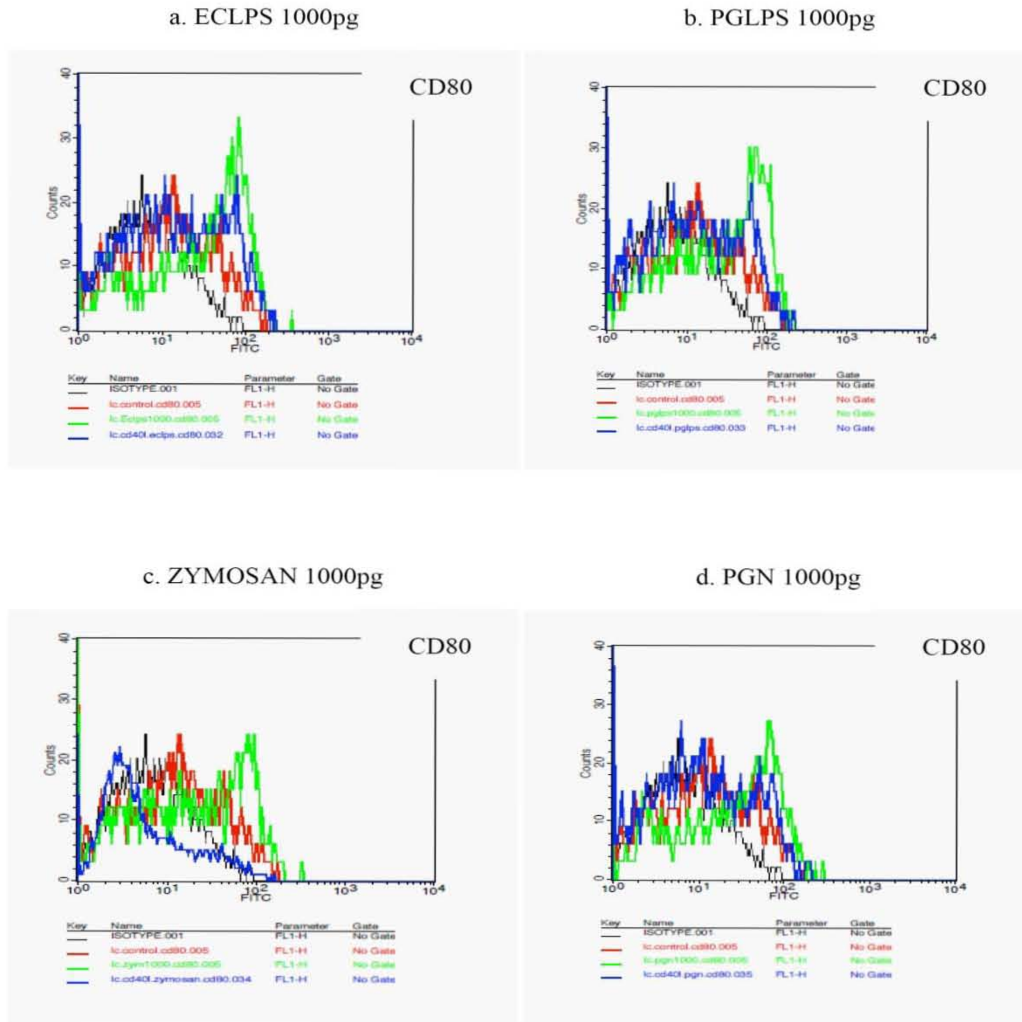


Figure Legend

Isotype

Untreated LC

LC + MAMP

CD40L conditioned LC + MAMP

As described in the experimental design the expression of CD80, on CD40L treated Langerhans cells, upon stimulation with the four different MAMPs, was analyzed with flow cytometry (Figure 10). CD80 was expressed constitutively in Langerhans cells (Figure 10).

PGLPS 1000pg:

Langerhans cells stimulated with 1000pg dose of PGLPS increased surface expression of CD80 substantially (Figure 10b). This inducible expression was statistically significant. Interestingly, when Langerhans cells were conditioned with CD40L, and subsequently challenged with 1000pg of PGLPS, they up-regulate the surface expression of CD80 above constitutive levels and CD40L conditioned LC but not as much as PGLPS challenged LC (Figure 10b).

ECLPS 1000pg:

Langerhans cells stimulated with 1000pg dose of ECLPS increased surface expression of CD80 substantially (Figure 10a). This inducible expression was statistically significant. Interestingly, when the Langerhans cells were conditioned with CD40L, and subsequently challenged with 1000pg of ECLPS, they failed to provide an additive effect to ECLPS induced increase in the surface expression of CD80 (Figure 10a) but up regulated CD80 expression more than just CD40L conditioned LC.

Zymosan 1000pg:

Langerhans cells stimulated with 1000pg dose of zymosan increased surface expression of CD80 substantially (Figure 10c). This inducible expression was statistically significant. Interestingly, when the Langerhans cells were conditioned with CD40L, and subsequently challenged with 1000pg of zymosan, they failed to up-regulate the surface expression of CD80 (Figure 10c) and in fact down regulated CD80 expression on these cells.

Peptidoglycan (PGN) 1000pg:

Langerhans cells stimulated with 1000pg dose of PGN increased surface expression of CD80 substantially (Figure 10d). This inducible expression was statistically significant.

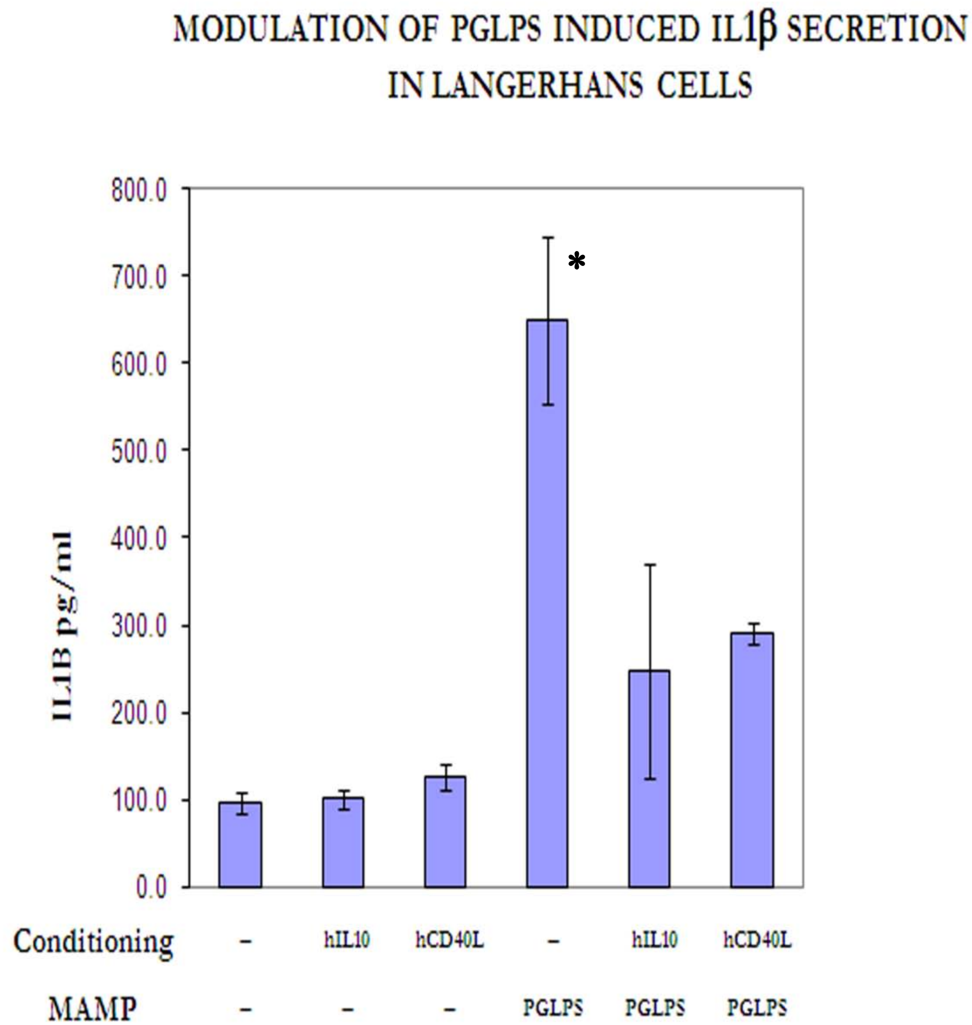
Interestingly, when the Langerhans cells were conditioned with CD40L, and subsequently challenged with 1000pg of PGN, they up-regulate the surface expression of CD80 above constitutive levels and just CD40L conditioned LC but not as much as PGN challenged LC (Figure 10d).

Cytokines

Effect of immune-modulation on cytokine secretion by Langerhans cells in response to MAMPs:

Effect of immune-modulation on IL-1β secretion by Langerhans cells in response to MAMPs:

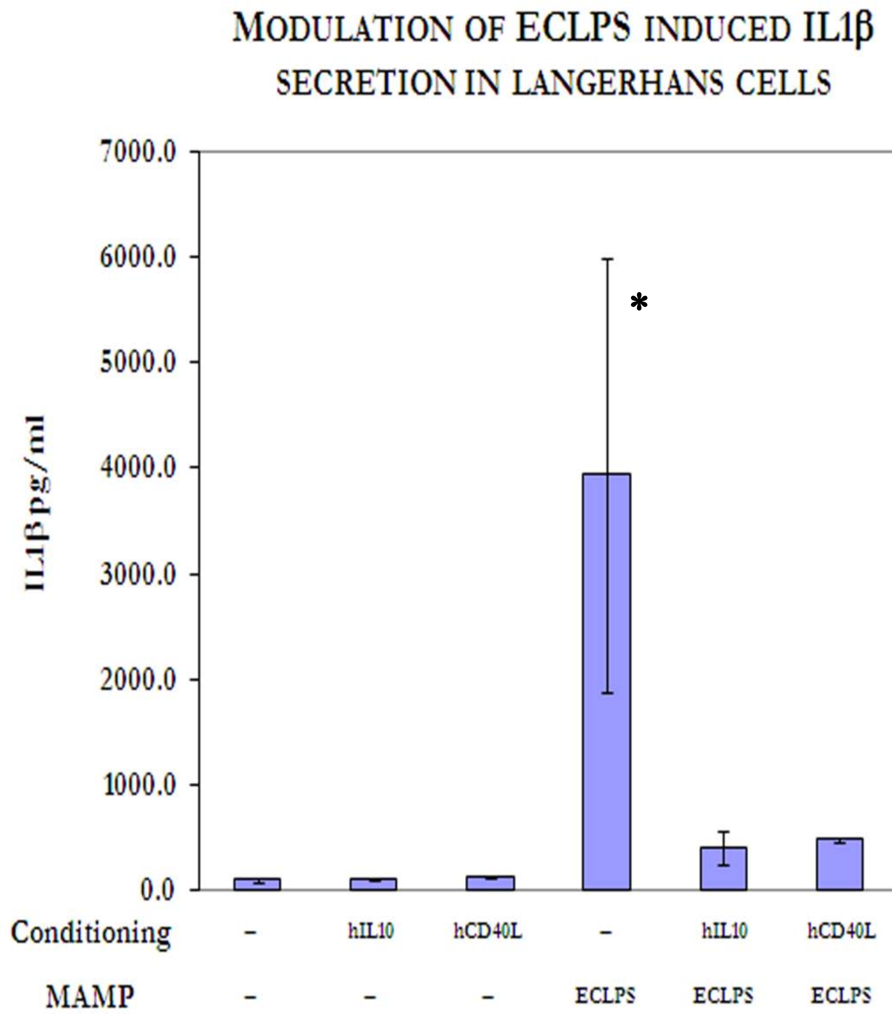
Figure IV-11: Effect of immune-modulation on IL-1β secretion by Langerhans cell in response to PGLPS



∗ – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls

The modulation of PGLPS induced IL-1β secretion from Langerhans cells was measured with help of a flow-cytometry based bead assay. The results are depicted in Figure 11. Langerhans cells secrete constitutively low levels of IL-1β. rhIL-10 or CD40L conditioning alone doesn't cause any change in the amounts of IL-1β secretion. PGLPS challenged Langerhans cells secrete 6 fold more IL-1β as compared to unstimulated Langerhans cells. rhIL-10 / CD40L conditioned and PGLPS stimulated Langerhans cells on the other hand secrete only 2 fold more IL-1β as compared to unstimulated Langerhans cells. The statistical significance was test with a paired student's T test with equal variance.

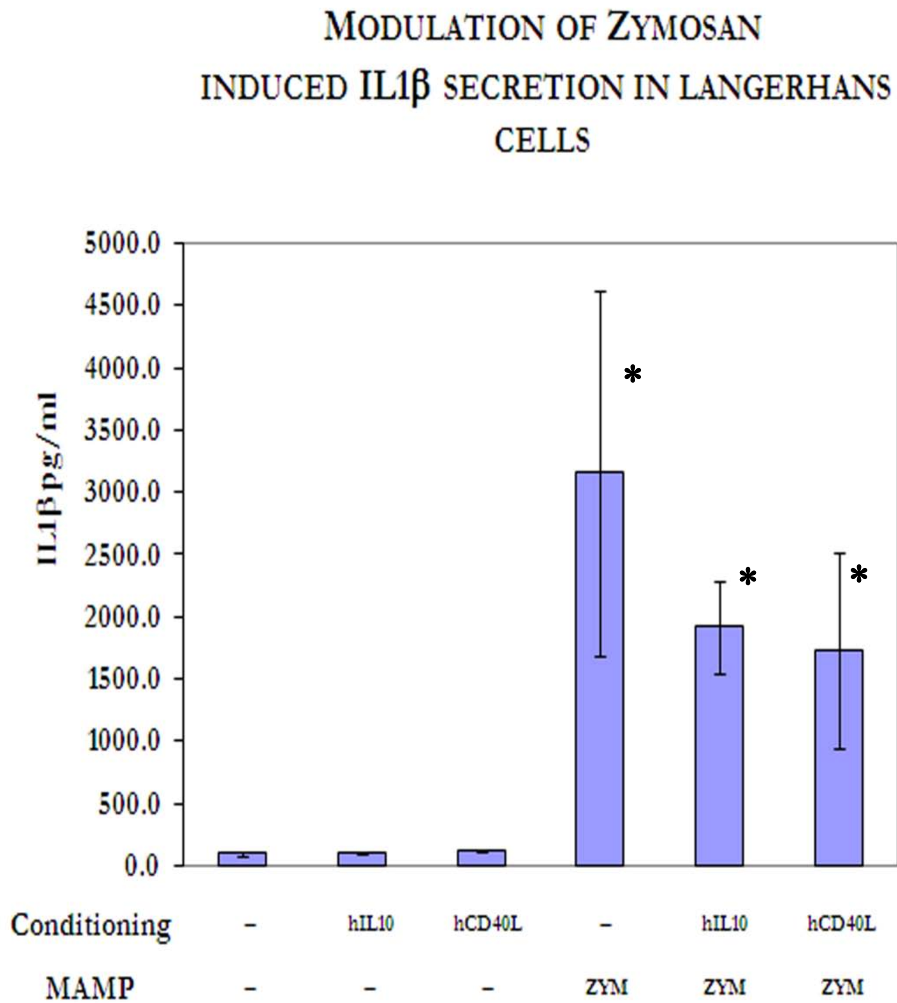
Figure IV-12: Effect of cytokine conditioning on IL-1β secretion by Langerhans cell in response to ECLPS



* – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls

The modulation of ECLPS induced IL-1β secretion from Langerhans cells was measured with help of a flow-cytometry based bead assay. The results are depicted in Figure 12. Langerhans cells secrete constitutively low levels of IL-1β. rhIL-10 or CD40L conditioning alone doesn't cause any change in the amounts of IL-1β secretion. ECLPS challenged Langerhans cells secrete 10-60 fold more IL-1β as compared to unstimulated Langerhans cells. rhIL-10 / CD40L conditioned and PGLPS stimulated Langerhans cells on the other hand secrete only 4-5 fold more IL-1β as compared to unstimulated Langerhans cells.

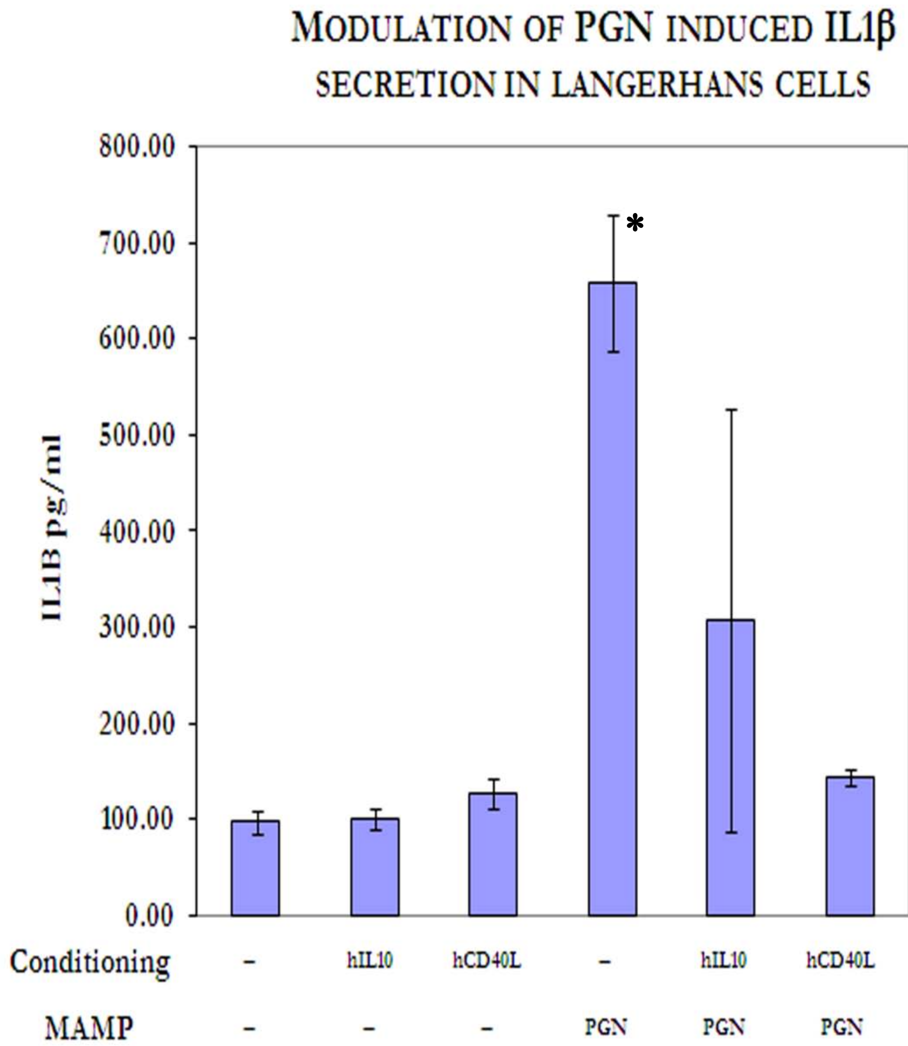
Figure IV-13: Effect of cytokine conditioning on IL-1β secretion by Langerhans cell in response to Zymosan



∗ – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls

The modulation of Zymosan induced IL-1β secretion from Langerhans cells was measured with help of a flow-cytometry based bead assay. The results are depicted in Figure 13. Langerhans cells secrete constitutively low levels of IL-1β. rhIL-10 or CD40L conditioning alone doesn't cause any change in the amounts of IL-1β secretion. Zymosan challenged Langerhans cells secrete 15-45 fold more IL-1β as compared to unstimulated Langerhans cells. Interestingly, rhIL-10 / CD40L conditioned and PGLPS stimulated Langerhans cells on the other hand secrete approximately 20 fold more IL-1β as compared to unstimulated Langerhans cells. Thus Zymosan, as compared to the other MAMPs, was able to overcome suppression of rhIL10 and CD40L mediated modulation of Langerhans cells.

Figure IV-14: Effect of cytokine conditioning on IL-1β secretion by Langerhans cell in response to PGN

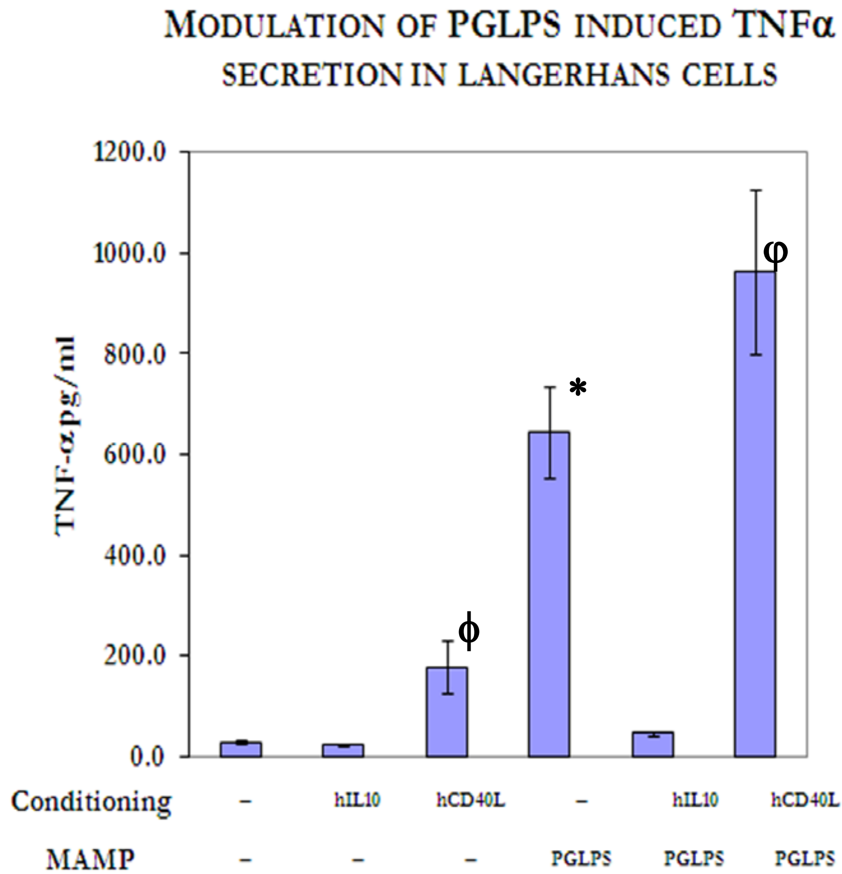


* – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls

The modulation of PGN induced IL-1β secretion from Langerhans cells was measured with help of a flow-cytometry based bead assay. The results are depicted in Figure 14. Langerhans cells secrete constitutively low levels of IL-1β. rhIL-10 or CD40L conditioning alone doesn't cause any change in the amounts of IL-1β secretion. PGN challenged Langerhans cells secrete approximately 60-70 fold more IL-1β as compared to unstimulated Langerhans cells. rhIL-10 conditioned and PGLPS stimulated Langerhans cells on the other hand secrete on an average 3 fold more IL-1β as compared to unstimulated Langerhans cell although this was not statistically significant from constitutive expression levels. CD40L conditioned and PGLPS stimulated Langerhans cells on did not up-regulate IL-1β secretion as compared to unstimulated Langerhans cells.

Effect of immune-modulation on TNF-α secretion by Langerhans cells in response to MAMPS:

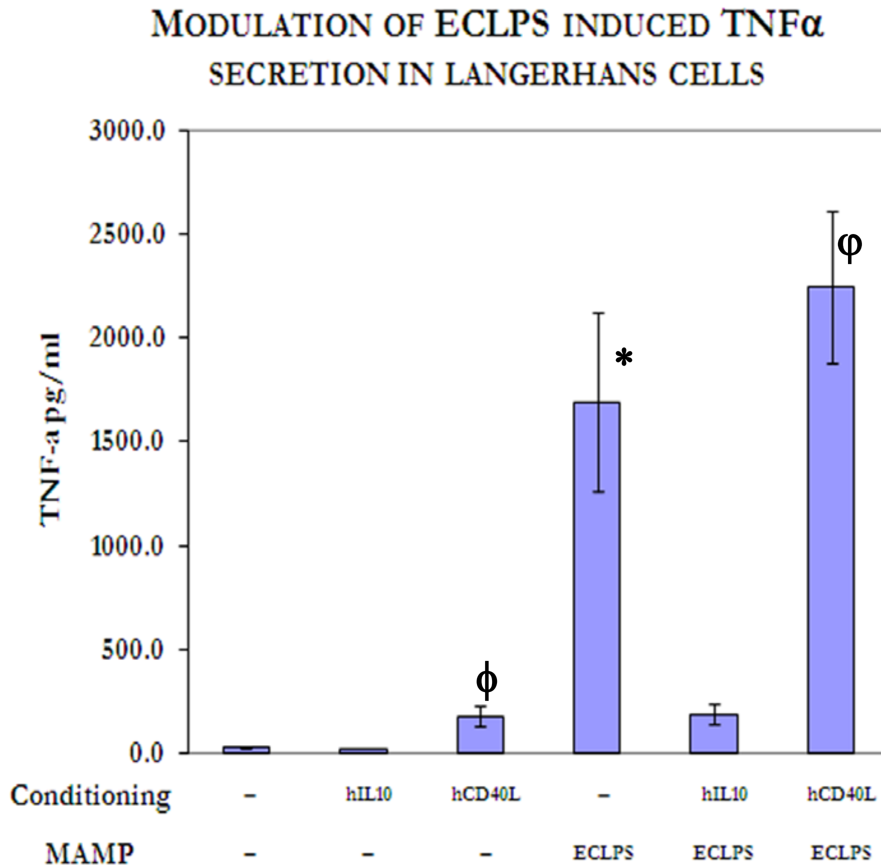
Figure IV-15: Effect of cytokine conditioning on LC TNF-α secretion in response to PGLPS



∗ – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls ϕ – Statistical significance with a P value of $\leq$ 0.05 as compared to MAMP treated LCs φ – Statistical significance with a P value of $\leq$ 0.05 as compared to MAMP treated LCs

The modulation of PGLPS induced TNF-α secretion from Langerhans cells was measured with help of a flow-cytometry based bead assay. The results are depicted in Figure 15. Langerhans cells secrete constitutively low levels of TNF-α. rhIL-10 conditioning alone doesn't cause any change in the amounts of TNF-α secretion. CD40L conditioning causes a 2- fold up regulation of TNF-α secretion. PGLPS challenged Langerhans cells secrete 6 fold more TNF-α as compared to unstimulated Langerhans cells. rhIL-10 conditioned and PGLPS stimulated Langerhans cells doesn't up regulate TNF-α secretion as compared to unstimulated Langerhans cells. CD40L conditioned and PGLPS stimulated Langerhans cells on the other hand secrete 9.5 fold more TNF-α as compared to unstimulated Langerhans cells.

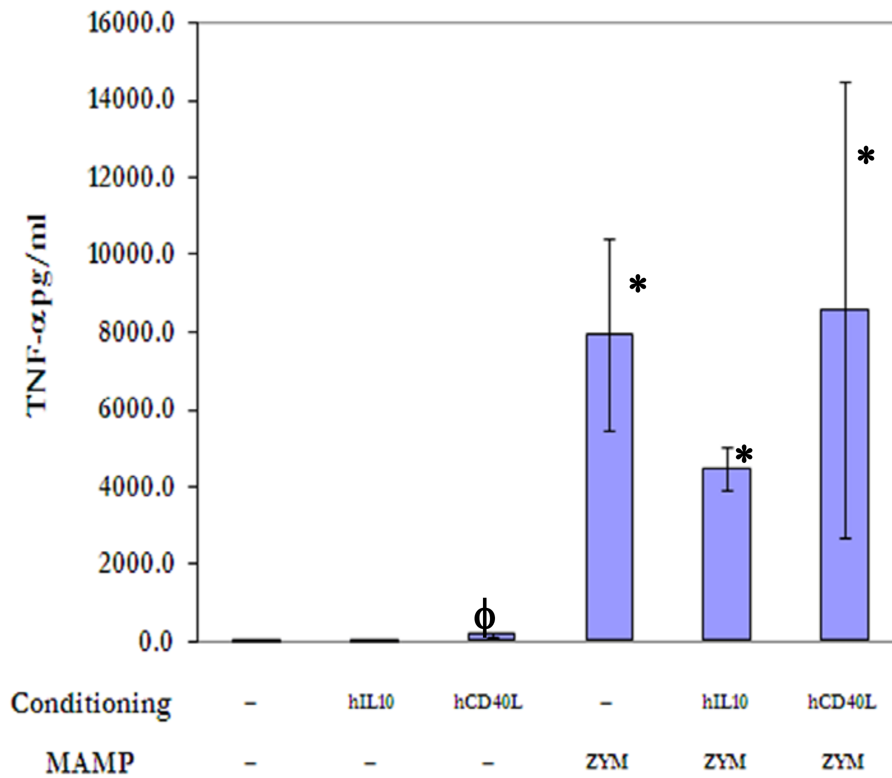
Figure IV-16: Effect of cytokine conditioning on LC TNF-α secretion in response to ECLPS



∗ – Statistical significance with a P value of ≤ 0.05 as compared to untreated controls ϕ – Statistical significance with a P value of ≤ 0.05 as compared to MAMP treated LCs φ – Statistical significance with a P value of ≤ 0.05 as compared to MAMP treated LCs

The modulation of ECLPS induced TNF-α secretion from Langerhans cells was measured with help of a flow-cytometry based bead assay. The results are depicted in Figure 16. Langerhans cells secrete constitutively low levels of TNF-α. rhIL-10 conditioning alone doesn't cause any change in the amounts of TNF-α secretion. CD40L conditioning causes a 2- fold up regulation of TNF-α secretion. ECLPS challenged Langerhans cells secrete 16 fold more TNF-α as compared to unstimulated Langerhans cells. rhIL-10 conditioned and ECLPS stimulated Langerhans cells doesn't up regulate TNF-α secretion as compared to unstimulated Langerhans cells. CD40L conditioned and ECLPS stimulated Langerhans cells on the other hand 20 fold more TNF-α as compared to unstimulated Langerhans cells.

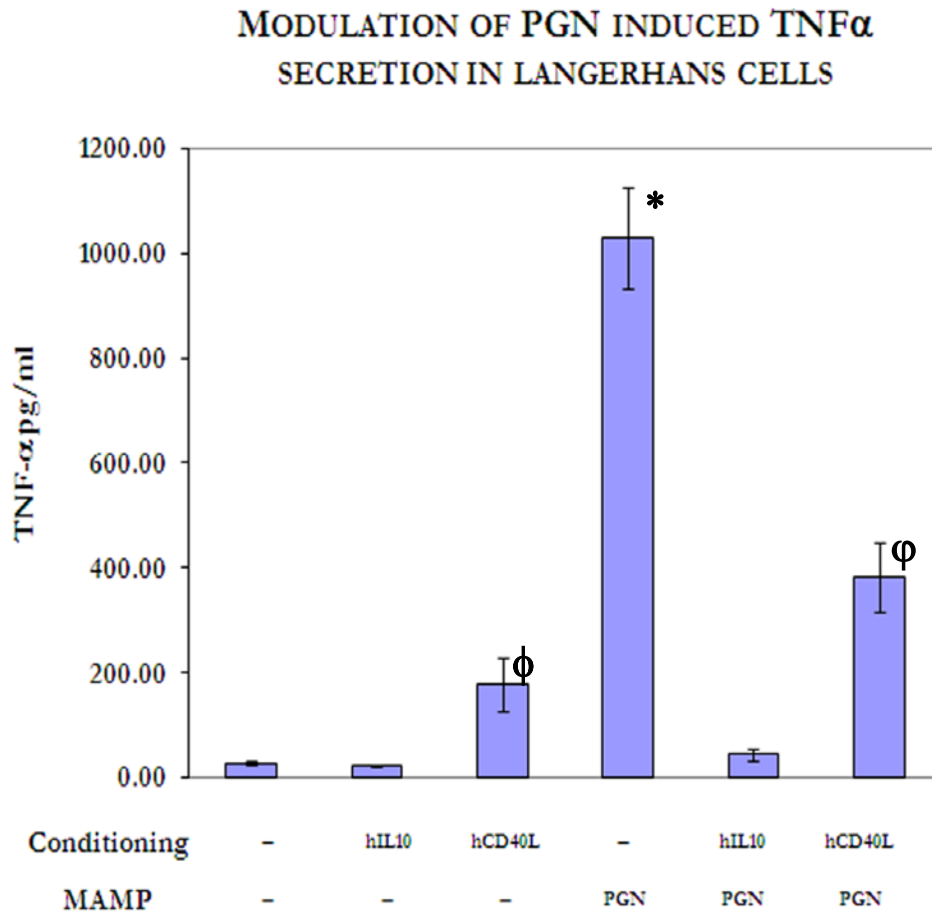
Figure IV-17: Effect of cytokine conditioning on LC TNF-α secretion in response to Zymosan



∗ – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls ϕ – Statistical significance with a P value of $\leq$ 0.05 as compared to MAMP treated LCs

The modulation of Zymosan induced TNF-α secretion from Langerhans cells was measured with help of a flow-cytometry based bead assay. The results are depicted in Figure 17. Langerhans cells secrete constitutively low levels of TNF-α. rhIL-10 conditioning alone doesn't cause any change in the amounts of TNF-α secretion. CD40L conditioning causes a 2- fold up regulation of TNF-α secretion. Interestingly Zymosan challenged Langerhans cells secrete high amounts of TNF-α almost 80 fold more TNF-α as compared to unstimulated Langerhans cells. rhIL-10 conditioned and Zymosan stimulated Langerhans cells suppresses TNF-α secretion as compared to unstimulated Langerhans cells but only to a limited extent. rhIL-10 conditioning and Zymosan stimulated Langerhans cells secrete 40 fold more than untreated LCs and 2 fold less than just Zymosan stimulated LCs. CD40L conditioned and Zymosan stimulated Langerhans cells on the other hand secreted 100 fold more TNF-α as compared to unstimulated Langerhans cells.

Figure IV-18: Effect of cytokine conditioning on LC TNF-α secretion in response to PGN

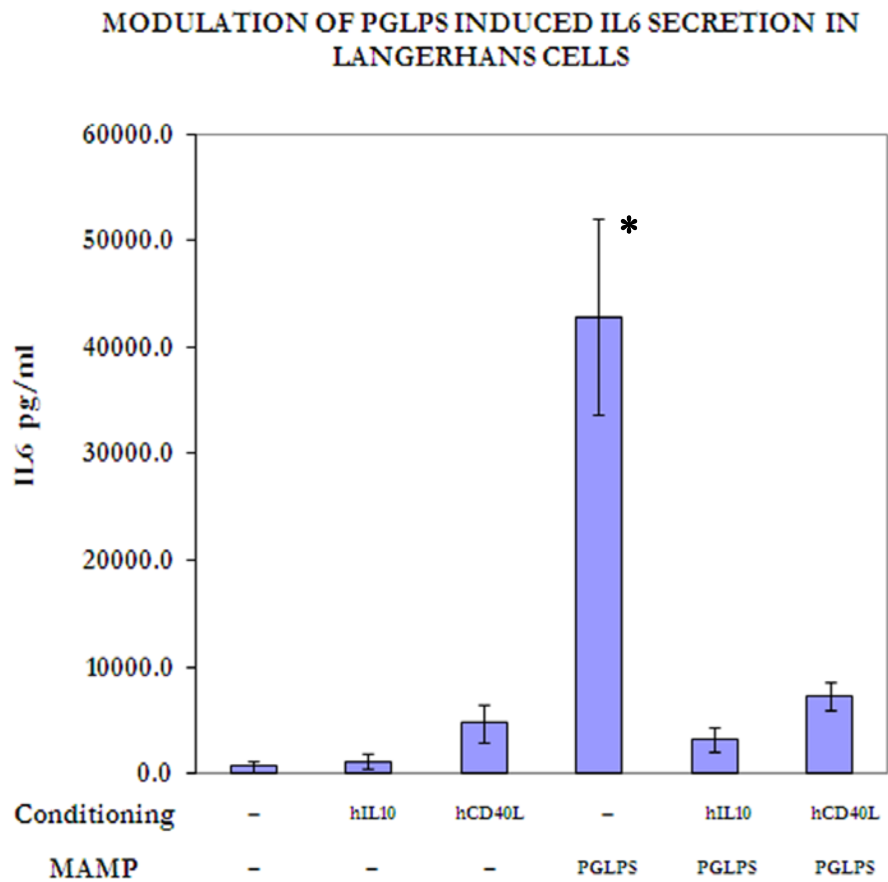


∗ – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls ϕ – Statistical significance with a P value of $\leq$ 0.05 as compared to MAMP treated LCs φ – Statistical significance with a P value of $\leq$ 0.05 as compared to MAMP treated LCs

The modulation of PGN induced TNF-α secretion from Langerhans cells was measured with help of a flow-cytometry based bead assay. The results are depicted in Figure 18. Langerhans cells secrete constitutively low levels of TNF-α. rhIL-10 conditioning alone doesn't cause any change in the amounts of TNF-α secretion. CD40L conditioning causes a 2- fold up regulation of TNF-α secretion. PGN challenged Langerhans cells secrete 10 fold more TNF-α as compared to unstimulated Langerhans cells. rhIL-10 conditioned and PGN stimulated Langerhans cells doesn't up regulate TNF-α secretion as compared to unstimulated Langerhans cells. Interestingly unlike the other MAMPs CD40L conditioned and PGN stimulated Langerhans cells secreted only 4 fold more TNF-α as compared to unstimulated Langerhans cells.

Effect of immune-modulation on IL-6 secretion by Langerhans cells in response to MAMPS:

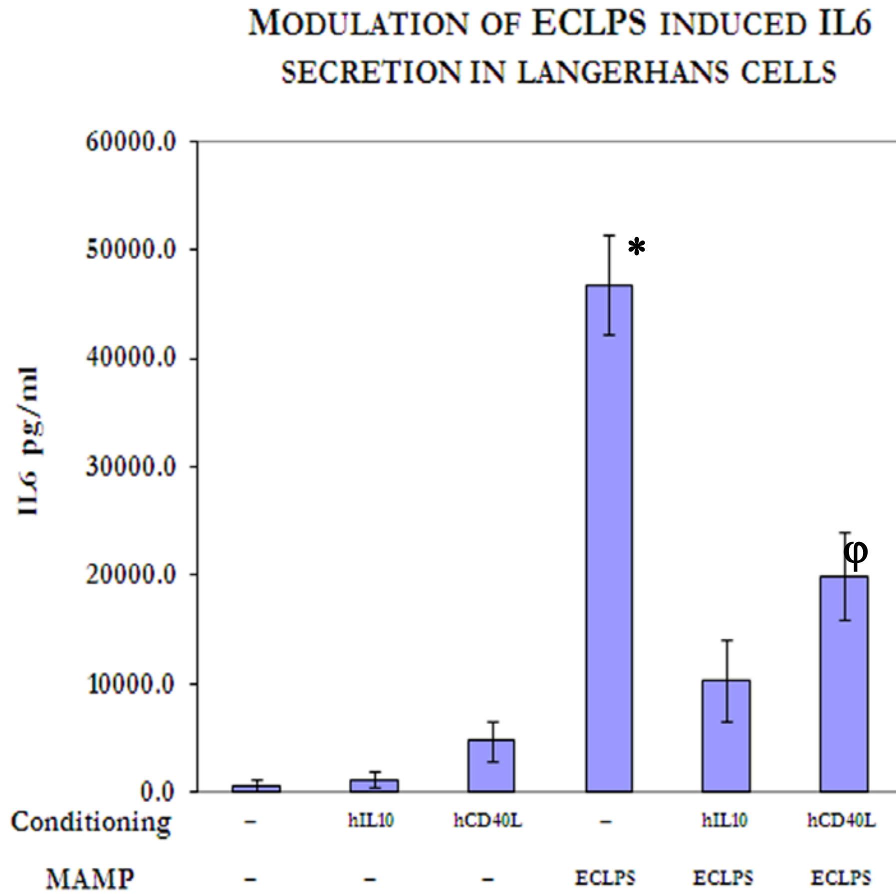
Figure IV-19: Effect of cytokine conditioning on IL-6 secretion by Langerhans cell in response to PGLPS



* – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls

The modulation of PGLPS induced IL-6 secretion from Langerhans cells was measured with help of a flow-cytometry based bead assay. The results are depicted in Figure 19. Langerhans cells secrete constitutively low levels of IL-6. rhIL-10 conditioning alone doesn't cause any change in the amounts of IL-6 secretion. On other hand CD40L conditioning alone causes a modest up regulation of IL-6 secretion (4 fold). PGLPS challenged Langerhans cells secrete 40 fold more IL-6 as compared to unstimulated Langerhans cells. rhIL-10 conditioned and PGLPS stimulated Langerhans cells on the other hand secrete only 3 fold more IL-6 as compared to unstimulated Langerhans cells. While, CD40L conditioned and PGLPS stimulated Langerhans cells secrete 7 fold more IL-6 as compared to unstimulated Langerhans cells.

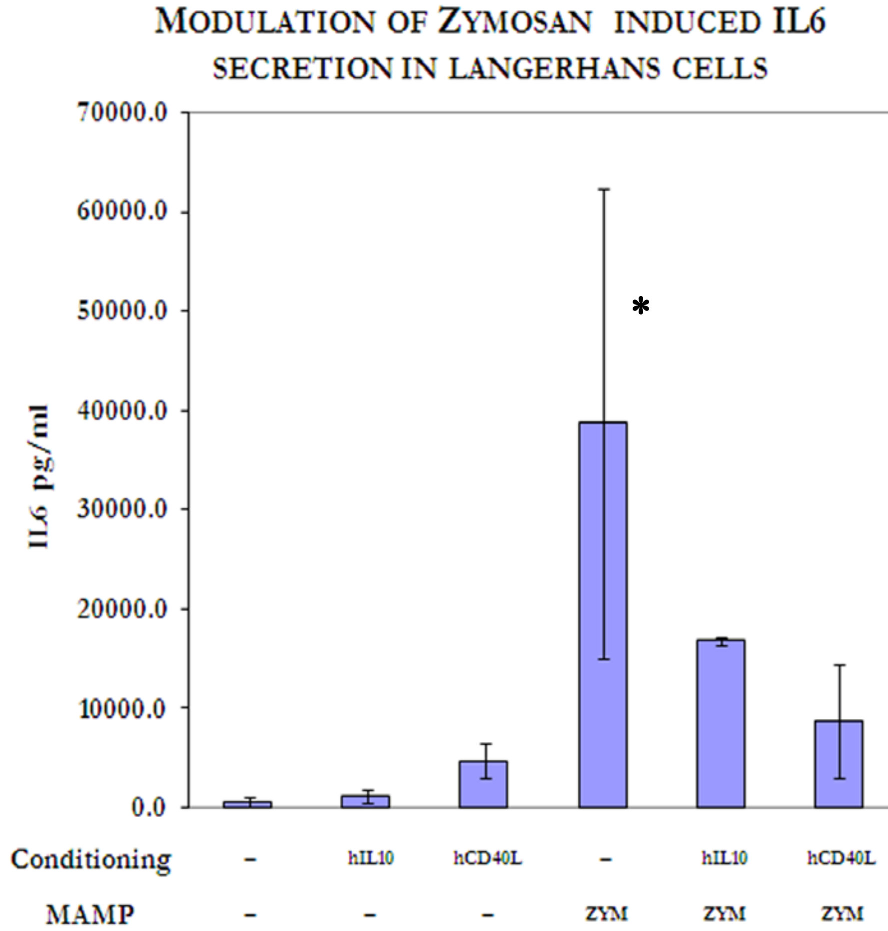
Figure IV-20: Effect of cytokine conditioning on IL-6 secretion by Langerhans cell in response to ECLPS



∗ – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls φ – Statistical significance with a P value of $\leq$ 0.05 as compared to MAMP treated LCs

The modulation of ECLPS induced IL-6 secretion from Langerhans cells was measured with help of a flow-cytometry based bead assay. The results are depicted in Figure 20. Langerhans cells secrete constitutively low levels of IL-6. rhIL-10 conditioning alone doesn't cause any change in the amounts of IL-6 secretion. On other hand CD40L conditioning alone causes a modest up regulation of IL-6 secretion (4 fold). ECLPS challenged Langerhans cells secrete 45 fold more IL-6 as compared to unstimulated Langerhans cells. rhIL-10 conditioned and ECLPS stimulated Langerhans cells on the other hand secrete 9 fold more IL-6 as compared to unstimulated Langerhans cells. While, CD40L conditioned and ECLPS stimulated Langerhans cells secrete 19 fold more IL-6 as compared to unstimulated Langerhans cells.

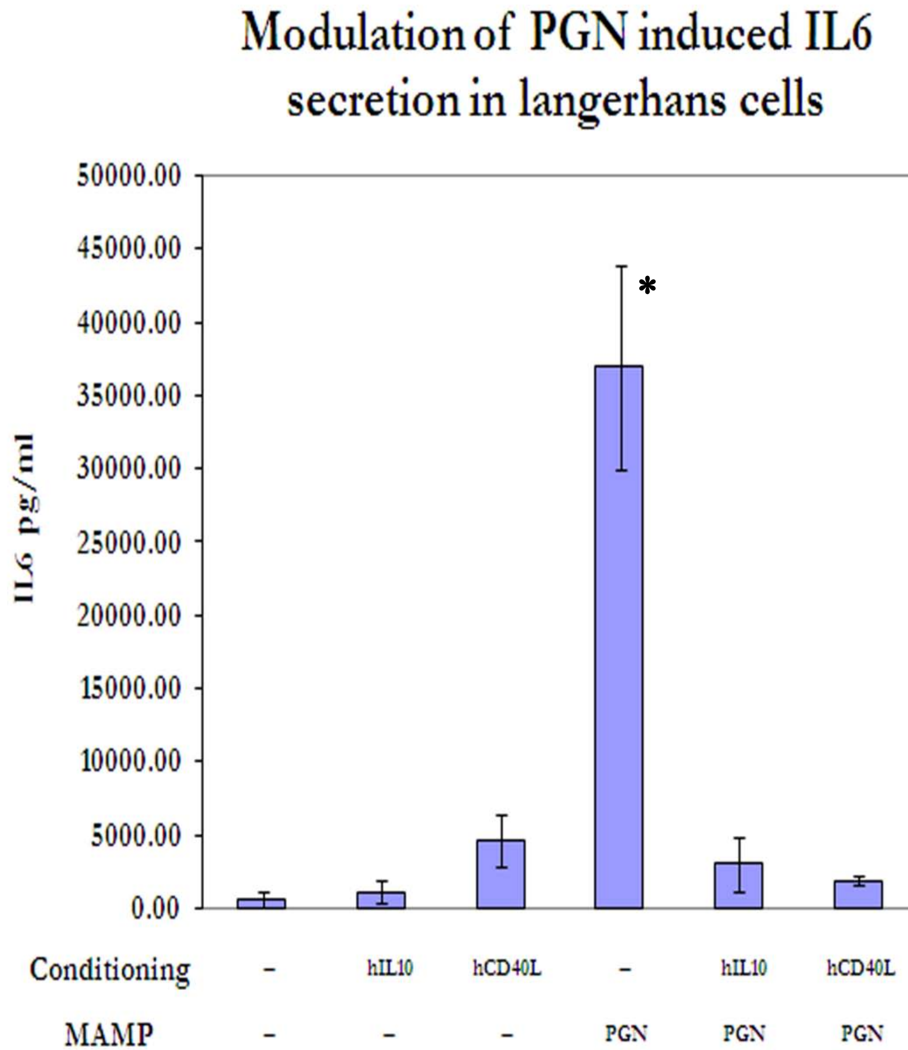
Figure IV-21: Effect of cytokine conditioning on IL-6 secretion by Langerhans cell in response to Zymosan



∗ – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls

The modulation of Zymosan induced IL-6 secretion from Langerhans cells was measured with help of a flow-cytometry based bead assay. The results are depicted in Figure 21. Langerhans cells secrete constitutively low levels of IL-6. rhIL-10 conditioning alone doesn't cause any change in the amounts of IL-6 secretion. On other hand CD40L conditioning alone causes a modest up regulation of IL-6 secretion (4 fold). Zymosan challenged Langerhans cells secrete 38 fold more IL-6 as compared to unstimulated Langerhans cells. rhIL-10 conditioned and Zymosan stimulated Langerhans cells on the other hand secrete 15 fold more IL-6 as compared to unstimulated Langerhans cells. While, CD40L conditioned and Zymosan stimulated Langerhans cells secrete 8 fold more IL-6 as compared to unstimulated Langerhans cells.

Figure IV-22: Effect of cytokine conditioning on IL-6 secretion by Langerhans cell in response to PGN

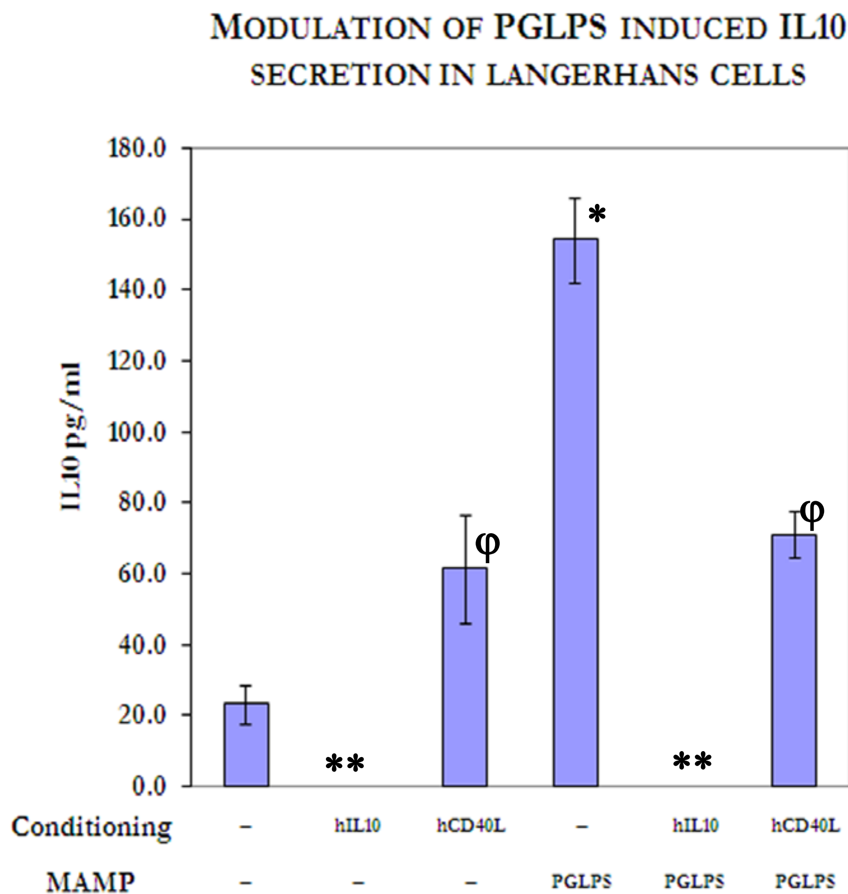


$*$ – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls

The modulation of PGN induced IL-6 secretion from Langerhans cells was measured with help of a flow-cytometry based bead assay. The results are depicted in Figure 22. Langerhans cells secrete constitutively low levels of IL-6. rhIL-10 conditioning alone doesn't cause any change in the amounts of IL-6 secretion. On other hand CD40L conditioning alone causes a modest up regulation of IL-6 secretion (4 fold). PGN challenged Langerhans cells secrete 35 fold more IL-6 as compared to unstimulated Langerhans cells. rhIL-10 conditioned and PGN stimulated Langerhans cells on the other hand secrete only 3 fold more IL-6 as compared to unstimulated Langerhans cells. While, CD40L conditioned and PGN stimulated Langerhans cells secrete almost constitutive levels of IL-6 similar to unstimulated Langerhans cells.

Effect of immune-modulation on IL-10 secretion by Langerhans cells in response to MAMPS:

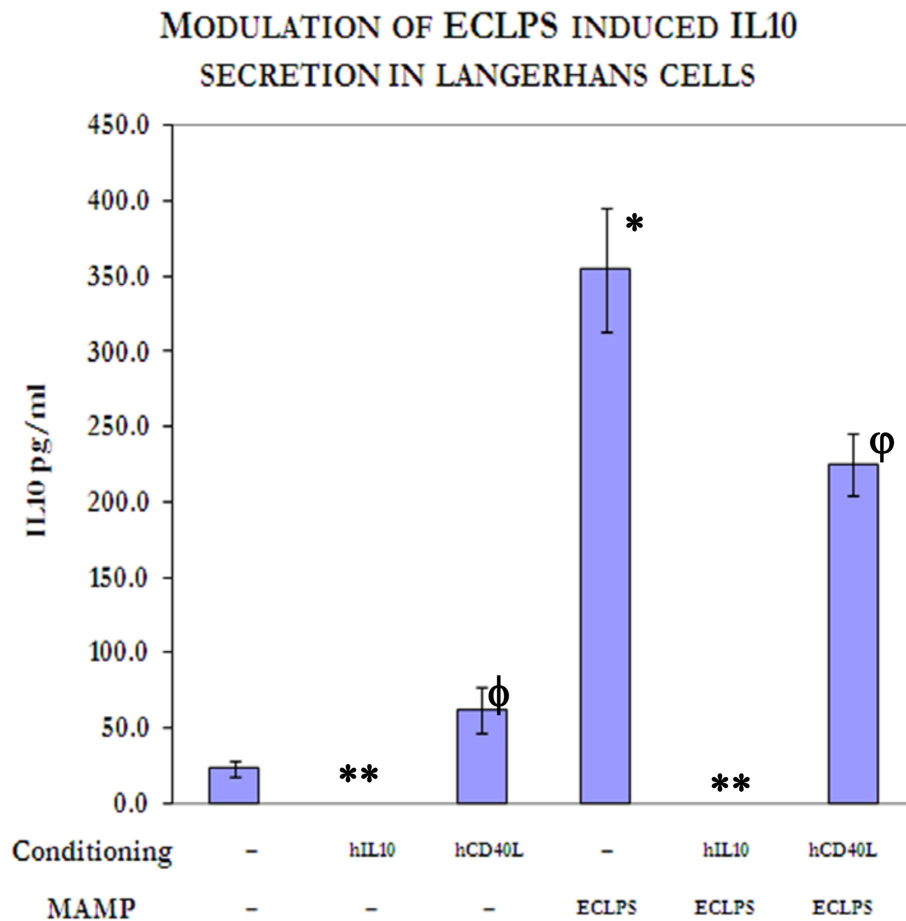
Figure IV-23: Effect of cytokine conditioning on IL-10 secretion by Langerhans cell in response to PGLPS



∗ – Statistical significance with a P value of ≤ 0.05 as compared to untreated controls; φ – Statistical significance with a P value of ≤ 0.05 as compared to MAMP treated LCs; ∗∗ – hIL10 was exogenously added hence not depicted in the graph.

The modulation of PGLPS induced IL-10 secretion from Langerhans cells was measured with help of a flow-cytometry based bead assay. The results are depicted in Figure 23. Langerhans cells secrete constitutively low levels of IL-10. CD40L conditioning alone causes a modest up regulation of IL-10 secretion (3 fold). PGLPS challenged Langerhans cells secrete 8 fold more IL-10 as compared to unstimulated Langerhans cells. CD40L conditioned and PGLPS stimulated Langerhans cells secrete 3 fold more IL-10 compared to unstimulated Langerhans cells and similar to CD40L conditioned Langerhans cells.

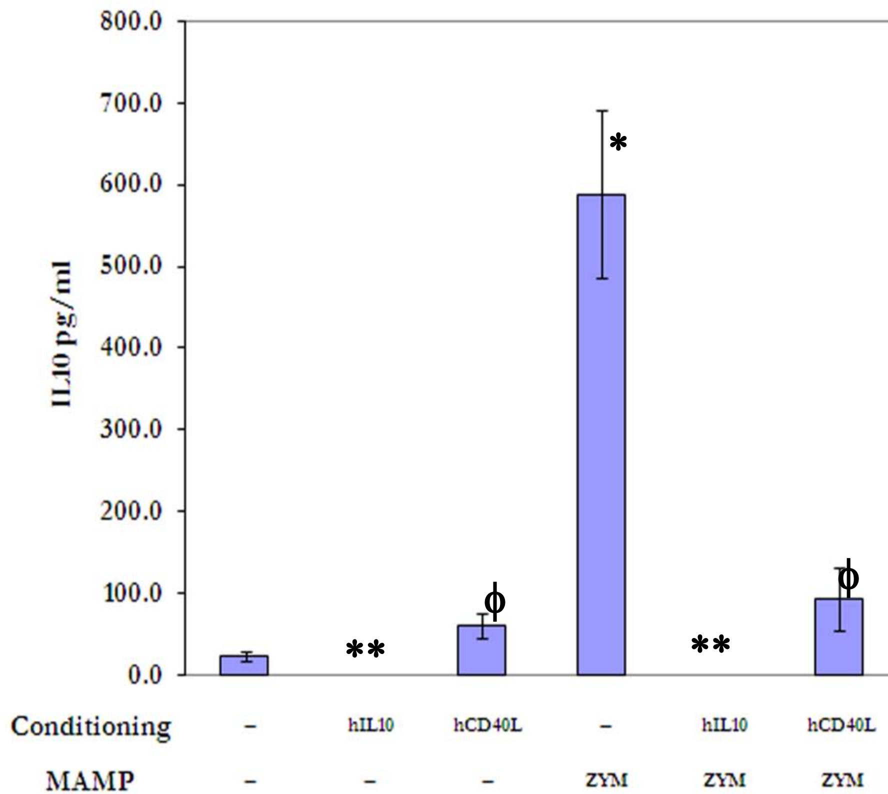
Figure IV-24: Effect of cytokine conditioning on IL-10 secretion by Langerhans cell in response to ECLPS



∗ – Statistical significance with a P value of ≤ 0.05 as compared to untreated controls ϕ – Statistical significance with a P value of ≤ 0.05 as compared to MAMP treated LCs φ – Statistical significance with a P value of ≤ 0.05 as compared to MAMP treated LCs; ∗∗ – hIL10 was exogenously added hence not depicted in the graph.

The modulation of ECLPS induced IL-10 secretion from Langerhans cells was measured with help of a flow-cytometry based bead assay. The results are depicted in Figure 24. Langerhans cells secrete constitutively low levels of IL-10. CD40L conditioning alone causes a modest up regulation of IL-10 secretion (3 fold). ECLPS challenged Langerhans cells secrete 20 fold more IL-10 as compared to unstimulated Langerhans cells. CD40L conditioned and ECLPS stimulated Langerhans cells secrete 10 fold more IL-10 compared to unstimulated Langerhans cells.

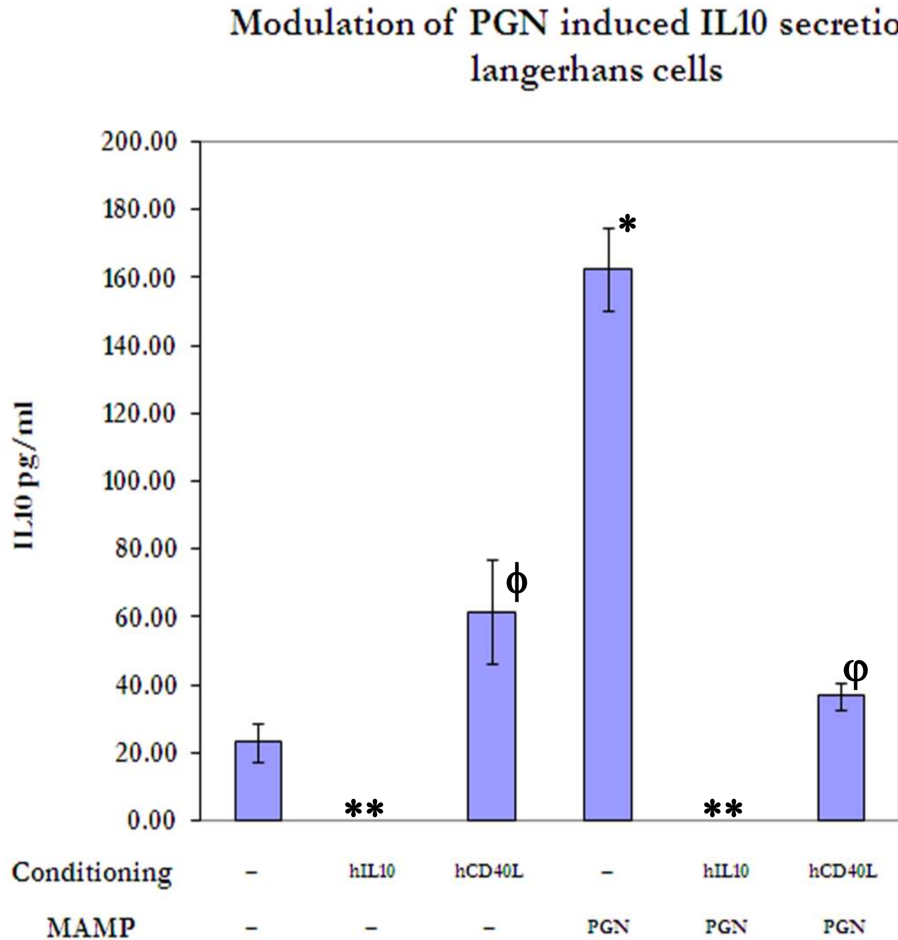
Figure IV-25: Effect of cytokine conditioning on IL-10 secretion by Langerhans cell in response to Zymosan



∗ – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls ϕ – Statistical significance with a P value of $\leq$ 0.05 as compared to MAMP treated LCs; ∗∗ – hIL10 was exogenously added hence not depicted in the graph.

The modulation of Zymosan induced IL-10 secretion from Langerhans cells was measured with help of a flow-cytometry based bead assay. The results are depicted in Figure 25. Langerhans cells secrete constitutively low levels of IL-10. CD40L conditioning alone causes a modest up regulation of IL-10 secretion (3 fold). Zymosan challenged Langerhans cells secrete 30 fold more IL-10 as compared to unstimulated Langerhans cells. CD40L conditioned and Zymosan stimulated Langerhans cells secrete 3 fold more IL-10 compared to unstimulated Langerhans cells and similar to CD40L conditioned Langerhans cells.

Figure IV-26: Effect of cytokine conditioning on IL-10 secretion by Langerhans cell in response to PGN



∗ – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls ϕ – Statistical significance with a P value of $\leq$ 0.05 as compared to MAMP treated LCs φ – Statistical significance with a P value of $\leq$ 0.05 as compared to MAMP treated LCs; ∗∗ – hIL10 was exogenously added and hence not depicted in the graph.

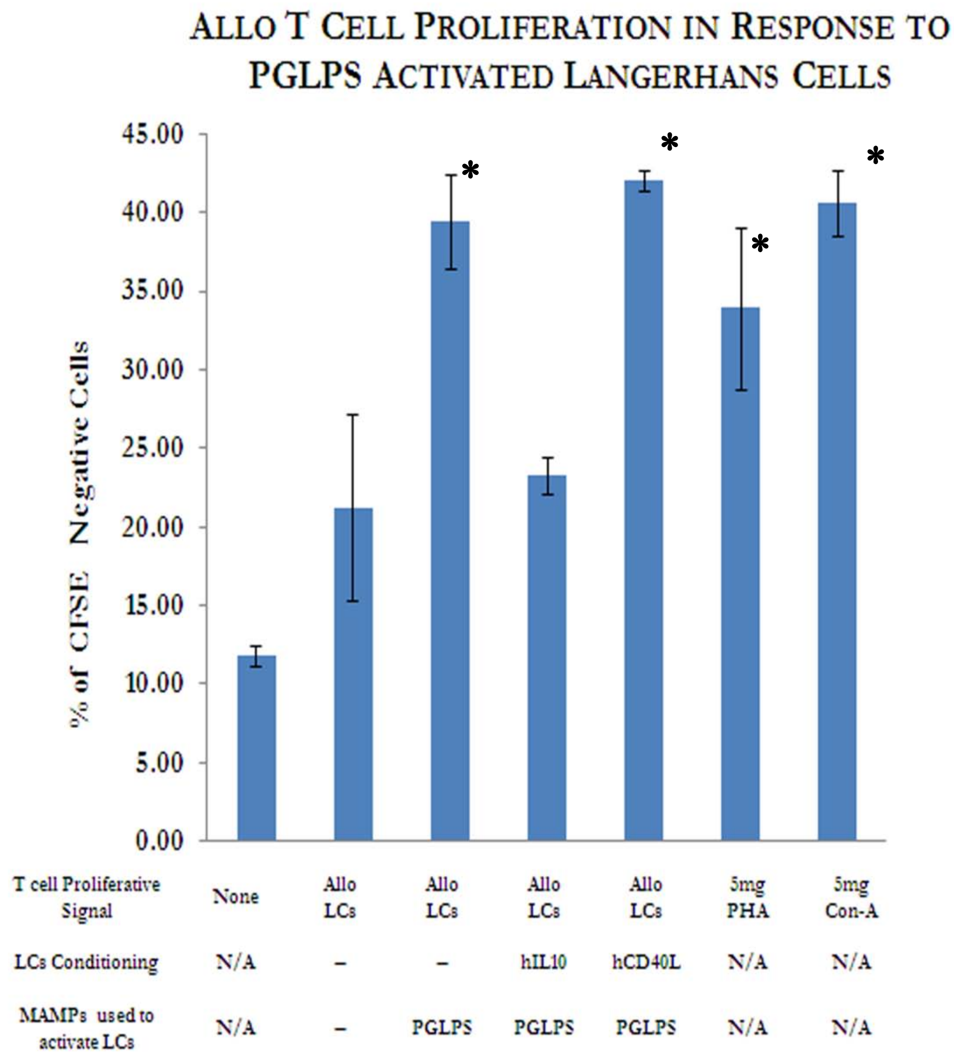
The modulation of PGN induced IL-10 secretion from Langerhans cells was measured with help of a flow-cytometry based bead assay. The results are depicted in Figure 26. Langerhans cells secrete constitutively low levels of IL-10. CD40L conditioning alone causes a modest up regulation of IL-10 secretion (3 fold). PGN challenged Langerhans cells secrete 8 fold more IL-10 as compared to unstimulated Langerhans cells. CD40L conditioned and PGN stimulated Langerhans cells secrete 2 fold more IL-10 compared to unstimulated Langerhans cells.

Induction of T cell response

Allo T cell response

Allo T cell proliferating capacity of MAMPs challenged LPS under different micro-environment al conditions were analyzed with help of the CFSE protocol discussed before. The following section described the results of the analysis. The figures 27 - figure 30 shows in the Y axis the increase in percentage of CFSE negative Allo T cells after co-culturing these cells with the differentially activated Langerhans cells. The X axis describes the different conditioning agents the LCs were before being subjected to MAMPs challenge which are also labeled in the x axis. 5µg PHA and 5µg concavalin A was used as positive controls for analyzing T cell proliferation. T cells alone or T cells with untreated Langerhans cells were used as negative controls. The statistical significance of the differences between the experimental conditions was analyzed with a help of a paired student T Test with equal variance. The results show that allo-T cells had a basal proliferative rate around 11% CFSE negative T cells in the medium. Allo-T cells cultured with untreated Langerhans cells induced a proliferation rate of approximately 21% CFSE negative T cells. Concavalin and PHA induced a proliferation response of about 35% to 40% CFSE negative T cells respectively.

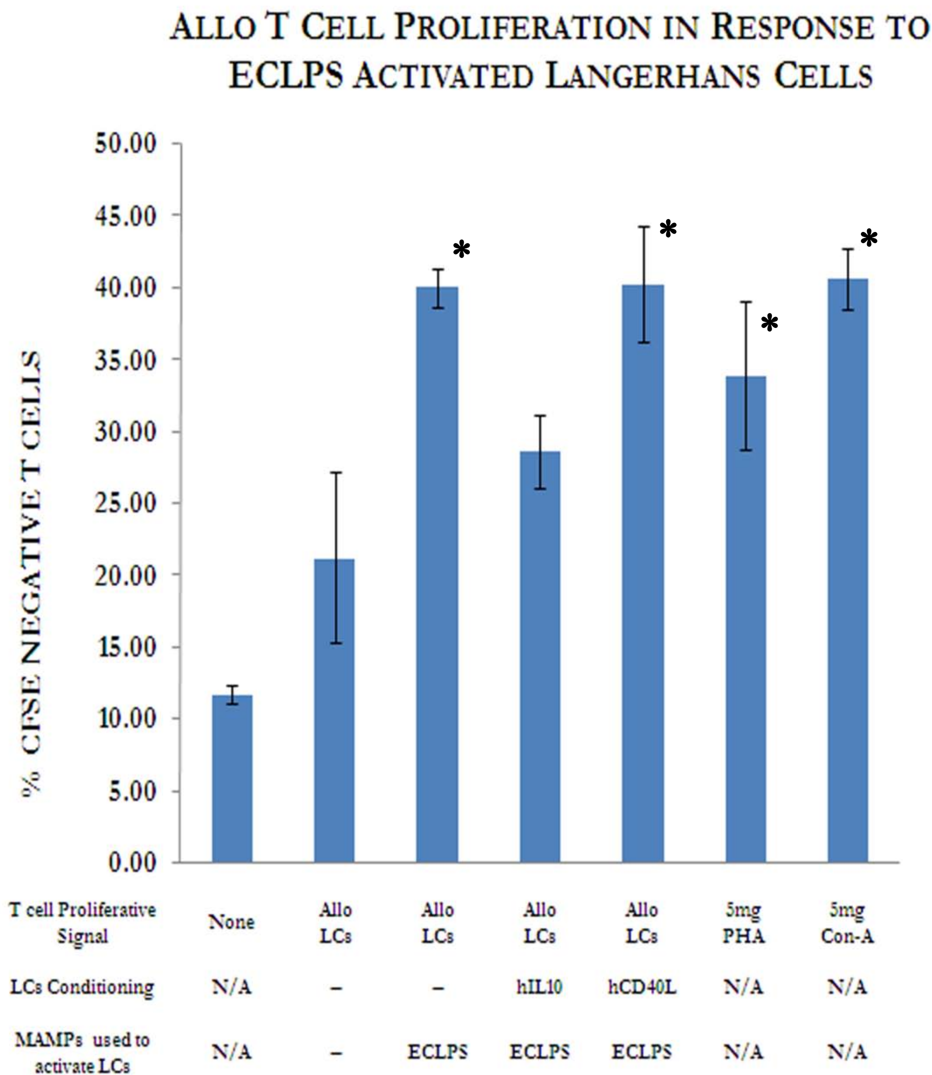
Figure IV-27: Effect of cytokine conditioning on Allo T cell proliferation by Langerhans cell activated by PGLPS



∗ – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls

The Allo-T cell proliferative capacity of differentially conditioned and PGLPS challenged LCs is depicted in figure 27. We show here that PGLPS challenged LCs when co-cultured with allo T cells induces an T cell proliferative response similar to PHA or Concavalin A (39% CFSE negative T cells). hIL-10 conditioning of the LCs prior to PGLPS challenge leads to reduction in the allo T cell proliferative capacity bringing it down to almost similar to untreated LCs (20% CFSE negative T cells). CD40L conditioning of LCs before PGLPS challenge leads to a slight increase in the allo T cell proliferative capacity (42% CFSE negative T cells).

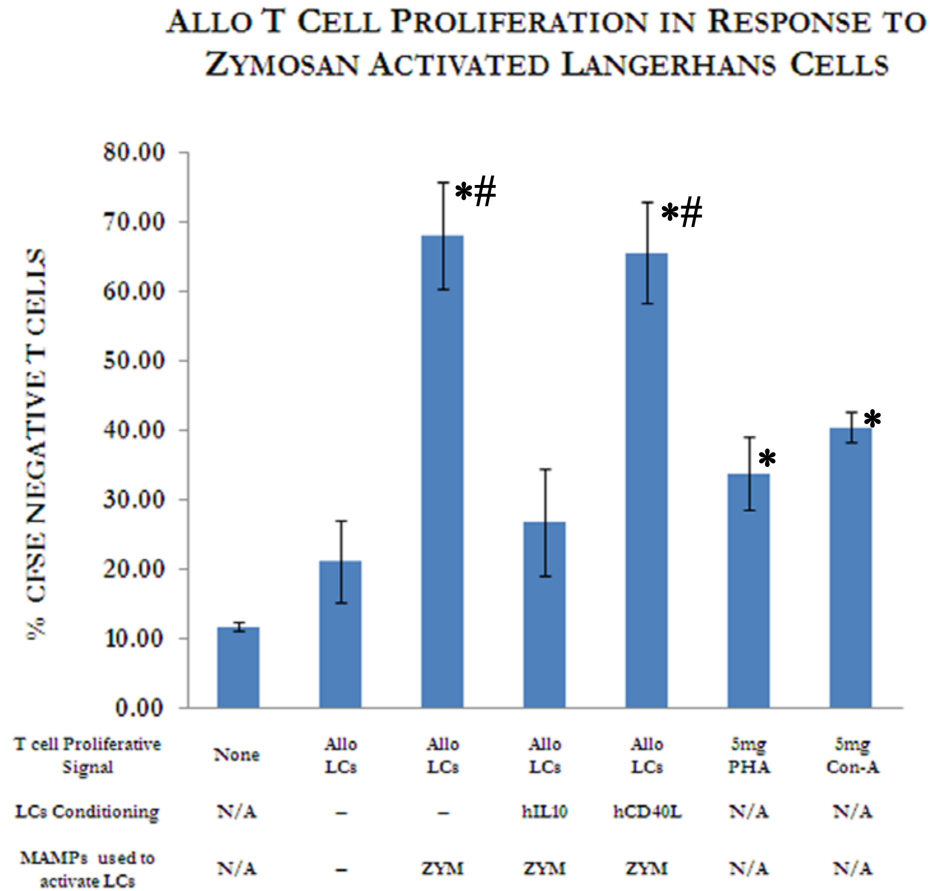
Figure IV-28: Effect of cytokine conditioning on Allo T cell proliferation by Langerhans cell activated by ECLPS



* – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls

The allo T cell proliferative capacity of differentially conditioned and ECLPS challenged LCs is depicted in figure 28. We show here that ECLPS challenged LCs when co-cultured with allo T cells induces a T cell proliferative response similar to PHA or Concavalin A (41% CFSE negative T cells). hIL-10 conditioning of the LCs prior to ECLPS challenge leads to reduction in the allo T cell proliferative capacity (30% CFSE negative T cells) but not to the background levels. CD40L conditioning of LCs before ECLPS challenge doesn't contribute to any additional increase in allo T cell proliferative capacity (41% CFSE negative T cells).

Figure IV-29: Effect of cytokine conditioning on Allo T cell proliferation by Langerhans cell activated by Zymosan

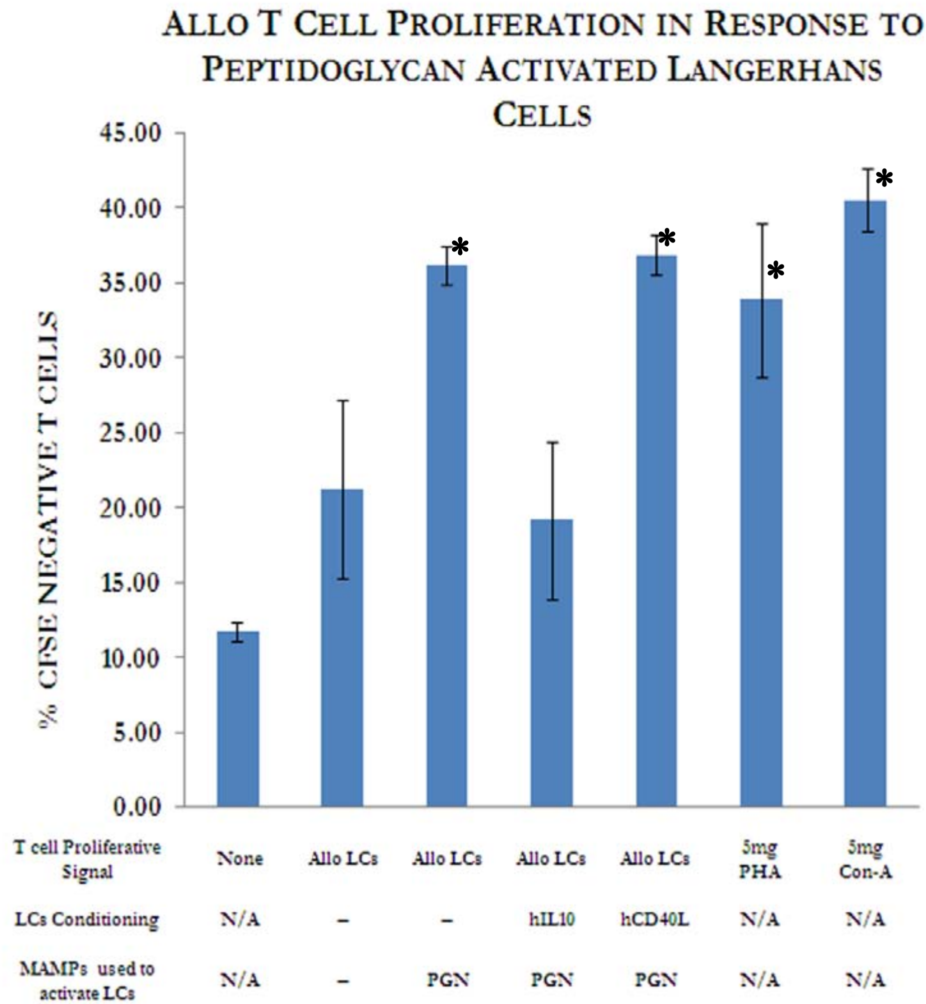


∗ – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls; ∗# – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls

The allo T cell proliferative capacity of differentially conditioned and Zymosan challenged LCs is depicted in figure 29. We show here that Zymosan challenged LCs when co-cultured with allo T cells induces a robust T cell proliferative response even more than PHA or Concavalin A (69 % CFSE negative T cells). hIL-10 conditioning of the LCs prior to Zymosan challenge leads to reduction in the allo T cell proliferative capacity (25% CFSE negative T cells) almost similar to the background levels.

CD40L conditioning of LCs before Zymosan challenge doesn't contribute to any additional increase in allo T cell proliferative capacity as compared to zymosan challenge alone (68% CFSE negative T cells).

Figure IV-30: Effect of cytokine conditioning on Allo T cell proliferation by Langerhans cell activated by PGN



∗ – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls

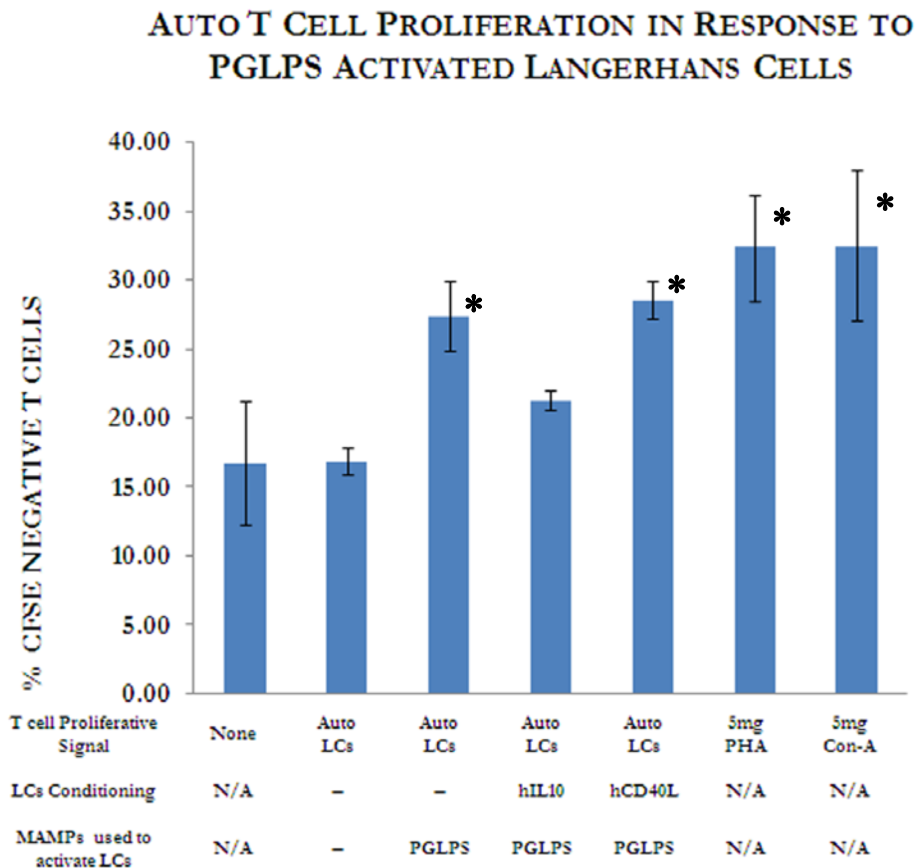
The allo T cell proliferative capacity of differentially conditioned and PGN challenged LCs is depicted in figure 30. We show here that PGN challenged LCs when co-cultured with allo-T cells induces a T cell proliferative response similar to PHA or Concavalin A (35% CFSE negative T cells). hIL-10 conditioning of the LCs prior to PGN challenge leads to reduction in the allo T cell proliferative capacity (19% CFSE negative T cells) slightly less than background levels. CD40L conditioning of LCs before PGN challenge doesn't contribute to any additional increase in allo T cell proliferative capacity (36% CFSE negative T cells).

Auto T cell response

Auto T cell proliferating capacity of MAMPs challenged LPS under different micro-environment al conditions were analyzed with help of the CFSE protocol discussed before. The following section described the results of the analysis. The figures 31 - figure 34 shows in the Y axis the increase in percentage of CFSE negative Auto T cells after co-culturing these cells with the differentially activated Langerhans cells. The X axis describes the different conditioning agents the LCs were before being subjected to MAMPs challenge which are also labeled in the x axis. 5µg PHA and 5µg concavalin A was used as positive controls for analyzing T cell proliferation. T cells alone or T cells with untreated Langerhans cells were used as negative controls. The statistical significance of the differences between the experimental conditions was analyzed with a help of a paired student T Test with equal variance.

The results show that auto-T cells had a basal proliferative rate (15% CFSE negative T cells) in the medium. Auto-T cells cultured with untreated Langerhans cells did not induce any auto-T cell proliferation (16% CFSE negative T cells). Concavalin and PHA induced a proliferation response of about 32% CFSE negative T cells.

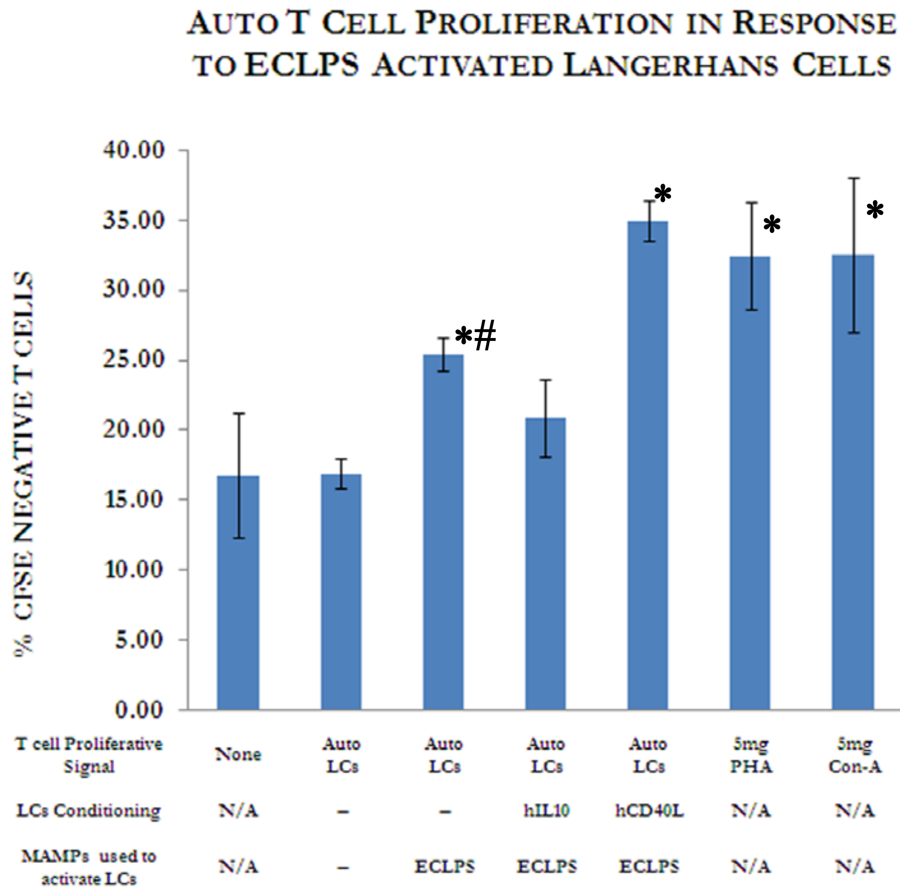
Figure IV-31: Effect of cytokine conditioning on Auto T cell proliferation by Langerhans cell activated by PGLPS



∗ – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls

The Auto-T cell proliferative capacity of differentially conditioning and PGLPS challenged LCs is depicted in figure 31. We show here that PGLPS challenged LCs when co-cultured with auto T cells induces a modest auto T cell proliferative response which was less than PHA or Concavalin A (27 % CFSE negative T cells). hIL-10 conditioning of the LCs prior to PGLPS challenge leads to reduction in the T cell proliferative capacity bringing it down to almost similar to untreated LCs (20% CFSE negative T cells). CD40L conditioned LCs before PGLPS challenge did not add to the increase in T cell proliferative capacity (26% CFSE negative T cells).

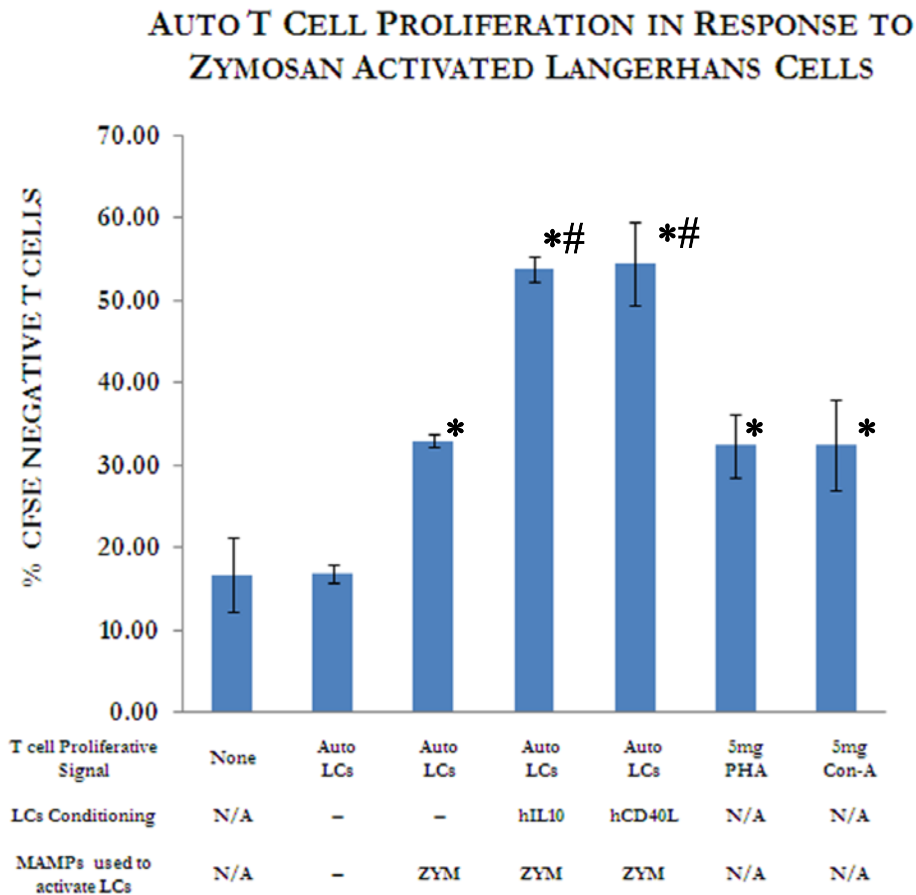
Figure IV-32: Effect of cytokine conditioning on Auto T cell proliferation by Langerhans cell activated by ECLPS



∗ – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls; ∗# – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls

The auto T cell proliferative capacity of differentially conditioning and ECLPS challenged LCs is depicted in figure 32. We show here that ECLPS challenged LCs when co-cultured with auto T cells induces a modest T cell proliferative response lesser than PHA or Concavalin A (25% CFSE negative T cells). hIL-10 conditioning of the LCs prior to ECLPS challenge leads to reduction in the T cell proliferative capacity (21% CFSE negative T cells) almost to the background levels.CD40L conditioning of LCs before ECLPS challenge increases in T cell proliferative capacity (35% CFSE negative T cells).

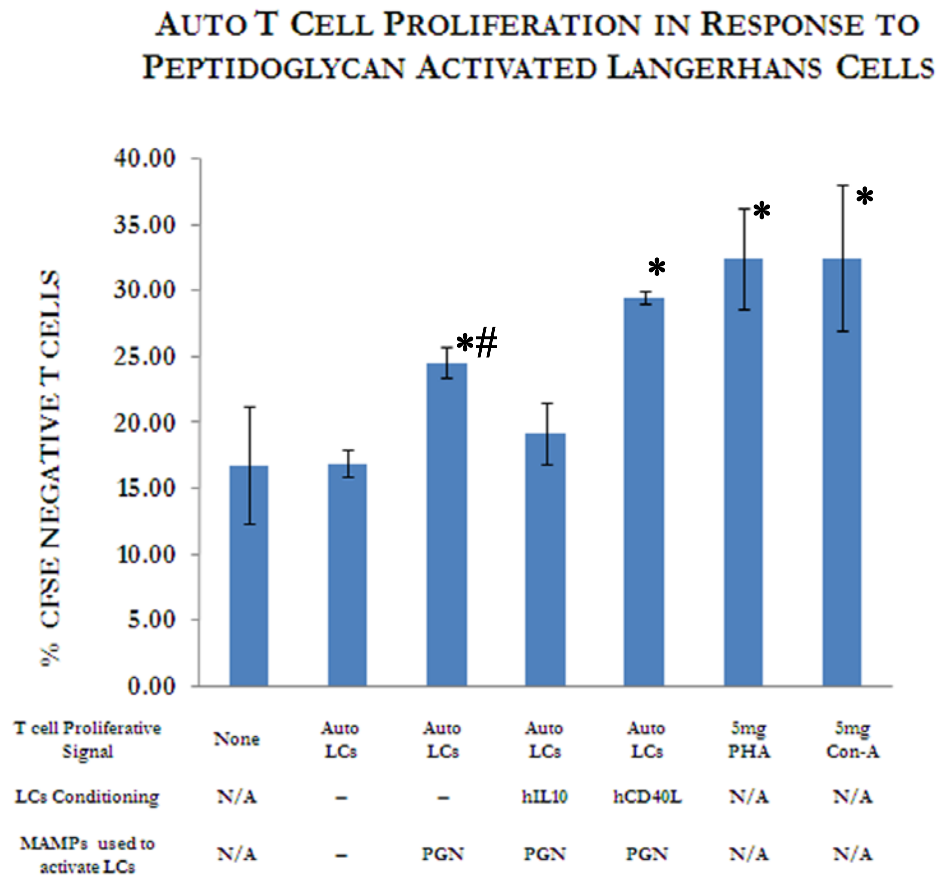
Figure IV-33: Effect of cytokine conditioning on Auto T cell proliferation by Langerhans cell activated by Zymosan



∗ – Statistical significance with a P value of ≤ 0.05 as compared to untreated controls*;* ∗# – Statistical significance with a P value of ≤ 0.05 as compared to untreated controls

The auto T cell proliferative capacity of differentially conditioned and Zymosan challenged LCs is depicted in figure 33. We show here that Zymosan challenged LCs when co-cultured with auto T cells induces a robust T cell proliferative response similar to PHA or Concavalin A (33% CFSE negative T cells). hIL-10 conditioning of the LCs prior to Zymosan challenge leads to a robust increase in the T cell proliferative capacity (52% CFSE negative T cells) even more than PHA or Concavalin A treatment. CD40L conditioning of LCs before Zymosan challenge also leads to a robust increase in T cell proliferative capacity as compared to zymosan challenge alone (52% CFSE negative T cells).

Figure IV-34: Effect of cytokine conditioning on Auto T cell proliferation by Langerhans cell activated by PGLPS



∗ – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls; ∗# – Statistical significance with a P value of $\leq$ 0.05 as compared to untreated controls

The auto T cell proliferative capacity of differentially conditioned and PGN challenged LCs is depicted in figure 34. We show here that PGN challenged LCs when co-cultured with auto T cells induces a modest T cell proliferative response less than PHA or Concavalin A (24% CFSE negative T cells). hIL-10 conditioning of the LCs prior to PGN challenge leads to reduction in the auto T cell proliferative capacity (17 % CFSE negative T cells) almost similar to background levels. CD40L conditioning of LCs before PGN challenge causes a slight increase in auto T cell proliferative capacity (31% CFSE negative T cells).

Summary of Results II

Surface markers of LC activation:

Our results indicate that conditioning of LCs with help of rhIL-10 makes LCs unresponsive to MAMP challenge (figure 3, 6, and 9) as evidenced by the lack of up regulation of activation marker CD83 (figure 3) and co stimulatory molecules CD86 (figure 6) and CD80 (figure 9) in response to any of the four different MAMPs after rhIL-10 conditioning. In fact rhIL-10 conditioning of LCs results in a modest down regulation of CD83 (figure 2) and profound downregulation of CD86 (figure 5) below constitutive levels and remains at these lower levels after MAMP stimulation (figure 3 and 6). CD80 levels remain similar to constitutive levels after rhIL-10 conditioning (figure 8), and MAMP challenge of these conditioned cells also express CD80 at constitutive levels except for zymosan challenge which induces a downregulation of CD80 (figure 9).

CD40L, TNF-α and IL1β conditioning fails to up regulate CD83 (figure 2) and CD80 (Figure 8) in LC, but CD40L and TNF α induce a slight up regulation of CD86 (figure 5).

CD40L conditioned LCs when challenged with MAMPs resulted in the following interesting and unexpected results.

1. CD40L conditioned LC when challenged with any of the four different MAMPs fails to up regulate CD83 (figure 4) and CD86 (figure 7). In fact Zymosan challenge of the CD40L conditioned LC induce a downregulation of CD83 and CD 86 as seen in figure 4 and 7 respectively.
2. The different MAMPs induced a differential CD80 expression on CD40L conditioned LCs as seen in figure 10. ECLPS induced up regulation of CD80 remain unaffected by CD40L conditioning of LCs. On the other hand CD40L conditioned LCs express less CD80 when challenged with PGLPS or PGN as compared to just PGLPS or PGN stimulated LC that are not conditioned with CD40L, indicating an interference of PGLPS and PGN signaling in CD40L conditioned LC. Zymosan again induces a downregulation of CD80 expression in CD40L conditioned LC.

Summary of the effect of conditioning on cytokine secretion by LC

IL-1β:

Both rhIL-10 conditioned LCs and CD40L conditioned LC secrete less IL-1β when challenged with any of the four MAMPs as compared to unconditioned LCs challenged with the MAMPs but more than conditioned LCs with no MAMP challenge, indicating there is some interference in the MAMP induced IL-1β gene expression when the LCs are conditioned with either rhIL-10 or CD40L as shown in figures 11- 14. LC conditioned with rhIL-10 fail to secrete any IL-1β when stimulated with ECLPS.

IL-10:

Similar to IL-1β, both rhIL-10 conditioned LCs and CD40L conditioned LC secrete less IL-10 when challenged with any of the four MAMPs as compared to unconditioned LCs challenged with the MAMPs but more than CD40L conditioned LCs with no MAMP challenge, indicating there is some interference in the MAMP induced IL-10 gene expression when the LCs are conditioned with either rhIL-10 or CD40L as shown in figures 23-26. CD40L conditioning by itself slightly up regulates IL-10 secretion.

IL-6:

The CD40L conditioning of LC also results in interference in the MAMPs induced IL-6 gene expression as seen in figures 19-22. MAMPs activated LC secreted more IL-6 than CD40L conditioned LC challenged with MAMPs, LC conditioned with rhIL-10 fail to secrete any IL-6 when stimulated with PGLPS and PGN and only a very slight up regulation of IL-6 in response to ECLPS. rhIL-10 conditioning fails to abolish zymosan induced IL-6 in LC although a modest reduction does occur compared to unconditioned LC challenged with zymosan.

TNF-α:

TNF-α secretion from LCs differed from the other cytokines as seen in figures 15-18. There was no interference in MAMPs induced TNF-α expression in CD40L conditioned LCs except for PGN challenge. In fact CD40L conditioning appears to enhance TNF-α secretion in response to all the MAMPs challenge except for PGN. PGN challenge of CD40L conditioned LC result in reduced TNF-α as compared to no conditioning. CD40L conditioning increases TNF-α secretion in LCs. rhIL-10 conditioning on the other hand completely abolishes MAMPs induced TNF-α secretion by the LC.

Allo and Auto T cell proliferation:

As seen in figures 26- 30 and figures 31-34 CD40L pre-conditioning doesn't add to the allo and auto T cell proliferative capability of the MAMPs activated LC. On the other hand rhIL10 conditioning considerably reduced the allo and auto T cell proliferative capacity of LC even though they are challenged with the MAMPs.

Chapter V

Aim - III

Effect of a conditioning micro-environment on immune responses of Langerhans cells to Oral MAMPS

Introduction:

Background & rationale:

The results for Aim I & Aim II indicated that the $CD34^+$ derived $Langerin^+$ LC can respond to different MAMPs through the repertoire of PRRs that they express. LC are also capable of inducing a modest T cell proliferation after being activated by MAMPs through the co-stimulatory activity and the cytokines that they secrete. Furthermore these MAMPs induced activation, cytokine response and ability to proliferate T cells can be modulated by immune modulators like CD40L and IL-10.

Although hIL-10 is a regulatory cytokine whose main function is containment and eventual termination of an inflammatory response, it also plays a role in B cell development and differentiation [112]. In the context of periodontal disease such a stimulatory response may not be desirable. vIL-10 cannot enhance class II MHC molecules on B cells, and it cannot proliferate thymocytes and mast cells like cellular IL-10 [113, 151]. It also doesn't have co-stimulatory activity on

T cells. Thus vIL-10 has some advantages over hIL-10, hence we wanted to explore if the modulatory effect of hIL-10 can be reproduced with vIL-10.

Furthermore the context of the micro-environment in which the immune activation is important for the eventual outcome of tolerance vs. immuno-stimulation as discussed in detail in the introduction section. Keratinocytes the primary and predominant cell of the epithelium has been shown to have key roles in immune responses and in fact they are also considered as key mediators of inflammation [6].These cells are also known to co-ordinate their action with LC. Hence we were interested in exploring LC modulation in the context of an epithelial micro-environment. Thus we formulated the following specific aim and hypothesis to study the above stated objectives.

Aim - III:

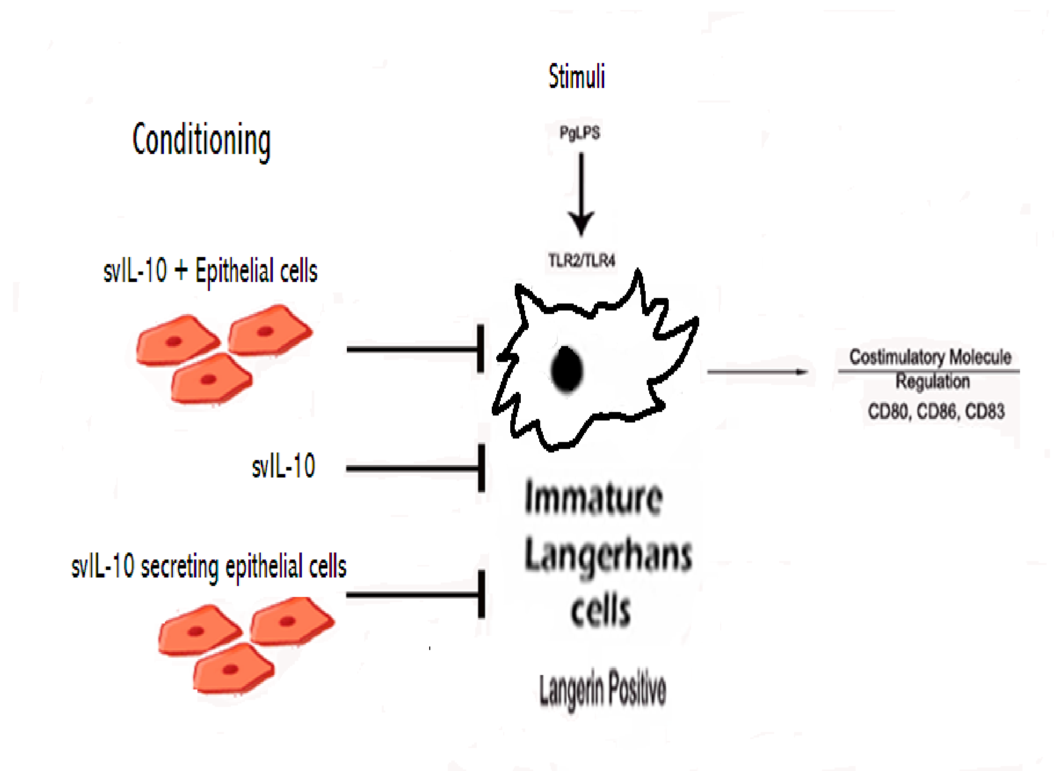
To develop a genetically engineered conditioning epithelial cell micro-environmental model to study and modulate Langerhans cell responses in vitro

Specific Hypotheses:

Expression of activation markers, antigen presenting molecules by LCs in response to microbial ligand can be modulated by genetically engineered conditioning epithelial cell micro-environment

Experimental Design

Figure V-1: Experimental Design for AIM III



Conditioning Protocol:

Isolated immature Langerhans cells are conditioned with one of the following conditioning protocol. Cell culture supernatants of svIL-10 secreting epithelial cells (secreting at a constant rate of approximately 50ng/hr/ml/million cells were collected after 1 hour incubation in fresh culture media. These supernatants were then analyzed with ELISA to confirm the concentration of svIL-10 present in them (50ng/ml). The Langerhans cells were incubated for 24 hrs in a 5% CO_2 incubator at 37^0C along with either the svIL-10 containing

medium adjusted with fresh culture medium to have a final concentration of 50ng/ml alone or with epithelial cells and the medium with a final concentration of 50ng/ml of svIL-10. LC are also incubated with epithelial cells which secrete a svIL-10 at a constant rate of 50ng/hr/ml/million cells Thus conditioned cells were then challenged with 1000pg of PGLPS as per the challenge protocol described in a later section.

Selective inhibitors of LPS activation:

To elucidate if the action of IL-10 (both rhIL-10 as well as svIL-10) is through inhibition of the NF kappa B (NF-κB) pathway or the p38 MAPK pathway LCs were inhibited with 2 selective inhibitors of these different pathways and then subsequently challenged with 1000pg of LPS as per the challenge protocol described in the following section. Towards this SB202190 which is a selective inhibitor of p38 MAPK [177, 185] pathways was used along with PDTC which is widely used as an inhibitor of NF-κB activation [176, 178, 179].

Challenge Protocol:

The conditioned Langerhans cells were the further challenged with 1000pg of PGLPS i.e., the cells were incubated with 1000pg of PGLPS for 24 hours at 37^0C in a 5% CO_2 incubator as seen in chapter III and IV.

Experimental Readouts:

The modulation of innate immune response by the conditioning protocol was analyzed by elucidating the change in co-stimulatory molecule expression by the LC.

Co-stimulatory molecules:

We hypothesized that conditioning of Langerhans cells will change the response of the Langerhans cells to stimulation by PGLPS just as was seen with hIL-10 in chapter IV. Thus we expected that the Langerhans cells will become unresponsive to PGLPS in the presence of svIL-10. This unresponsiveness will be observed even in the presence of epithelial keratinocytes. This down-modulation or up-regulation of activation of Langerhans cells can be assessed by measuring the regulation of co-stimulatory molecules expressed on the surface of Langerhans cells. We decided to use flow-cytometry based analysis and quantification of the change in expression of CD83, CD86, and CD80 to assess

the response of Langerhans cells. Thus, the cells were incubated with PGLPS for 24 hours after conditioning with the conditioning micro-environment and were then spun down, stained with fluorescence conjugated antibodies, and then fixed. Appropriate negative controls were included. These cells were then analyzed with help of flow-cytometry as described in the materials and methods chapter.

Results:

Co-stimulatory Molecules

CD83

Figure V-2: Effect of micro-environmental conditioning on CD83 expression on Langerhans cell response to MAMPs

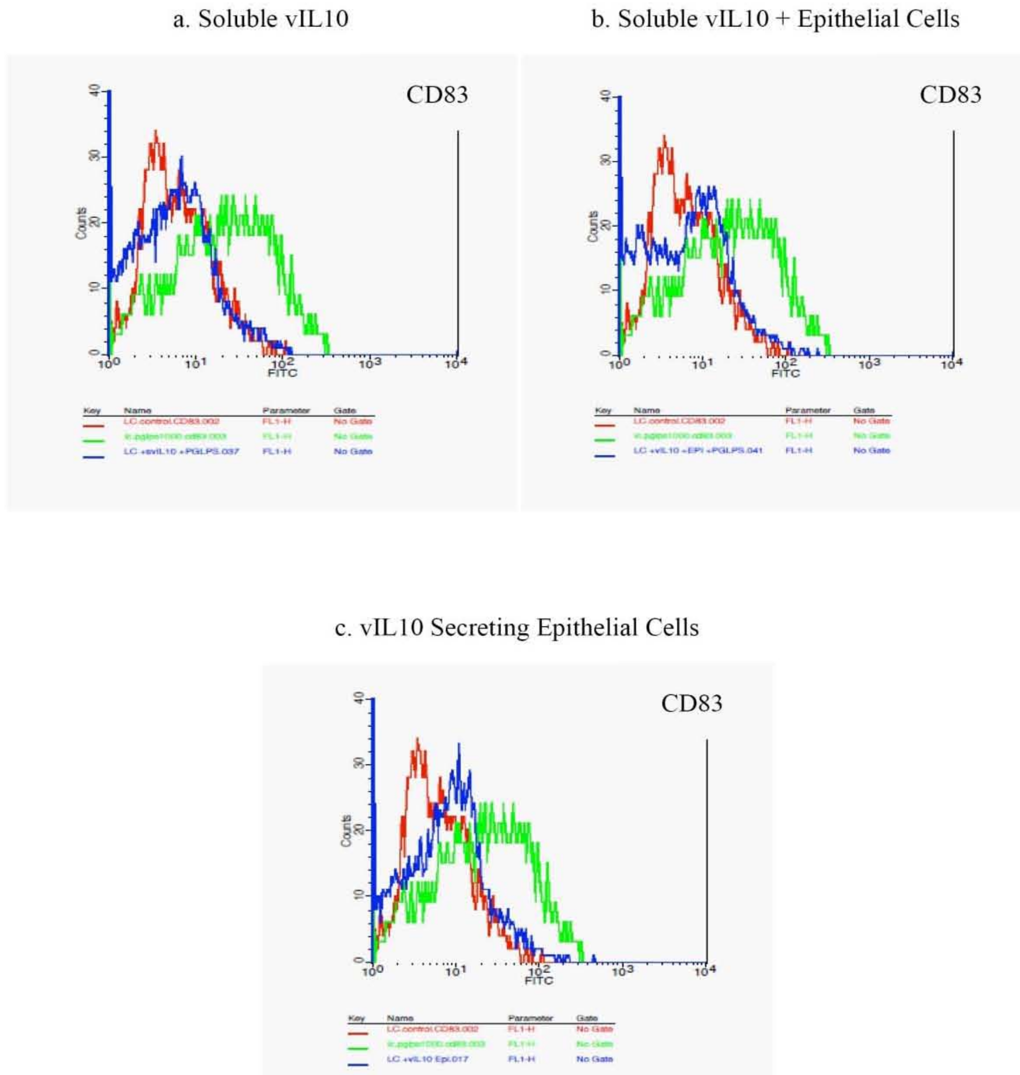


Figure Legend

Isotype

Untreated LC

LC + MAMP

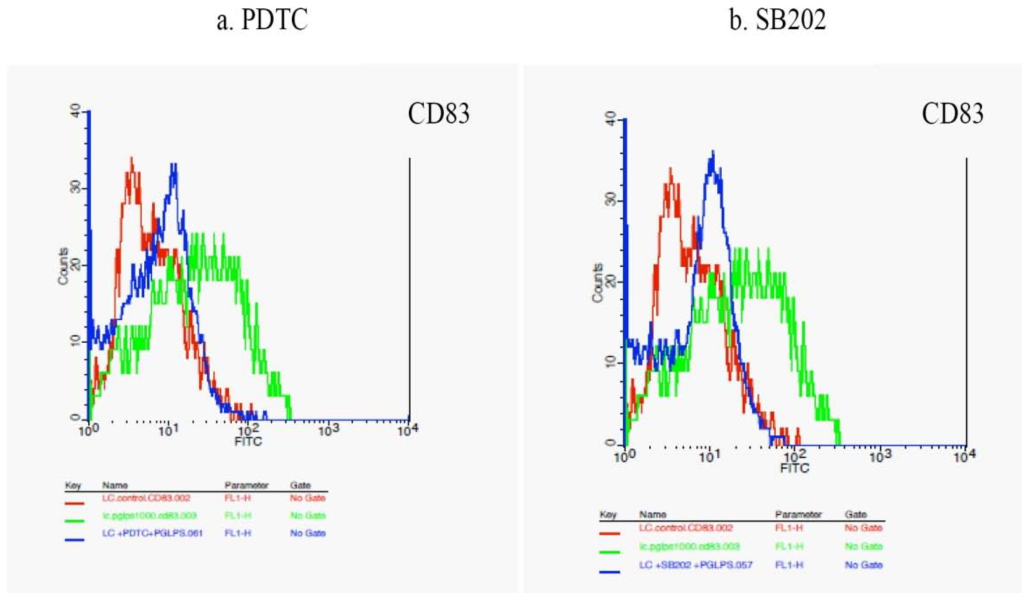
Conditioned LC + MAMP

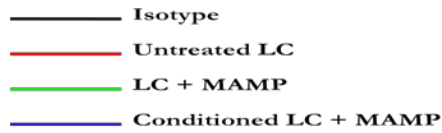
As seen in earlier chapters Langerhans cells stimulated with 1000pg dose of PGLPS increased surface expression of CD83 substantially (Figure 2). When the Langerhans cells were conditioned with svIL-10, and subsequently challenged with 1000pg of PGLPS, they failed to up-regulate the surface expression of CD83 (figure 2a).

Similarly when the Langerhans cells are conditioned with svIL-10 in an epithelial micro-environment, and subsequently challenged with 1000pg of PGLPS, CD83 up regulation by PGLPS is inhibited and it remains at constitutive levels (figure 2b).

When Langerhans cells are conditioned by co-culturing them with vIL-10 secreting epithelium and subsequently challenged with 1000pg of PGLPS, CD83 up regulation by PGLPS is again inhibited and the expression remains similar to constitutive levels (figure 2c).

Figure V-3: Effect of Blocking PGLPS Downstream Signaling on CD83 expression Langerhans Cells



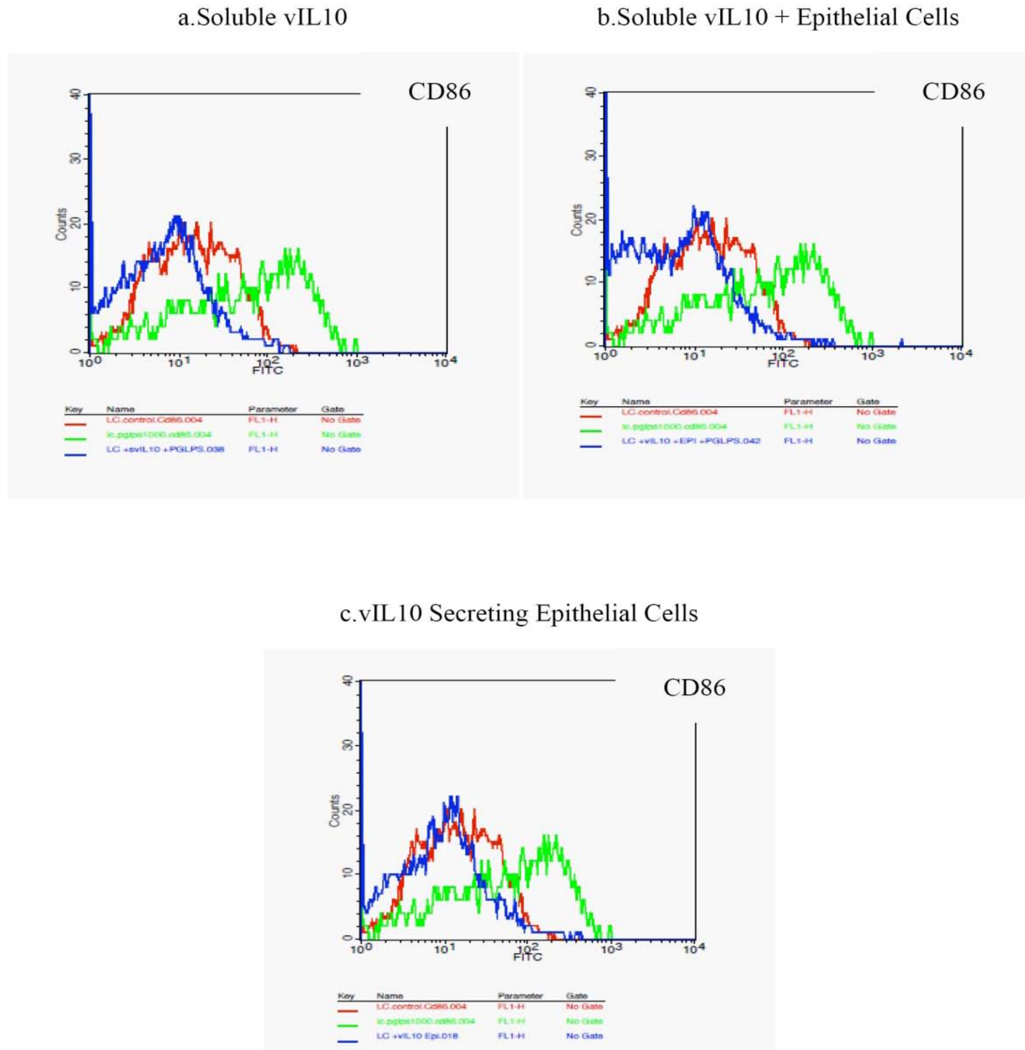


Langerhans cells which were treated with PDTC and subsequently challenged with PGLPS completely abolished PGLPS mediated up regulation of CD83 expression as shown in figure 3a.

Similarly Langerhans cells when treated with SB202 and subsequently challenged with PGLPS remain completely unresponsive to PGLPS mediated up regulation of CD83 expression as shown in figure 3b.

CD86

Figure V-4: Effect of micro-environmental conditioning on CD86 expression on Langerhans cell response to MAMPs



As seen in earlier chapters Langerhans cells stimulated with 1000pg dose of PGLPS increased surface expression of CD86 substantially (Figure 4). When the Langerhans cells were conditioned with svIL-10, and subsequently challenged with 1000pg of PGLPS, they failed to up-regulate the surface expression of CD86 and in fact the expression of CD86 was down regulated well below constitutive levels (figure 4a).

Similarly when the Langerhans cells are conditioned with svIL-10 in an epithelial micro-environment, and subsequently challenged with 1000pg of PGLPS, CD86 up regulation by PGLPS is inhibited and is actually down regulated well below constitutive levels (figure 4b).

When Langerhans cells are conditioned by co-culturing them with vIL-10 secreting epithelium and subsequently challenged with 1000pg of PGLPS, CD86 up regulation by PGLPS is again inhibited and more over CD86 expression was down regulated below constitutive levels (figure 4c).

Figure V-5: Effect of Blocking PGLPS Downstream Signaling on CD86 expression Langerhans Cells

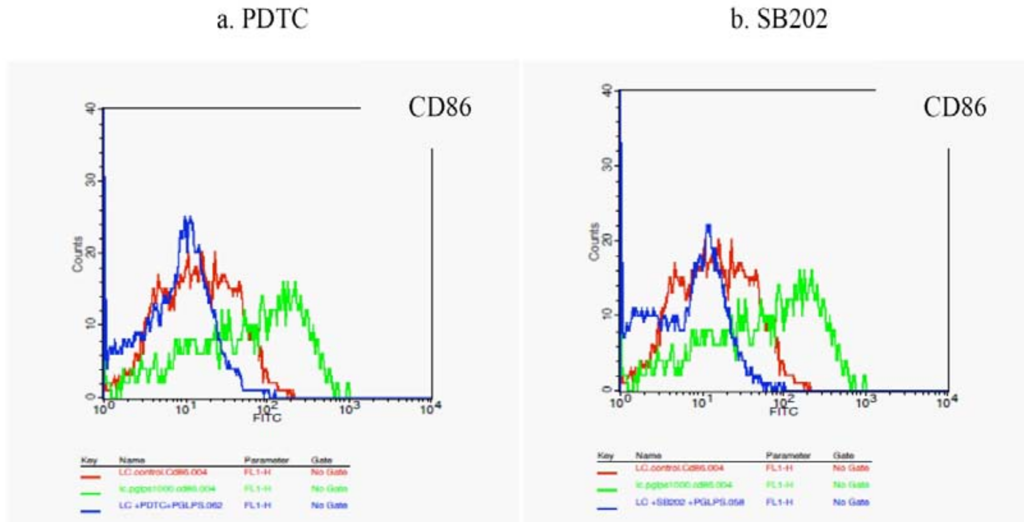


Figure Legend

- Isotype
- Untreated LC
- LC + MAMP
- Conditioned LC + MAMP

Langerhans cells which were treated with PDTC and subsequently challenged with PGLPS completely abolished PGLPS mediated up regulation of CD86 expression as shown in figure 5a. In fact treatment with PDTC causes a significant downregulation of CD86 expression on these Langerhans cells.

Similarly Langerhans cells when treated with SB202 and subsequently challenged with PGLPS remain completely unresponsive to PGLPS mediated up regulation of CD86 expression as shown in figure 5b. In fact treatment with SB202 causes a significant downregulation of CD86 expression on these Langerhans cells.

CD80

Figure V-6: Effect of micro-environmental conditioning on CD80 expression on Langerhans cell response to MAMPs

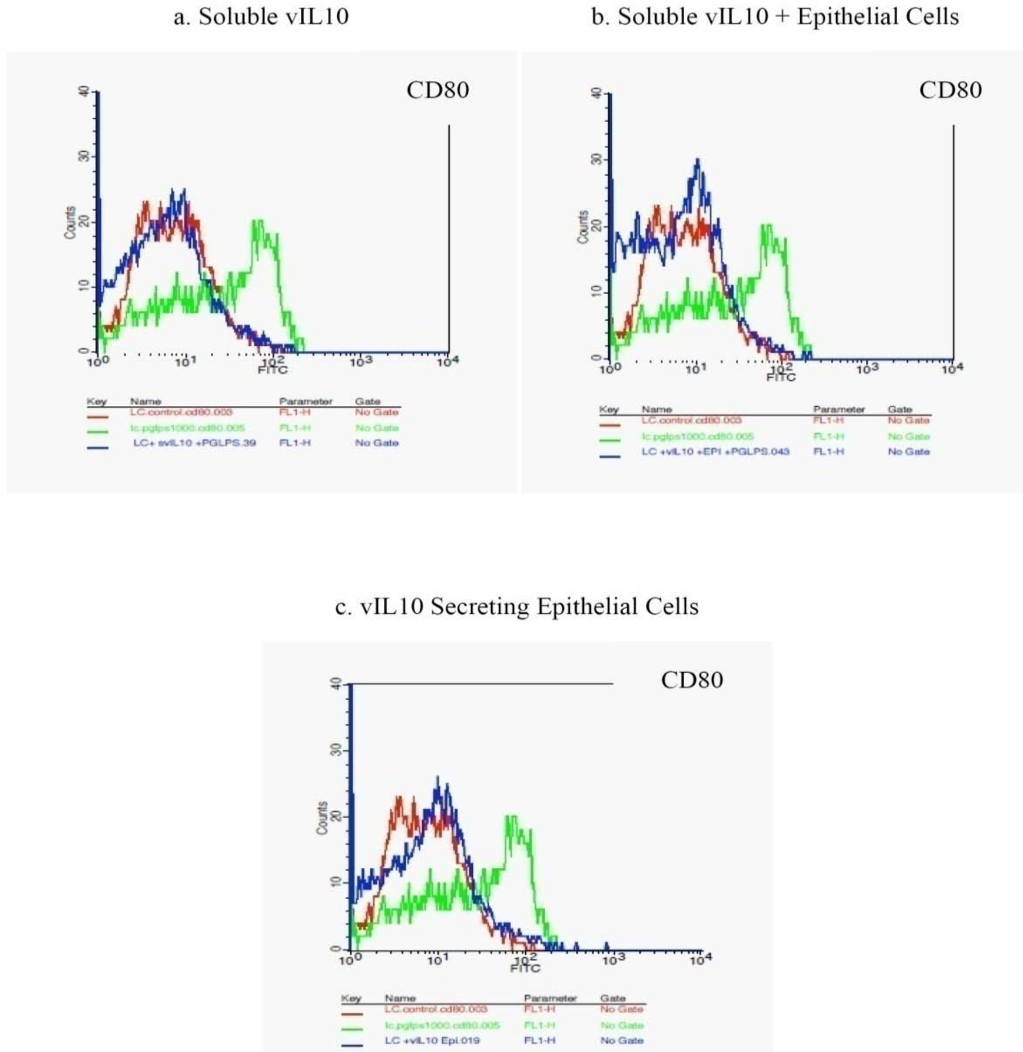


Figure Legend

- Isotype
- Untreated LC
- LC + MAMP
- Conditioned LC + MAMP

Langerhans cells stimulated with 1000pg dose of PGLPS increased surface expression of CD80 substantially (Figure 6) as shown in earlier chapters. When the Langerhans cells were conditioned with svIL-10, and subsequently challenged with 1000pg of PGLPS, they failed to up-regulate the surface expression of CD80 (figure 6a).

Similarly when the Langerhans cells are conditioned with svIL-10 in an epithelial micro-environment, and subsequently challenged with 1000pg of PGLPS, CD80 up regulation by PGLPS is inhibited and it remains at constitutive levels (figure 6b).

When Langerhans cells are conditioned by co-culturing them with vIL-10 secreting epithelium and subsequently challenged with 1000pg of PGLPS, CD80 up regulation by PGLPS is again inhibited and the expression remains similar to constitutive levels (figure 6c).

Figure V-7: Effect of Blocking PGLPS Downstream Signaling on CD80 expression Langerhans Cells

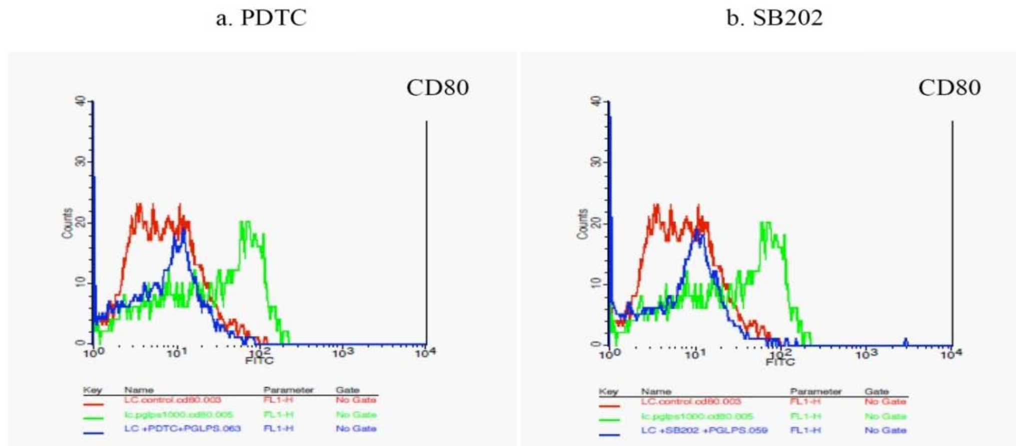


Figure Legend

- Isotype
- Untreated LC
- LC + MAMP
- Conditioned LC + MAMP

Langerhans cells which were treated with PDTC and subsequently challenged with PGLPS completely abolished PGLPS mediated up regulation of CD80 expression as shown in figure 7a. In fact treatment with PDTC causes a slight downregulation of CD80 expression on these Langerhans cells.

Similarly Langerhans cells when treated with SB202 and subsequently challenged with PGLPS remain completely unresponsive to PGLPS mediated up regulation of CD80 expression as shown in figure 7b. In fact treatment with SB202 causes a slight downregulation of CD80 expression on these Langerhans cells.

Summary of Results III

The results of conditioning with either svIL-10; or a modulating epithelial micro-environments in which svIL-10 is exogenous added; or a modulating svIL-10 secreting epithelial micro-environment; suggest that all the three conditioning are similar to the results obtained with rhIL-10 conditioning. In other words, MAMP induced CD83 and CD80 activation is completely abolished and remains at constitutive levels as depicted in figures 2 and 6. CD86 on other hand is down-regulated by all the three conditioning environment below constitutive levels and MAMPs challenge fail to up regulate CD86 expression and remains lower than constitutive levels as shown in figure 4.

P38 MAPK inhibitor SB202190 and NF-κB inhibitor PDTC also cause a similar abolishment of MAMP mediated up regulation of CD83 (figure 3) and CD80 (figure 7) and down regulation of CD86 (figure 5) in LCs indicating that IL-10 acts through these pathways to modulate the expression of co-stimulatory and activation markers.

DISCUSSION

Langerhans cells: the ambassadors of the immune system

Langerhans cells have been described as the sentinels of the epithelium [53]. The evolving fascinating story of LC suggests that these cells have a more complex role than just alerting the host to unwanted pathogens. LC/DC can negotiate the interaction of the host with surrounding environment in a calibrated way so as to enable the host to determine the exact type of response that is warranted to the environmental challenge. In other words LC/DC can play a crucial role in the determination of tolerance vs. immunogenic response based on the micro-environmental factors that they are presented with [186].

The sequential signals provided by antigen presenting cells, like LC/DC, to T cells determine the type of immune response that is generated. The first signal, which determines the specificity of the immune response, is mediated by the interaction of the T cell receptor with the MHC molecule with specific antigen. The second signal determines whether a response is necessary and it involves co-stimulatory or co-inhibitory responses involving interaction of CD80 and CD86 molecules with either CD28 or CTLA on T Cells. In other words, absence of co-stimulation or presence of co-inhibition could make T cells unresponsive to the specific antigen presented by the MHC molecules and is one

of the many ways by which peripheral tolerance or anergy is generated. The third signal is mediated through cytokines secreted by the dendritic cell which stimulates $CD4^+$ T cells to develop into T_H1, T_H2, or T_R cells [14, 99]. The sequential signals provided by the antigen presenting cells are triggered by MAMP activation of PRRs expressed by LCs/DCs [187].

The type of MAMPs that trigger the PRR plays a role in the polarization of LCs/DCs into triggering the different T cells response. The microbial triggered activation of LCs/DCs into different T helper polarizing APCs is explained by the activation model of T cell polarization.

Additionally the context in which LCs/DCs activation occurs provides instruction in the polarization of APC function. For example the presence of IFN-γ or IL-10 in the micro-environment instructs the APCs to polarize the T cell response to T_H1 or T_H2 type of response. This is described as the instructional model of T cell polarization [188].

An additional level of regulation in the LC/DC polarization is the expression of the PRRs. Dendritic cells are currently accepted as comprising 3 distinct subpopulations, including two within the myeloid lineage (Langerhans cells and dendritic cells) and one within the lymphoid lineage (plasmacytoid dendritic cells) [186]. An important caveat in this concept is that these DC subsets exhibit functional plasticity despite the occurrence of a certain degree of dendritic cell sub lineage commitment [189] which can be taken advantage of in the

immuno-modulation of these cells. In general human LCs have been shown to express all TLRs except TLR9 [188, 190]. On the other hand plasmacytoid DC express TLR1, TLR6, TLR7, and TLR9 [188, 190].

Thus lack of expression of PRRs can make DC/LC to be of a polarizing phenotype at the outset. In fact in vitro generated monocyte derived Langerhans cells do not express TLR2 and TLR4 and have been shown to be unresponsive to bacterial PRRs [191].

As mentioned in earlier sections the role of Langerhans cells in oral mucosal inflammatory diseases including periodontal diseases have been restricted mostly to structural and histo-morphometric studies. The existing studies on LCs in the oral mucosal context has been summarized by Cutler and Jotwani (2006) [186] and are as follows.

1. The highest Langerhans cells numbers are found in non-keratinized mucosa of the soft palate, ventral tongue, lip, and vestibule, while the lowest counts are found in keratinized mucosa of the hard palate and gingiva [71]

2. Langerhans cells in the gingival epithelium are very responsive to the accumulation of bacterial plaque (*i.e.*, the biofilm), migrating into the site during early gingivitis [83, 192] and migrating out as the gingivitis becomes more chronic (*i.e.*, after 21 days) [193] ;

3. Oral mucosal Langerhans cells are also responsive to nickel in patients with nickel allergies [194], to oral Candida sp. [195, 196] , oral lichen planus [85,

197-200]; lichenoid drug eruptions [201], rhomboid median glossitis [202], Verruciform xanthoma [203], HIV infection [204], oral squamous cell carcinoma [205] oral skin grafts [206], and hairy leukoplakia of the tongue [207];

4. Oral mucosal Langerhans cells appear properly oriented to sample the oral fluids and bacteria, with their dendrites toward the surface [76] ;

5. Oral mucosal bacteria (*e.g.*, *P. gingivalis* [208]) and viruses (*e.g.*, HIV [86]) gain access to Langerhans cells *in situ*;

6. Langerhans cells from oral mucosa co-express activation/maturation markers [209] and contribute to the mature CD83+ dendritic cell pool in the lamina propria during chronic adult periodontitis *in situ* [81]; B-cells also contribute to the CD83+ pool in the lamina propria [210];

7. Langerhans cells isolated *ex vivo* induce a stronger allogeneic mixed-lymphocyte reaction response than do Langerhans cells from normal skin [211, 212]; and

8. Langerhans cells lie close to epithelial γδ- T-cell receptors in health [213], but in disease (chronic adult periodontitis) come close to lamina propria CD4+ T-cells [81].

Not many studies have been done on the interaction and the subsequent immune response of LC with oral microbes or their MAMPs.

Thus we studied the immune response of $CD34^+$ derived $Langerin^+$ Langerhans cells to oral MAMPs.

Effect of MAMPs on LC innate and immuno-stimulatory function:

Porphyromonas *gingivalis* is an important putative periodontal pathogen [208]. One of the important MAMPS of P. *gingivalis*, PGLPS has been extensively studied in the context of dermal dendritic cells by many groups [106-108, 172]. The interaction of PGLPS with Langerhans cells is largely unknown. Hence we wanted to compare this interaction with other oral MAMPs like Peptidoglycan, zymosan and the relatively well studied non-oral MAMP ECLPS. Here we have shown that $CD34^+$ Langerin+ Langerhans cells are relatively potent immunogenic cells unlike monocytes derived LCs and may be a better representative of oral mucosal Langerhans cells.

MAMP mediated regulation of co-stimulatory molecules and activation markers in LC

LC constitutively express high levels of MHC class II molecules. This has been utilized for phenotypic identification of LCs by many groups as discussed in the introduction section. We have shown here through flow cytometry that is indeed the case with $CD34^+$ derived $Langerin^+$ LC. LC when stimulated with the four different MAMPs in different doses all up regulates MHC class II molecules slightly but not in a statistically significant manner. Thus LC are primed to provide the first signal for T cell activation.

LC when stimulated with the four different MAMPs in different doses all up regulate co-stimulatory molecules CD86 and CD80 and the activation marker CD83. CD86 up regulation is greater than CD80 up regulation in LC stimulated with any of the four MAMPs. This indicates a slight bias of these LC towards a T_H1 type of response. The up regulation of CD83, CD86, and CD80 doesn't appear to be dose dependent based on the different doses of MAMPs used. The LC activation of CD83, CD86, and CD80 is consistent with MAMP activation of their different respective PRRs.

All TLRs have a core signaling pathway which leads to the activation of NF-κB and MAPKs. This core signaling begins with the recruitment of cytosolic

adapter molecule MyD88 to TIR domain of TLR, which is the intracellular domain of TLRs. MYD88 also has a TIR domain which interacts homo-typically with TIR domain of TLR. It also interacts with a death domain and through these interactions recruits member of the IL-1R-associated kinase (IRAK) family of serine/threonine kinases to the TLR/MyD88 complex [214]. Phosphorylation of IRAK -1 by IRAK 4 initiates a signaling cascade that utilizes TRAF6. This signaling ultimately leads to the activation of NF-κB, p38 MAPK and Jun kinase [188, 215-217].The activation of the NF-κB transcription factors leads to the up-regulated expression of CD86, CD80, and CD83 genes [218].

ECLPS and PGLPS which interact with LC through TLR4 and TLR4/2 respectively have been shown to up regulate the NF-κB pathway. We have shown here the presence and inducible up regulation of TLR4 transcripts in LCs. We were unable to detect any TLR2 transcripts in the LCs through real time PCR, although we did observe weak constitutive expression of TLR2 in the surface of LC by flow cytometry.

Nevertheless, Darveau et al have demonstrated that HEK cells transfected with TLR4 or TLR2 and bone marrow cells obtained from TLR2$^{-/-}$ and TLR4$^{-/-}$ mice responded to *P. gingivalis* LPS , demonstrating that *P. gingivalis* LPS can utilize both TLR2 and TLR4 [219]. They attribute the TLR4 signaling to the different Lipid A moieties present in LPS preparations. Other groups like Ogawa

et al assert that PGLPS signals through TLR4 through biochemical and functional studies [220].

Thus PGLPS up regulate co-stimulatory molecules and DC activation marker CD83 through TLR4 and possibly through TLR2. TLR4 and TLR2 expression has been demonstrated in LC freshly isolated from the epidermis [63]. The expression of low amounts TLR2 and absence of TLR4 has been demonstrated in monocytes derived LCs [221], but as discussed in the introduction true LCs are myeloid derived and hence it is not clear if monocytes derived LC can be truly representative of LCs in vivo.

PGN has been shown to be recognized by NOD1 [222-224] and NOD2 [224-226] through segments in its core structure. Langerhans are efficient phagocytic, and macro-pinocytic cells [59]. Thus they can take up extracellular antigens and deliver them into the cytosol where they can be recognized by intracellular PRRs like NOD1 and NOD2 etc. Interestingly just like TLRs the downstream signaling of NODs have been shown to be mediated through the NF-κB pathway. Nod1 and Nod2 physically interact with a common downstream signaling molecule, RIP2 (also known as RICK or CARDIAK), through homophilic CARD–CARD interactions [227]. RIP2 leads to the activation of NF-kB by interacting with I-κB kinase-gamma (IKKγ or NEMO) [228]. Nod1 has also been reported to activate JNK in response to invasive bacteria [49]. We have shown here the constitutive and inducible expression of NOD1 and NOD2 in the

LCs. Thus we can speculate that the up regulation of the analyzed co-stimulatory molecules and activation markers in LCs in response to PGN is mediated through these receptors.

Zymosan, a yeast cell wall derivative, has been shown to be recognized by dectin-1, a C-type Lectin receptor for β-glucans [229-233] which expressed in murine [232] and human [233] DCs in conjunction with TLR2 [234]. Zymosan has been shown to be actively phagocytosed by LCs [59]. Zymosan has been shown to induce regulatory DCs in mice that secrete abundant IL-10, but little or no IL-6 and IL-12 (p70), and induce impaired T cell responses. Such regulatory DCs appear to be induced via activation of TLR2- and dectin-1–dependent activation of ERK MAPK, which promotes IL-10 production [235].

Dectin 1 also has an immuno-receptor tyrosine-based activation motif (ITAM)-like motif in its intracellular portion that activates the kinase SYK (spleen tyrosine kinase) [236]. Dectin -1 and TLR2 appears to co-localize on binding with Zymosan [237] and could possibly form a signaling complex [238]. Dectin-1 has also been found to be associated with the tetraspanin CD63 indicating that it might be part of a tetraspanin-mediated supra-molecular signaling complex, such as has been implicated in T- and B-cell receptor signaling [238]. The TLR2–TLR6 ligand in zymosan is unknown, as these TLRs do not recognize β-glucan11 [234], but the pro-inflammatory cytokine secretion by zymosan triggered cells could be through NF-κB pathway [238]. Dectin 1

might also be able to trigger pro-inflammatory cytokine production independently of TLRs in certain cells, although the role of SYK in these responses has not been determined [239]. In addition, dectin-1-mediated phagocytoses is both TLR and SYK independent in macrophages [240, 241] indicating that novel signaling pathways are involved [238].

As discussed earlier we were unable to show the up regulation of up TLR2 and only weak constitutive expression of TLR2 in LC. We were also unable to show Dectin 1 in LC. The up regulation of co-stimulatory molecules indicates the involvement of NF-κB activation. This could possibly indicate the presence of novel signaling pathways in these LC which activate the NF-κB. The signaling pathways involved in Zymosan activation of LCs needs to be elucidated.

Thus we show here MAMP activation of LC makes them competent to provide the second signal necessary for T cell activation.

MAMP mediated regulation of cytokine secretion in LC:

LC stimulated with ECLPS or Zymosan secreted approximately 4 fold more IL-1β as compared to LC stimulated with PGLPS or PGN. LC stimulated with Zymosan secreted approximately 4 fold more IL-10 as compared to LC stimulated with PGLPS or PGN. LC stimulated with ECLPS on the other hand

secreted approximately 3 fold more IL-10 as compared to LC stimulated with PGLPS and PGN. Zymosan induced a profound TNF-α secretory response when used to stimulate the LCs. Zymosan stimulated LC secreted almost 4 fold more TNF-α as compared to LC stimulated with PGLPS or PGN. ECLPS stimulated LC secreted 2 fold more TNF-α as compared to LC stimulated with PGLPS or PGN. IL-6 and IL-8 secretion increased dramatically from constitutive levels after stimulation with four different MAMPs. The increase in levels was approximately the same irrespective of the MAMP used. The difference in the cytokine profile illustrates the difference in the signaling of the different PRRs that recognize these MAMPs.

TLR2, TLR4, NOD1, and NOD2 all have a core signaling through NF-κB and MAPK pathways resulting in the up regulation of pro-inflammatory cytokines. Thus levels of IL-6, IL8 remain more or less the same between the different MAMPs due to their transcription control through NF-κB and MAPK.

IL1β secretion from Zymosan and ECLPS are approximately the same and it reflects the multiple pathways that these ligands stimulate. Zymosan is known to co-localize with TLR2 and DEC-1 [237] which can both signal through the NF-κB pathway, thus the increase secretion of IL-1b could be due to the summation of these signals. Alternatively zymosan –DEC1 mediated activation could trigger cytokine independently of TLRs in these LC [238].

ECLPS activates through TLR4. TLR4 mediated signaling differs from other TLRs and especially differs from TLR2 due to difference in the adaptor molecules. TLR2 signals through MyD88 and ultimately results in the activation of NF-κB, p38 MAPK and Jun kinase [188, 215-217]. TLR4 recruits 3 additional adaptor proteins leading to difference in gene expression. MAL/TIRAP, TRIF/TICAM1 and TRAM [188, 215, 242-244].PGLPS induced secretion of IL-1β through activation of NF-κB. PGN on the other hand can induce IL-1β secretion through both NF-κB and caspase -1 mediated up regulation of IL-1β [245].

Differences in TNF-α secretion by different MAMPs can be similarly explained. Zymosan induces a much higher amount of TNF-α as compared to other MAMPs. This difference could potentially be explained by the additional triggering of NFAT by dectin 1 leading to increased secretion of TNF-α as previously shown by others [246]. The higher amount of IL-10 secretion triggered by Zymosan is depended on SyK (spleen tyrosine kinase) signaling [236-238, 240, 246].

Thus in general MAMP activation lead to up regulation of pro-inflammatory cytokine secretion, with the notable exception of up regulation of IL-10 especially with Zymosan. The IL-10 up regulation is part of the simultaneous feedback loop that is secreted in response to PRR activation as a

preparation to reduce the inflammation so as to avoid collateral damage to the tissue as discussed in the introduction section. Thus Langerhans are capable of producing the third signal to induce T cell proliferation.

T cell Proliferation:

The LC are able to induce a modest allo and auto T cell proliferation when they are stimulated with the different MAMPs. The activation of LC by MAMPs results in three signals that are necessary for the proliferation of T cells. High amounts of surface expression of HLADRII / MHC class II molecules, significant up regulation of co-stimulatory molecules and activation markers and profound up regulation of cytokines induce the modest allo and auto T cell proliferation. The increased up regulation of IL-10 by zymosan explains the higher amount of proliferation noted with zymosan stimulated LC as IL-10 acts as a growth factor for T cells [151].

Modulation of LC response by micro-environmental factors:

As discussed in the introduction hIL-10 and vIL-10 have the ability to condition antigen presenting cells and polarize them towards an anergic or tolerogenic pathways. We screened a number of immuno-stimulatory cytokines so as to use them as a positive control for immune modulation. In other words the cytokine would provide an additive effect on MAMP induced activation of LC. LC stimulated with CD40L secreted higher amounts of TNF-α as compared to rhIL-10 and rhIL-1β. LC conditioned with TNF-α caused a profound increase in IL-10 secretion, while CD40L stimulation led to only a modest up regulation of IL-10.

LC conditioned with CD40L profoundly increased IL-6 and secreted IL-6 in a slightly higher level than TNF-α and much higher than rhIL10 and rhIL-1β. There was only a slight up regulation of IL-1β secretion in response to CD40L or TNF-α conditioning. Interaction between CD40L and CD40 expressed on LC surface leads to the activation of NFκB pathways. This and the fact that CD40L induced higher inflammatory cytokine profile than the other conditioning agents used helped us make the choice of using CD40L as an additive to MAMP signaling.

Modulation of innate and immuno-stimulatory functions of LC activation in response to MAMPs by IL-10:

IL-10 exerts it's inhibitory effect on MAMP activated pro-inflammatory gene expression by several mechanisms [112] such as the activation of the heme oxygenase-1 carbon monoxide pathway [247] and inhibition of the NF-κB pathway [248], the inhibition of AKT activity [249] and the induction of B cell lymphoma-3 (Bcl-3) [250]. The ability of IL-10 to inhibit LPS-induced gene expression has been shown to be transcriptionally mediated via the inhibition of the NF-κB pathway or a post-transcriptional mechanism via destabilizing mRNA [251].

IL-10 mediates its inhibitory effects by binding to its receptor complex, which induces the activation of the cytoplasmic receptor-associated tyrosine kinases, JAK1 and Tyk2 [112], followed by signal transducer and activator of transcription 3 (STAT3) phosphorylation, homo-dimerization and translocation to the nucleus, where it binds to STAT-binding elements in the promoters of various IL-10-inducible genes [251].

Additionally IL-10 has been shown to inhibit LPS induced activation of monocytes through inhibition of p38 MAPK and ERK2 mediated signaling pathways [252]. Sato et al have shown that mitogen-activated protein kinases (MAPKs), extracellular signal regulated kinase 2 (ERK2), stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK), and p38*mapk*, are potentially

involved in IL-10-mediated selective suppression of TNF-α induced changes of the monocytes-derived DC properties [253]. Thus IL-10 suppresses almost all of the signaling pathways involved in MAMP activation of LC.

Hence it is not surprising that IL-10 is able to practically shut down LC response to all the four different MAMPs. As shown in the results section conditioning with hIL-10, vIL10 and conditioning vIL10 microenvironments are able to abolish MAMP induced activation of LC as evidenced by the suppression of CD83, CD80, and CD86 up regulation in these cells.

Moreover, these LCs fail to up regulate the secretion of pro-inflammatory cytokines like IL-1β, TNF-α, IL-6, and IL-8 in response to any of the MAMPs after conditioning with hIL-10.

IL-10 conditioning also inhibits auto T cell and allo T cell proliferation induced by MAMP activated LC similar to what has been demonstrated with DC previously by other groups [133, 140].

This conditioning effect of IL-10 murine epidermal Langerhans cell has been previously demonstrated by Ozawa et al [141]. Many studies have shown the conditioning and tolerogenic properties of IL-10 on dendritic cells [102, 111, 138, 249].

To our knowledge, almost no studies exist that demonstrate the tolerogenic potential of IL-10 on Langerhans cells especially in the context of oral MAMPs and the inflammatory diseases mediated by them.

IL-10 modulation of IL-6 is particularly important in two ways. IL-6 is implicated in the breakdown of T regulatory cell mediated tolerance [26]. Modulation of IL-6 secretion in the oral mucosa in response to periodontal opportunistic pathogens like P. *gingivalis* could prevent pathogenesis of periodontal tissue damaging T cell mediated inflammation. Moreover IL-6 directly mediates alveolar bone destruction in periodontitis [254]. Suppression of the IL-6 secretion could contribute towards the inhibition of progressive alveolar bone loss. In fact IL-10 has also been to suppress infection-stimulated bone resorption in vivo in murine model of periodontitis [105]. IL-1β and TNF-α have also been implicated in periodontal tissue destruction [255, 256]. Down modulation of these cytokines could thus provide a protective influence on the peridontium [255, 256].

Modulation of innate and immuno-stimulatory functions of LC activation in response to MAMPs by CD40L:

Human epidermal LC express functional CD40 and ligation of CD40 with CD40L leads to up regulation of CD86 on the surface of these LC [257]. CD34+ cord blood derived dendritic Langerhans cells express functional CD40 [258]. CD40 cross-linking activates these cells and induces morphologic & phenotypical changes, as well as secretion of a limited set of cytokines, namely TNF-a, IL-8, and macrophage inflammatory protein $1\text{-}\alpha$ [258].

We show here that highly purified LC not only up regulate TNF-α, but also IL-6 and to a limited extent IL-10 and IL-1β. This is consistent with activation of CD40 and subsequent downstream signaling through NFκB. Once CD40L binds to CD40 it associated with TRAF 2, 3, 5 and6 [259]. This in turn activates NFκB pathways and/or MAPK pathways including p38 MAPK, JNK, and ERK pathways [259]. CD40 in addition can bind and activate Janus family kinase 3, which results in signal transducer activation and activation of transcription [259].

CD40L also up regulated CD86 in LC which is consistent with earlier reports [258].

Traditional models in dendritic cell activation describe that final maturation of DC occurs when CD40L expressed on T cells bind to CD40 expressed on DC [258]. Recent evidence points towards a more complex nature of the interaction of CD40L with DC/LC [260]. Both CD40 as well as TLR-mediated proinflammatory signaling pathways use TRAF6 as adapter for signaling. This presents interesting possibilities for signaling cross-talk or cross-inhibition. Endotoxin tolerance where repeated stimuli with LPS induces tolerance [108] is observed with CD40 signaling as well [260]. Sinistro and colleagues found that stimulation of monocytes with LPS resulted in a reduced response to a subsequent stimulation via CD40 ligation, including reduced IL-12 and TNF-α production [261]. Importantly, similar results were seen when monocytes from patients undergoing sepsis were evaluated. Monocytes from septic patients showed similar defects in TNF-α , IL-12, and IL-1β production, as well reduced expression of CD80 and CD86 [262]. Suttles and Stout found that pre-incubation of monocytes/macrophages with CD154 results in strong suppression of a secondary LPS stimulus, indicating that the tolerance effect is bidirectional [260]. They also found that CD40 stimulation resulted in robust induction of SOCS3, which they suggest may be responsible for this inhibitory effect. They further reported that CD40 stimulation of macrophages also results in a greatly reduced response to a subsequent stimulation of CD40. They speculate

that could be due to a combination of receptor endocytosis, TRAF degradation, and induction SOCS3 expression.

We show here in LC the same phenomenon occurs, when LC are conditioned with CD40L and challenged with any of the four MAMPs, the LC exhibit a lack of the expected up regulation of CD83 and CD86, zymosan challenge actually induces a downregulation of CD83 and CD86. ECLPS challenged subsequent to CD40L ligation fails to up regulate CD80 as well. The other 3 MAMPs induce a down regulation of CD80. The CD40L pre conditioning does not induce any additional allo or auto T cell proliferative response by the LC.

Moreover IL-β, IL-10, IL-6 secretion by the CD40L conditioned LC subsequently challenged by MAMPs is reduced as compared to MAMPs stimulated LCs indicating an interference in these cytokine gene expression.

Contrary to the reports on monocytes/macrophages by Suttles et al, TNF-α secretion from LC is actually enhanced in response to CD40L conditioning and subsequent MAMPs challenge except for PGN challenge. This is an intriguing finding as to the need for preserving TNF-α function by the LC when they down modulate the other cytokines. One explanation for this could be that TNF-α is resistant to suppression by SOCS3 [263].

Alternatively CD40L has additional pathways which do not overlap with TLR4 signaling, for example the ERK pathways. The ERK pathway is important

for TNF-α gene expression in addition to NFκB pathways. Thus the TNF-α secretion could be preserved in these LCs despite the occurrence of endotoxin tolerance like mechanisms.

This preservation of TNF-α could be biologically significant as CD40L interaction with LCs is just a migratory signal for the LC and they migrate through action of cytokines like TNF-α on E-cadherin [264]. TNF-α in LC may be just a migratory cytokine and immature LCs can secrete high levels of TNF−α as has been previously reported [68]. This could explain the preservation of TNF-α secretion in LC, as it is an important cytokine for LC trafficking [70].

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