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***Immunomodulatory Effects Of Bovine Lactoferrin On Antigen
Presenting Cells***

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CHAPTER 2: INTRODUCTION

Lactoferrin

Structure

Lactoferrin (LF) is an 80 kDa glycoprotein belonging to the transferrin (Tf) family of iron-binding proteins [1]. It was first discovered more than 60 years ago as a “red protein” from milk [2], and has been the subject of intensive structural and functional studies since it was first purified from human and bovine milks simultaneously in three separate laboratories, in 1960 [3-5].

The amino acid sequence of LF was detected in 1984 [1]. The mature protein consists of a single polypeptide chain of about 690 amino acid residues with high homology among species. Current sequence databases annotate LF sequences from nine species: human, mouse, cow, horse, pig, goat, sheep, buffalo and camel. Crystallographic analysis of LF from different species revealed a highly conserved three-dimensional structure, but with differences between species [6]. The polypeptide is folded into two symmetric globular lobes, which represent its N- and C-terminal halves. These two lobes, namely N and C lobes, are linked by a short α -helix (H) of about 10-15 amino acid residues. Non-covalent interactions, mostly hydrophobic, provide a cushion between the two lobes, with C-terminal helix playing a large part. Both lobes have the same fold, consistent with their sequence identity of $\sim 40\%$. In each lobe, two α/β domains, meant as N1 and N2, or C1 and C2, enclose a deep cleft within which is the metal binding site [6] (**Figure 1**).

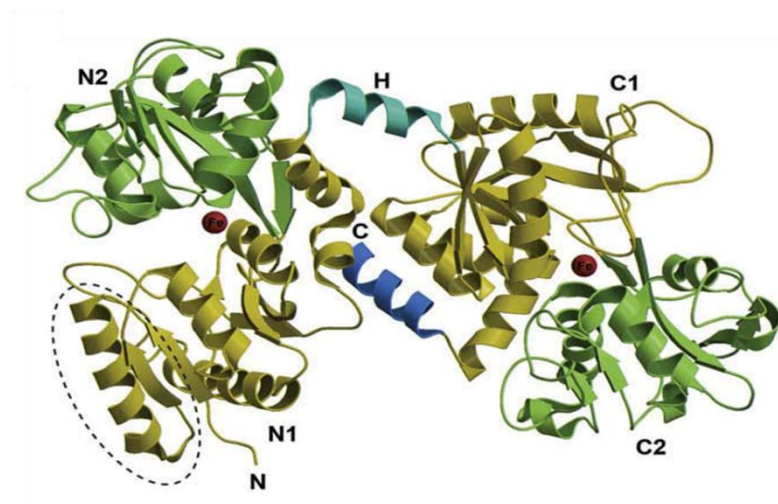


Figure 1. Polypeptide folding of human LF. (From Baker E.N. & Baker H.M., *Biochimie* 2009, 91: 3–10).

All LFs and Tfs so far characterized have essentially identical metal binding sites. Each site binds at a remarkably high affinity ($K_d=10^{-22}$ M), but reversibly, one Fe^{3+} ion, with Fe^{3+} binding being dependent on the concomitant and synergistic binding of carbonate, CO_3^{2-} [7]. LF can bind not only Fe^{3+} ions, but also Cu^{2+} , Zn^{2+} and Mn^{2+} ions with a lower affinity [6]. Despite their structural similarities, LF differs from Tf in several important aspects including biological location and iron binding capacity that likely contribute to its unique functional activities.

Isoforms

Biophysical studies have shown that a large conformational change in LF structure occurs during metal binding and release [8]. Because of its ability to reversibly bind Fe^{3+} ions, LF can exist free (apo-LF) or associated (holo-LF) with Fe^{3+} . Iron binding induces a “closed” structure (holo-LF), conformationally rigid and very stable, in which the two domains of each lobe enclose the bound Fe^{3+} ion, effectively sequestering it from the external environment. Conversely the apo-LF is an “open”

form, less stable and less compact, more flexible and prone to thermal denaturation and proteolysis [6].

The comprehension of the structural dynamics of LF is important for the understanding of its various biological activities. The conformational changes, which differentiate the holo and apo forms of LF, are simple domain movements. For this reason, the majority of the molecular surface remains the same, so that binding sites for receptors or bacteria, viruses, etc. are likely to be unaffected by iron status. The main difference is that the open binding cleft of the apo form offers additional possibilities for molecular interactions [6].

Surface properties

LF has been described as a molecule with a double face, composed of an internal portion, highly conserved between species and endowed with metal binding capacity, and a strongly cationic external surface [7]. In fact, all LFs show a strong cationic nature characterized by a high isoelectric point (pI~9) which differentiates this protein from Tfs (pI~5-6) and influences the ability of LF to bind to a variety of cell types and anionic molecules [6].

The positive charge, mainly concentrated at the N-terminus (1-7 amino acid), in the first helix (12-30 amino acid in the human LF) and in the region that connects the two lobes, is thought to be crucial for the majority of LF activities, including immunomodulation and lipopolysaccharide (LPS) binding [6]. In particular, the major basic region surrounds the N-terminus, which is responsible for DNA, heparin, LPS, glycosaminoglycans and ceruloplasmin binding [9-11] and the first helix, which includes the major portion of the lactoferricin domain, a potent bactericidal peptide [12].

All LFs are glycosylated with some differences between species [6]. Although glycosylation has no influence on LF properties such as iron binding and release, thermal stability and folding, most of the glycosylation sites are exposed on the external surface of the molecule and have been supposed to play a role in LF interaction with viruses, toxins, sialic acid-binding immunoglobulin superfamily lectins and C-type lectin receptors (CLRs) on immune cells [7,13,14].

Expression and release

LF is very largely distributed in the organism. It is secreted from epithelial cells into most exocrine fluids, including tears, saliva, vaginal fluids, semen, nasal and bronchial secretions, bile, gastrointestinal fluids, urine, sweat [15] and most highly in milk [5] where its concentration in humans varies between 1 to 7 g/l (mature milk and colostrum, respectively) [16], making it the second most abundant protein in milk, after caseins [17]. LF is also found in considerable amounts in secondary neutrophil granules ($15\mu\text{g}/10^6$ neutrophils) [18,19], where first appears at the stage of the pro-myelocyte [20].

Because of its widespread distribution in the organism, LF is considered a key component in the host first line defense system.

Similarities and differences of bovine and human LF

As described above, LF is produced by mucosal epithelial cells in various mammalian species. Among them, human (hLF) and bovine (bLF) LF have been shown to exhibit the highest degree of structural and functional similarity, and tested for clinical use in a variety of animal models and in clinical trials [21-31]. Although both preparations have been documented to be efficacious in different therapeutic settings, they may differ for some properties that are summarized in **Table 1** [32].

Bovine and hLF exhibit a high homology in nucleic acid and amino acid sequences (77% and 69%, respectively) as well as in disulfide bonding and secondary structure, and both lack of free sulfhydryls [1,33-35]. Moreover, bLF has an iron content approximately four-fold higher [36], but a lower thermo-resistance than the human counterpart [37]. Crystallography analysis showed that the most striking structural difference between hLF and bLF resides in the relative orientation of the two lobes [38]. Furthermore, hLF is more resistant to proteolysis than bLF probably due to less accessible cleavage sites to trypsin [39,40]. These proteins also differ in the number of potential glycosylation sites, with three and five N-linked glycosylation sites present in hLF and bLF respectively, but only a part of these potential sites are generally glycosylated, two in hLF and four in bLF [41,42]. A differential utilization of these sites results in distinct glycosylation variants. Furthermore, specie-specific differences in the structure of LF glycans were also reported [43] and have been associated with the hLF capacity to induce NF- κ B activation, a property not found for bLF [44]. Accordingly, the sugar composition influences also the ability of hLF and bLF to bind molecules. In this regard, it has been reported that bLF, but not hLF, bind to dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) on dendritic cells (DCs) [14,45].

In addition, both hLF and bLF contain a low- and high-affinity LPS-binding site [46] and have been shown to bind to bacterial porins thus favoring destabilization of bacteria outer membrane [47].

Table 1. Similarities and differences of human *versus* bovine LF

LF properties	Human <i>versus</i> bovine	References
Nucleic acid sequence homology	77%*	[1,34,35]
Amino acid sequence homology	69%*	[35]
Secondary structure	100%*	[33]
Disulfide bonding	100%*	[33]
Lobe orientation	different	[38]
Glycosylation sites	lower	[41,42]
N-acetyllactoseamine glycans	different	[43]
Iron content	lower	[36]
Thermoresistance	higher	[37]
Proteolysis resistance	higher	[39,40]
DC-SIGN binding	lower	[14]
NF- κ B activation	different	[44]
LPS binding	100%*	[46]
Porine binding	100%*	[47]

Summary of the principal experimental observations comparing human and bovine LF.

* indicates the percentage of similarity.

(From Latorre D. *et al.*, *Biochem Cell Biol* 2012, 90(3): 269-78)

LF Receptors on Antigen Presenting Cells

The cationic nature of LF accounts for its propensity to bind anionic molecules resulting in massive binding to mammalian cells and making identification of receptors involved in the biological roles of LF very difficult [48]. Although the identification of several putative LF receptors (LFRs) reveals considerable variations among species, tissues and cell types [49], there is still no clear evidence of a monospecific LFR since most molecular targets on the host cells are multiligand receptors and many of them were reported as signaling, endocytosis and nuclear targeting molecules [50,51].

Several studies have described a direct binding of LF to monocytes/macrophages. In this respect, mouse peritoneal macrophages (M ϕ) were the first cell type stated to

express LFRs in mammals [52], and then human monocytes were shown to bind to hLF in a reversible, saturable and specific manner [53].

At the surface of cells, the sulphated chains of proteoglycans are responsible for the low affinity and high density binding of LF (80% of total LF binding). Other important receptors are represented by low-density lipoprotein receptor-related proteins (LRPs), frequently referred to as scavenger receptors, widely expressed on several cell types, including M ϕ [54,55]. In addition, nucleolin, a multifunctional shuttling protein present in nucleus, cytoplasm, and on the surface of different cells including M ϕ [56], has been identified as LFR and involved, together with proteoglycans, in the endocytosis and nuclear targeting of LF [57]. Furthermore, it has been shown that hLF interacts directly with CD14, an LPS receptor involved in the activation of the immune system that exists both as a soluble protein (sCD14) found in serum [58], and as a membrane protein (mCD14), highly expressed on the surface of monocytes/M ϕ [59]. Recently, the multifunctional glycolytic protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH), localized on the surface of M ϕ , has been described as a novel LFR in M ϕ which mediates the trafficking of LF to the endosomal compartment [60].

In contrast to monocytes/M ϕ , no specific analysis has been yet carried out to define the nature of LFRs expressed on DCs. However, different studies have suggested that CLRs such as mannose receptor (MR) and DC-SIGN, may represent LFRs on this cellular type. In particular, it has been observed that the adjuvant effect of LF in skin Langerhans cells (LCs) is inhibited by blocking the MR, indicating this receptor as a putative site for LF interaction with this subset of DCs [13]. In addition, it has been shown that, by binding DC-SIGN, LF enhances adenoviral infection of monocyte-derived DCs (MD-DCs) [45] and prevents HIV-1 transmission to T cells [14].

As described above, the capacity of LF to interact with CLRs apparently depends on its sugar composition. In this respect, it has been demonstrated that although both bLF and hLF interact with the MR, it occurs to a different extent and strongly relates to the different composition in relevant sugars [42]. Likewise, hLF, in contrast to bLF, does not bind DC-SIGN, as assessed by its lack of capacity to increase adenoviral infection [45] and to prevent the DC-SIGN-mediated HIV-1 transfer to CD4⁺ T lymphocytes [14].

Since most of LFRs described above permit signaling, it has been supposed that the mere binding of LF to a cell surface may itself modulate biological effects [50].

LF Biological Functions

LF is an essential element of the innate immunity found only in mammals. This exclusive characteristic has suggested that this molecule could be involved in newborn nutrition and protection. However, in adult life, because of its structural features and capacity to respond to a variety of physiological and environmental changes, LF continues to exert a plethora of biological activities [61].

LF similarities to Tf have addressed initial research on LF function to its iron-binding properties: iron absorption, antimicrobial activity and modulation of iron metabolism during inflammation. However, subsequent studies have revealed a large number of other possible functions, many of which do not appear to involve iron binding.

It is now well accepted that LF, in addition to a strong and well characterized antimicrobial activity against a broad spectrum of bacteria, fungi, yeasts, viruses and parasites [62-64], also exhibits anticarcinogenic activities [25,65], plays a role in iron homeostasis [27,66,67] and bone remodeling [68], and shows immunomodulatory properties [32,54,69,70] (**Figure 2**).

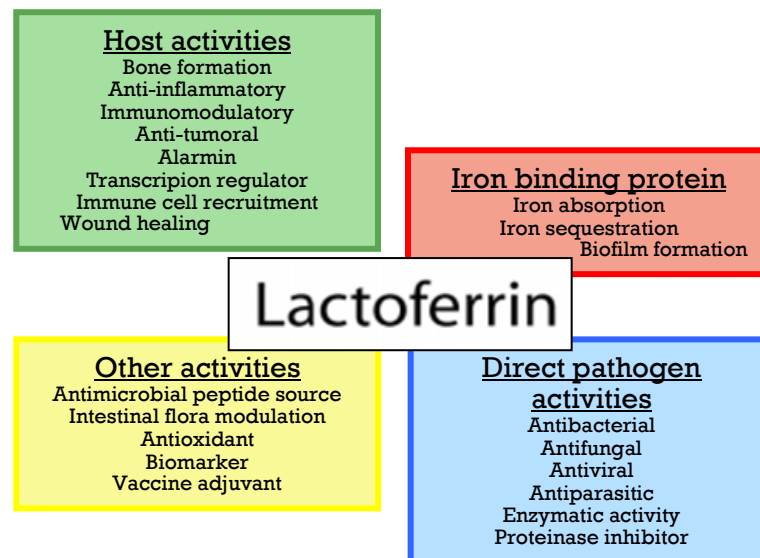


Figure 2. The wide range of functional properties described for LF. (From Vogel H.J., *Biochem Cell Biol* 2012, 90: 233-244)

Immunomodulatory functions

The ability of LF to modulate the overall immune response and to protect against microbial infection and septic shock has been largely described. In this respect, it is noteworthy that LF concentrations markedly increase in biological fluids and locally in patients suffering from inflammatory disorders [69]. Despite most LF is released from neutrophils at the inflammatory sites, activation of these cells starts during their recruitment, which mainly explains why LF concentration in plasma may greatly increase during inflammation [54]. This concentration is as low as 0.4 - 2 mg/l under normal conditions but increases up to 200 mg/l in septicaemia [71]. LF released in blood is rapidly cleared by the liver parenchymal cells [72] and, very recently, the scavenger receptor C-type lectin SRCL, widely expressed on endothelial cells, has been proposed as a major scavenger of LF both locally at sites of inflammation and systemically in the circulation [73]. According to these observations, it may be assumed that increased LF in plasma plays systemic effects on the immune system.

Since this molecule is released in the apo-form, at the site of inflammation its iron-scavenging properties can be directed against microbes together with the direct microbicidal activity of the molecule [62-64]. In addition to the antimicrobial properties, it has been suggested that LF can exert immunostimulatory effects as well as immunoinhibitory activities by modulating the overall immune response [32,54]. These apparently controversial LF functions rely not only on its capacity to sequester iron but also on its property to interact with molecular and cellular components of both host and pathogens including endotoxin and its receptors. In this respect, the ability of LF to bind LPS or limit its *in vitro* interaction with receptors (e.g. LPS-binding protein (LBP) and sCD14) can explain the versatility of LF molecule, which can efficiently suppress endotoxin-induced excessive immune reaction in sepsis or promote, in particular conditions, a protective response against pathogen challenge.

LF biological activity on Antigen Presenting Cells

Although the cellular and molecular mechanisms accounting for the immunomodulatory effects of LF are far from being fully elucidated yet, evidence indicates that the LF ability to directly interact with antigen presenting cells (APCs), i.e. monocytes/M ϕ and DCs, may play a critical role. At the functional level, LF modulates important aspects of APCs biology, including migration and cell activation, whereas at the molecular level it affects expression of soluble immune mediators, such as cytokines, chemokines and other effector molecules, thus contributing to the regulation of inflammation and immunity. Hence, LF can influence both innate and adaptive immune response.

LF interacts with monocyte and M ϕ , and modulates their functions during inflammatory and infectious processes. In particular, *in vitro* and *in vivo* studies have shown that LF is

able to increase phagocytosis and intracellular killing of different pathogens both in mouse and human monocytes/M ϕ [74-79]. Furthermore, LF has been described to induce the production of superoxide and pro-inflammatory molecules such as nitric oxide, TNF, IL-6, IL-12 and IL-8 [77,80-83]. In addition, bLF can stimulate IFN- β production by murine M ϕ thus mediating the host's antiviral response [84]. Although exposure of naïve M ϕ to LF results in the induction of some cytokines/chemokines, the effects of this molecule on already activated M ϕ (i.e. LPS-treated or pathogen infected) are more complex. In particular, LPS-induced production of IL-6, TNF, IL-1 β and IL-8 is inhibited by LF, both bovine and human or its fragment lactoferricin B, in various human monocytic cell lines [85-87]. Furthermore, it has been observed that addition of bLF to spleen M ϕ and J774A.1 murine cell line, stimulated with suboptimal LPS concentrations, increased production of IL-12, whereas the secretion of IL-10 was decreased [88]. Moreover, LF shows chemoattractant effect that seems to be specific for monocyte/M ϕ populations [89]. In this regard, a recent study has demonstrated that LF synthesized and released by apoptotic cells, selectively induces migration of mononuclear phagocytes but not of granulocytes, thus stimulating the resolution of inflammation and the tissue microenvironment repair [90].

Although the immunomodulatory effects of LF on monocytes/M ϕ have been largely investigated, limited information are currently available about the activity of this compound on DCs.

Some studies have been carried out to evaluate the role of LF in the regulation of epidermal LCs migration. LCs, a subset of DCs, are considered to play a pivotal role in the induction and regulation of cutaneous immune responses [91]. *In vivo* studies have shown that both intradermal injection and topical application of LF significantly inhibit

allergen-induced LC migration and accumulation at the site of inflammation by blocking the IL-1 β and subsequent TNF production [92-94].

Despite the LF anti-inflammatory properties exerted on LC subpopulation, recent studies have described this molecule as a novel maturation factor for human MD-DCs. Interestingly, recombinant hLF promotes the maturation and activation of these cells by up-regulating the expression of major histocompatibility complex (MHC) class II molecules, CD80, CD83, and CD86 and chemokine receptors (CXCR4 and CXCR7), the production of pro-inflammatory cytokines, and by increasing their capacity to trigger proliferation of allogenic T lymphocytes [89,95]. According to these results, among a panel of anti-inflammatory drugs tested for their capacity to revert nickel-induced maturation and cytokines secretion in MD-DCs, only recombinant hLF failed to inhibit DC maturation, as assessed by impaired CD86 up-modulation and CXCL8 production [96]. As described above, it has been reported that bLF prevents the MD-DC-mediated HIV-1 transmission by blocking DC-SIGN interaction with the viral surface glycoprotein gp120 [14]. Likewise, the efficacy of LF to prevent HIV-1 capture by DCs was reported for both R5 and X4 HIV strains [97].

Biological activity of LF-bound LPS: TLR4-dependent and -independent effects

Growing evidence suggests that the LPS-binding capacity of LF provides a dual advantage to the host immune response. In fact, on the one hand LF can directly sequester LPS or interfere with its interaction with cell surface receptors thus inhibiting the excessive host's response to endotoxin challenge whereas, on the other hand, it can take advantage of the bound LPS to trigger an immune response engaging specific LPS receptors. In this regard, the ability of LF to form complexes appears to account for at

least some of its immunomodulatory effects, positively or negatively affecting the immune response [32,54] (**Figure 3**).

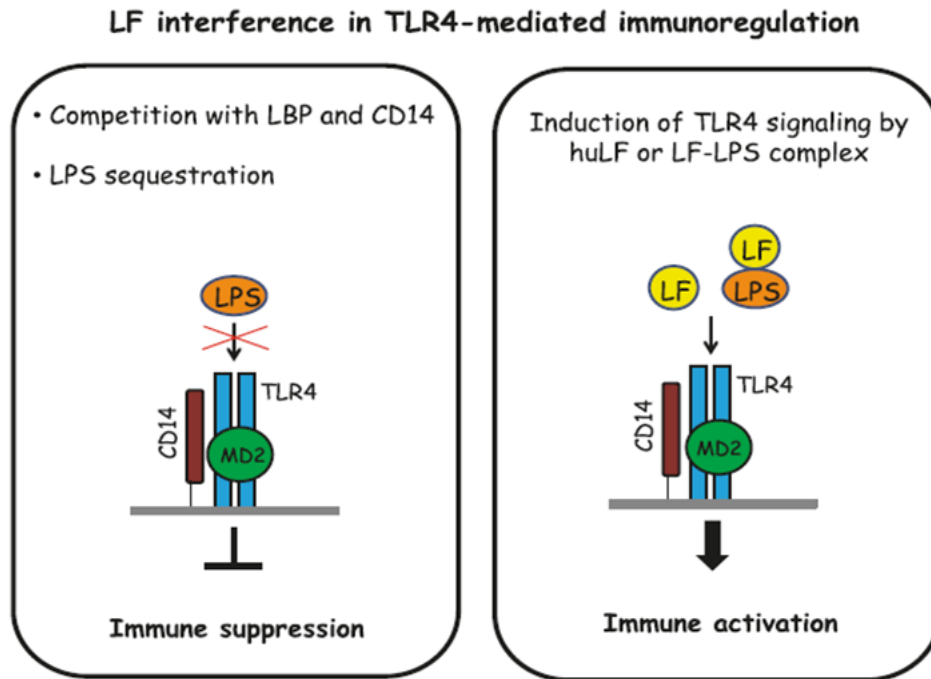


Figure 3. LF-LPS interplay in the modulation of the immune response. LF can directly bind LPS, neutralizing its activity, or compete with LPS receptors (i.e. CD14 and LBP) thus preventing its binding to Toll-like receptor 4 (TLR4) and the consequent inflammatory response. Conversely, LF, *per se* or complexed to LPS, can interact with TLR4 stimulating a protective immune response against pathogen challenge. (From Latorre D. et al., *Biochem Cell Biol* 2012, 90 (3): 269-78)

Several *in vitro* and *in vivo* studies have demonstrated that LF can inhibit, in a concentration- and time-dependent manner, a number of LPS-induced effects. In particular, *in vitro* studies have shown the LF capacity to inhibit the LPS-induced (i) cytokines production, i.e. TNF, IL-6, IL-1 β , IL-8 in different cell types [85-87,98-102]; (ii) E-selectin and ICAM-1 expression in human endothelial cells [101,103], (iii) proliferation, prostaglandin E₂ (PGE₂) production, cyclooxygenase-2 (COX-2) and matrix metalloproteinase 9 (MMP-9) expression in PBMCs [104].

Moreover, the capacity of LF to modulate the LPS-induced inflammatory process has been also well documented *in vivo*. The protective effect of exogenous LF against endotoxin shock in various animal models has been extensively reported [99,105-111]. In keeping with the anti-inflammatory effect of LF observed in *in vitro* studies, serum levels of LPS-induced pro-inflammatory factors such as IL-6, TNF, nitric oxide and PGE2 were found significantly reduced in LF-treated animals in comparison with untreated controls after LPS inoculation [108,112-118].

Although the mechanisms responsible for this anti-inflammatory activity have not been fully elucidated, at least some of them may be due to the LF ability to avidly bind LPS, thus blocking its interaction with cellular membranes or competing with LPS for binding to a common receptor.

However, other studies have proved that LF-bound LPS can retain the capacity to stimulate mouse and human cells [119-121]. Accordingly, the LPS bound to LF may contribute to LF biological activity by favouring its interaction with cell surface receptors. In particular, it has been reported that LF-LPS complexes can still prime human monocytes and murine M ϕ , stimulate B lymphocyte proliferation and exert adjuvant activity increasing humoral immune response in mice [119-121].

Furthermore, comparative studies, carried out with LPS responsive and LPS hyporesponsive mice, have demonstrated a strong dependency of the LF-LPS complex triggered signals on TLR4, leading to the conclusion that the immunostimulatory properties of LF could be due, at least in part, to LPS binding [120]. In particular, LF binds the lipid A portion of LPS via charge-charge interaction. As described above, the portion of LF that binds anionic molecules, including lipid A, is limited to its N-terminus arginine rich domain [10]. Thus, it is likely that bound LPS can still expose the unbound part of lipid A that is recognized by LPS receptors such as TLR4. Such a

LF-LPS complex recognition would result in M ϕ activation [120]. Of note, the lipid A backbone is also the epitope being recognized in the Limulus assay (LAL), the standard method for detection of endotoxin contamination, thus explaining why the LF-LPS complex is found to be LAL positive [120,122].

Collectively, these observations suggest that lipid A can be recognized even after LF-LPS complex has been formed, and that this complex retains the capacity to activate M ϕ through TLR4. Thus, LPS may represent an important structural component of LF molecule, likely involved in its stabilization or favouring its interaction with receptors and accessory molecules.

Despite LF binding to LPS represents an important aspect, it does not entirely account for all immunomodulatory effects of this molecule (**Table 2**) [32]. Accordingly, it has been reported that LF-induced IL-6 secretion and CD40 expression in murine peritoneal M ϕ is achieved via TLR4-independent and -dependent mechanisms, respectively, thus indicating potentially separate pathways for LF-mediated M ϕ events in innate immunity [82]. In keeping with these results, the capacity of LF to induce a type-I interferon (IFN) mediated antiviral state, but not TNF production, has been shown to rely on the function of TLR4 in responding cells. These results further suggests that this molecule may induce M ϕ activation via TLR4-dependent and -independent mechanisms [84].

Table 2. LF-mediated TLR4-dependent and -independent effects

	LF effects	References
TLR4-dependent	Type I IFN production in murine M ϕ	[84]
	Induction of CD40 expression in murine M ϕ	[82]
	NF- κ B activation in human monocytes and mouse fibroblasts	[44]
TLR4-independent	Induction of IL-6 production in murine M ϕ	[82]
	Induction of TNF in murine M ϕ	[84]

(From Latorre D. et al., Biochem Cell Biol 2012, 90 (3): 269-78)

Dendritic Cells

DCs Origin and Development

DCs are continuously generated from CD34⁺ hematopoietic stem cells (HSPCs) within the bone marrow or the umbilical-cord blood. CD34⁺ stem cells differentiate into common lymphoid progenitors (CLPs) or common myeloid progenitors (CMPs) which subsequently differentiate into different DC subtypes that differ in location, cytokines/chemokines production, receptors involved in antigen uptake and in cytokines/chemokines recognition, all factors involved in their immunological functions (**Figure 4**) [91]. CMPs can differentiate into LCs and interstitial DCs, localized in the skin epidermis and dermis, respectively [123]. In addition, two types of DC precursors are found in blood: monocytes and plasmacytoid DCs (pDCs) precursors, which belong to CMPs and CLPs, respectively. DC precursors differ from differentiated DCs both in morphological and functional properties. Indeed, they are characterized by the lack of DC morphology and migratory ability, by low expression of costimulatory molecules and by the failure to induce significant naïve T cells activation [123].

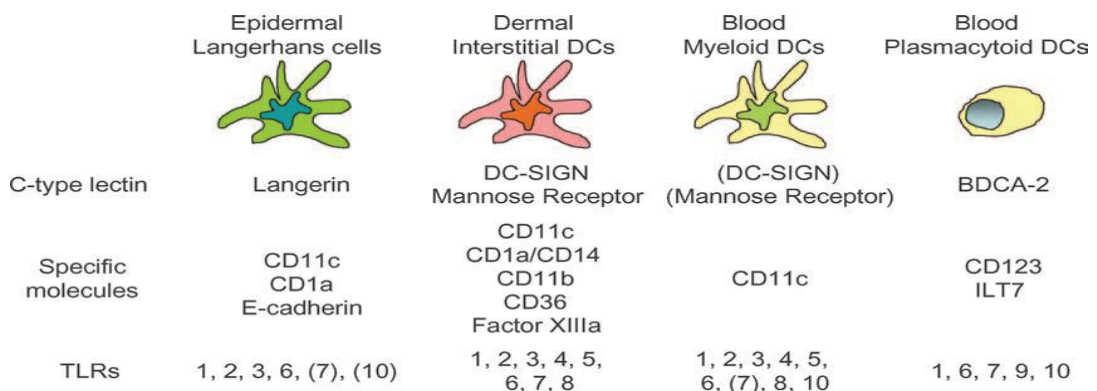


Figure 4. Human DC subsets *in vivo*. DC subsets differ in location and in the expression of a set of molecules involved in antigen uptake and microbe recognition. (From Ueno H. *et al.*, *Immunol Rev* 2007, 219:118-42)

The initial characterization of DC populations in humans has been delayed by their low frequency in blood (less than 1% of blood mononuclear cells). Hence, different experimental protocols have been investigated to induce *in vitro* DC generation from precursor cells [124-127]. Among them, the generation of DCs starting from human blood monocyte precursors cultured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4, is the most widely accepted experimental protocol to date [128]. The discovery of techniques to generate DCs *in vitro* has allowed the identification of many of their biological and molecular properties.

Role of DCs in the immune system

Antigen recognition and DCs maturation process

DCs are a heterogeneous population of immune cells of haematopoietic origin highly specialized in the handling of antigens (i.e. those from infectious agents and self) and their presentation to lymphocytes recognition.

Thanks to their ability to directly interact on the one hand with pathogens, as well as with various innate cell types (such as granulocytes, NK cells and NKT cells) and on the other hand with adaptive immune cells (T and B lymphocytes), DCs represent a critical link between innate and adaptive immunity and are essential for the development of antigen-specific immune responses. However, depending on their state of maturation and mode of activation, DCs are also essential for the induction and maintenance of immune tolerance [129,130].

Because of their high plasticity, the local microenvironment and the nature of the activating stimuli strongly influence the specific DCs function and, consequently, the type of adaptive immune response [91,131].

In the steady state, DCs reside both in peripheral tissues forming an interface with the external environment (i.e. skin, gut and lungs), and in lymphoid organs (i.e. thymus, bone marrow, spleen, lymph nodes, and Peyer's patches) [132].

In peripheral tissues, they are in an immature state and act as “immunological sensors” specialized in the capture of antigens, monitoring the microenvironment and alerting for potential dangerous signals. Hence, immature DCs (iDCs) scan peripheral tissues where they recognize, take up and efficiently process antigens for presentation, in association with MHC molecules, to naïve T lymphocytes at lymphoid organs [131].

Upon infection or tissue damage, iDCs are rapidly recruited by the “danger signals” from pathogens and cytokines/chemokines locally produced at the site of inflammation. Numerous agents derived from microbes, dying cells, cells of the innate and adaptive immune system can activate DCs (**Figure 5**). Potential dangerous signals are detected by DCs through pattern recognition receptors (PPRs), which bind to a limited set of conserved pathogens-associated molecular patterns (PAMPs) that are unique in the microbial world. PPRs include cell surface CLRs, intracytoplasmic NOD-like receptors (NLRs) and TLRs. CLRs expressed on DCs act as anchors for a large number of microbes including viruses, bacteria, parasites and fungi, and allow their internalization, but they also act as adhesion molecules between DCs and other cell types, including endothelial cells, T cells and neutrophils [132]. Among CLRs, MR and DC-SIGN are expressed by MD-DCs [133]. Moreover, NLRs comprise a large family of intracellular PPRs, all bearing a conserved NOD domain. Their location suggests a possible role in the detection of microbes escaping the surveillance of extracellular or endosomal receptors. NOD1 and NOD2 are the best-characterized NLRs. They recognize molecules produced during the synthesis or degradation of peptidoglycan [131]. Although several receptors recognize microbial structures, TLRs are the only PRRs

identified to date that directly mediate full DCs maturation. Thirteen different TLRs have been identified in mammals [134]. Some of them (TLRs 1-6 and 11) are expressed at the cell surface and recognize different products of bacterial, fungal or protozoan origin, including lipopeptides, LPS and peptidoglycans. Others, such as TLRs 3, 7, 8 and 9, are localized in the endoplasmic reticulum and recognize microbial nucleic acids [134]. TLRs can also bind a large set of endogenous ligands, including heat shock proteins (HSPs), hyaluronate and heparan sulfate (extracellular matrix breakdown products), fibronectin, high mobility group box 1 protein (HMGB1) and modified low-density lipoproteins [135]. In addition, lysates of dying cells also induce the maturation of DCs and enhance their antigen presentation ability. These endogenous activating molecules are collectively named damage-associated molecular pattern molecules (DAMPs) [91,132]. Distinct DC subsets display different TLRs. Focusing on MD-DCs, they express TLR1, TLR2, TLR3, TLR4, TLR5 and TLR8. During differentiation from monocytes to DCs, cells show an up-regulation of TLR3 expression while decreasing TLR1, TLR2, TLR4, TLR5 and TLR8 expression [136].

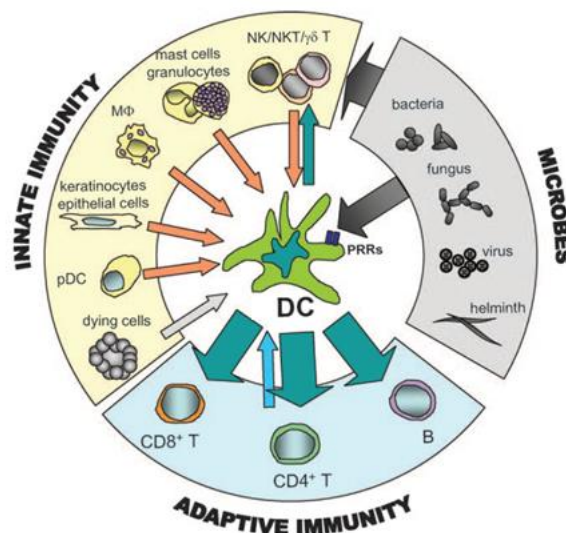


Figure 5. Regulation of DCs activation. DCs can be activated by numerous agents derived from microbes (PAMPs), dying cells (DAMPs), cells of innate immune system and cells of the adaptive immune system. Activated DCs migrate to the draining lymph nodes, where they encounter cells of the adaptive immune system. (From Ueno H. *et al.*, *Immunol Rev* 2007 219:118-42)

Immature DCs can use several pathways to take up antigens: (i) macropinocytosis, (ii) receptor-mediated endocytosis via CLRs or type I (CD64) and II (CD36) Fc receptors for immune complexes or opsonized particles, and (iii) phagocytosis of particles such as apoptotic and necrotic cell fragments, viruses and bacteria, including mycobacteria as well as intracellular parasites [137]. Generally, captured protein antigens are presented by MHCs classical molecules (class I and II) that stimulate T α/β lymphocytes while lipid antigens are presented by MHC non-classical molecules, such as CD1, and stimulate primarily T γ/δ lymphocytes and NKT cells [138].

Upon antigen recognition and processing, DCs undergo a complex process of maturation involving a series of coordinate events, which lead to their phenotypic and functional changes. Maturation is a terminal differentiation process that transforms DCs from cells specialized for antigen capture into cells specialized for T-cell stimulation. The events that take place during the maturation process are essential for understanding the control of immunity and tolerance [139]. During this process, DCs decrease their phagocytic capability, increase their efficacy to present processed antigens in the context of MHC molecules and acquire a migratory phenotype associated with the up-regulation of the G protein-coupled receptor CCR7, which is the dominant mediator of DC mobilization to the T cell compartment of lymphoid organs [140]. In particular, DCs undergo (i) changes in morphology such as loss of adhesive structures, cytoskeleton reorganization and the acquisition of high cellular motility; (ii) loss of endocytic/phagocytic receptors; (iii) up-regulation of costimulatory molecules, such as CD40, CD80, CD86, and MHC class I and II molecule expression, (iv) up-regulation CCR7 and down-regulation of CCR6 expression and (vi) secretion of polarizing cytokines and chemokines [91].

Unlike other APCs, DCs are highly specialized for homing efficiently to the T cell zones of lymphoid organs and optimal interactions with T lymphocytes. Indeed, in the steady state, iDCs migrate at a low rate from peripheral tissues via the blood or lymph to the T-cell regions of the lymphoid organs without undergoing activation. Then, they present self-antigens to lymphocytes in the absence of costimulation thereby leading to peripheral tolerance [130]. Before activation, iDCs can express CCR1, CCR2, CCR5, CCR6, CXCR1, CXCR2 and CXCR4, with their expression pattern differing somewhat among DC subsets. The migration of mature DCs (mDCs) from the sites of antigen capture to T-cell regions of draining lymph nodes, where they contact naïve or memory T cells, plays a key role in initiating a specific immune response [141]. In this manner, DCs form the key link between innate and adaptive immunity (**Figure 6**).

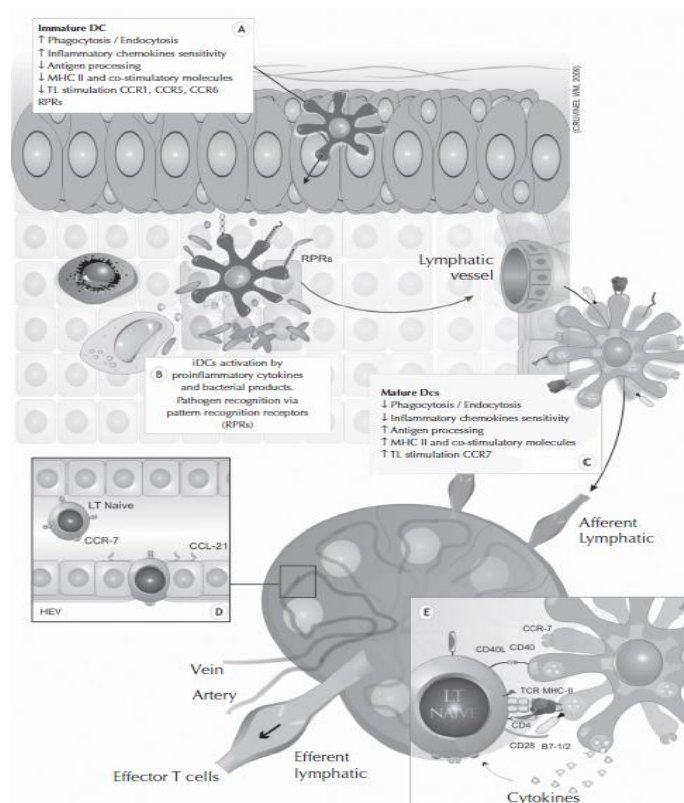


Figure 6. DCs and generation of antigen specific T lymphocytes. (A) Features of iDCs. (B) Activation and uptake of pathogens through cytokine microenvironment and interaction with PPRs, with consequent migration of DCs to lymph nodes. (C) Maturation of DCs. (D) Migration of naïve T cells to paracortical area of lymph node. Entry through high endothelial venules (HEV) and chemokine-driven migration of lymphoid tissue. (E) Presentation of processed antigens to T lymphocytes (LT), generating activated effector cells. (From Cruvinel W. de M. et al., *Bras J Rheumatol* 2010, 50 (4): 434-61)

Stimulation of the adaptive immune responses

In the lymph nodes, mDCs present on their surface a set of antigenic epitopes in association with MHC molecules that reflect the antigenic situation at the site of infection. Through the expression of MHC class I and MHC class II molecules, DCs are able to interact with naïve $CD8^+$ T cytotoxic and naïve $CD4^+$ T lymphocytes, respectively [141]. The signals that lead to T cell activation are generated at the level of the immunological synapse, a specialized area of contact between T cells and DCs, where adhesion molecules and T cell receptors (TCRs) form distinct supramolecular complexes [130,142]. Three main signals are required before a naïve T lymphocyte becomes an effector cell (**Figure 7**). The first signal is induced by the cross-linking of TCR triggered by the appropriate peptide-MHC complex presented on mDCs (signal 1). The second signal (costimulation) is mainly induced by the interaction of CD28 on the T cell surface and the costimulatory molecules CD80 (B7-1) and CD86 (B7-2) on the surface of mDC (Signal 2). The third signal is directed by DC-derived cytokines and enables the differentiation of T cells into effector (Signal 3) [133,141]

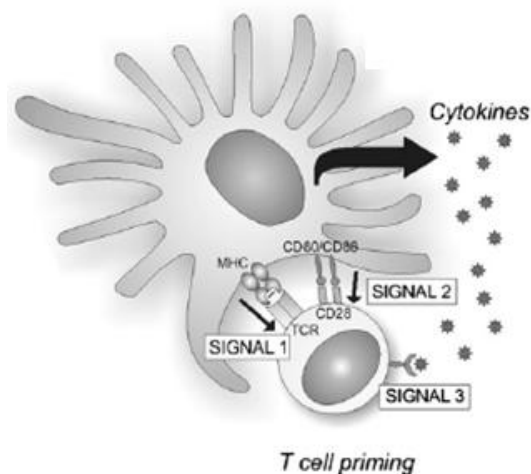


Figure 7. Signals required for T cells activation and polarization. Upon maturation, DCs become capable to activate naïve T cells (signals 1 and 2) promoting the differentiation of newly activated T lymphocytes into effector cells (signal 3). (From Sabattè J. et al., *Cytokine & Growth Factor Reviews* 2007, 18: 5–17)

Effective priming of naïve $CD4^+$ T cells results in their clonal expansion and differentiation into cytokine-secreting effector and memory T cells. Hence, naïve $CD4^+$ T cells may differentiate into one of several lineages of effector T helper (Th) cells, including Th1, Th2, Th17, Th22 and T regulatory cells (Treg). The expansion of a specific $CD4^+$ effector/Treg cell subset is largely determined by the expression of a set of transcription factors essential for their differentiation, mainly induced by the cytokines produced by mDCs during the T cell activation process, and by their pattern of cytokine production [143] (**Figure 8**). Thus, lymphotoxin- α (LT α) and IFN- γ -producing Th1 cells require the presence of IFN- γ and IL-12 released by mDCs and the lineage-specifying T-box transcription factor (T-bet) for their differentiation. Th1 cells induce delayed type hypersensitivity (DTH) reactions that are protective against intracellular bacteria, fungi and protozoa, but can also be responsible for autoimmune disorders such as experimental autoimmune encephalomyelitis (EAE) and experimental autoimmune uveitis (EAU) [144]. Furthermore, in the presence of IL-2 and IL-4 released by mDCs, naïve $CD4^+$ T cells can polarize into Th2 lymphocytes that express the trans-acting T-cell-specific transcription factor (GATA-3) and, because of its ability to produce IL-4, IL-5, IL-9 and IL-13, are protective against extracellular parasites, but can also be responsible for allergic disorders. Likewise, in the presence of TGF β , IL-6 and IL-21 (in mice) or of IL-1 and IL-23 (in humans), naïve Th cells express retinoic acid-related orphan receptor (ROR) γ t and differentiate into Th17 cells that produce IL-17A, IL-17F, and IL-21 and are involved in the protection against extracellular bacteria and fungi, but can also be responsible for autoimmune disorders, such as EAE, EAU and collagen-induced arthritis (CIA) [144]. Recently, it has been shown that in the presence of TNF and IL-6, naïve Th cells can express aryl hydrocarbon receptor (AHR) and differentiate into IL-22-producing Th22 cells that, thanks to their production of IL-

22 and expression of skin homing chemokine receptors (CCR4 and CCR10), have been hypothesized to be important in skin homeostasis and inflammation [144]. Moreover, some studies have also described IL-9-producing Th9 cells, and T follicular helper (Tfh) cells as separate lineages. However, it is still a matter of debate whether they may represent diversity within Th lineage, rather than separate lineages [143].

In addition to the cytokines produced, effector T cells can be distinguished by their differential expression of chemokine receptors that direct them to distinct inflammatory environments [143,145].

During Th cell differentiation toward one lineage, the other lineage fate is usually suppressed through several mechanisms. In particular, the transcription factors expressed in one lineage also suppress the production of cytokines of other lineages [143]. Despite the fact that Th cell polarization has been thought as an irreversible process of differentiation, current evidence describes Th cells as plastic populations that can be reprogrammed into other lineages in the presence of appropriate stimulation [143,144]. For instance, it has been observed that Th2 cells can differentiate into Th9 cells in the presence of IL-4 and TGF β [146] and that Th17 cells can shift to Th1 cells in the presence of IL-12 [147,148].

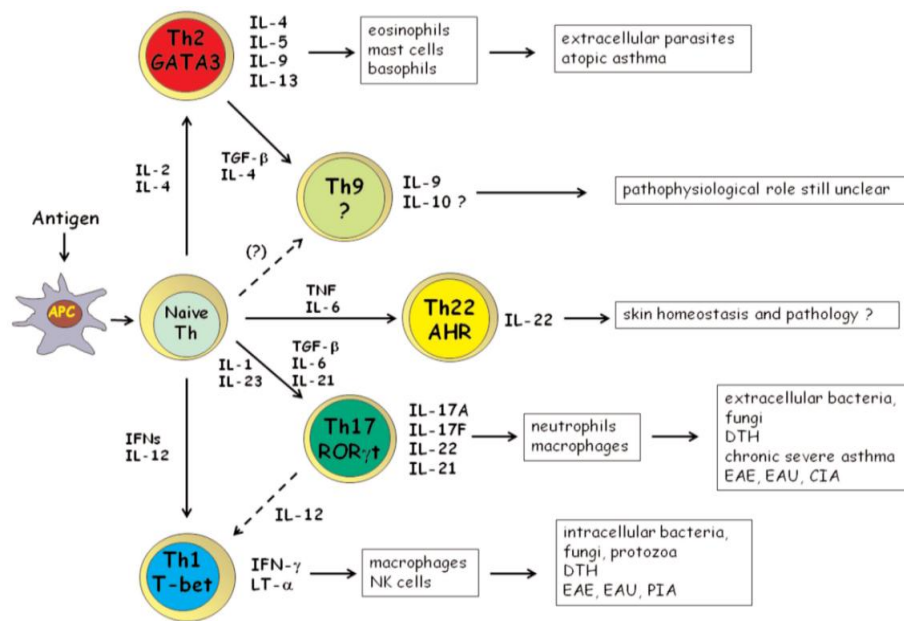


Figure 8. Main populations of CD4⁺ effector T cells. (From Annunziato F. & Romagnani S., *Arthritis Research & Therapy* 2009, 11: 257)

Induction of immune tolerance

In addition to their well-known role as the most powerful stimulators of adaptive immunity, DCs are also essential for the maintenance of immunological tolerance (both central and peripheral tolerance) to self-antigens. Central and peripheral tolerance act in a synergic way to prevent autoimmunity without inhibiting the ability of immune system to be activated in case of dangerous signals.

Central tolerance is operated in the thymus by DCs and medullary epithelial cells through negative selection, a process where most of the T cells, which recognize self-antigens at high affinity, are deleted at an immature stage of their development. However, potentially harmful self-reactive T cells can escape the wall of central tolerance and can promote autoimmune diseases. In order to prevent this process, peripheral tolerance serves as a backup mechanism to promote systemic tolerance to such autoreactive immune cells. In addition, it is also critical in suppressing immune

responses to innocuous external antigens or non-pathogenic organisms in the lung and digestive tract as well as at the immune privileged areas such as the brain, the anterior chamber of the eye, the testis, and the fetus. Breakdown of central or peripheral tolerance leads to autoimmune diseases such as type 1 diabetes, multiple sclerosis, inflammatory bowel disease, and rheumatoid arthritis [149].

DCs can promote peripheral tolerance by several mechanisms such as the induction of T-cell anergy or deletion, and the expansion of Treg. As described above, under homeostatic conditions, peripheral DCs typically display an immature phenotype so that they express only moderate levels of MHC class II and no, or very low, levels of costimulatory molecules. Thus, iDCs can inhibit the immune response of autoreactive T cells through the induction of (i) T cell anergy by presenting the antigen on their surface in the absence of costimulatory molecules or (ii) peripheral deletion of T cells by inducing their apoptosis due to excessive amount of antigen presented for extended periods of time. In addition, naïve T CD4⁺ cells can be induced to differentiate into Treg (iTreg), when DCs present antigens in the presence of cytokines such as TGF-β and IL-10, and other factors such as retinoic acid or vitamin D3 [150].

Furthermore, Treg arising in thymus are known as natural occurring Treg cells (nTreg) which are CD4⁺ CD25⁺ T cells and express the transcription factors Helios and Foxp3, necessary for the maintenance of their suppressive function [150]. DCs ability to induce and interact with Treg is critical for their tolerogenic effect. Conversely, Treg can promote the tolerogenic phenotype and capacity of DCs [149].

Several studies have been carried out to characterize phenotypical and functional features of tolerogenic DCs as well as the factors involved in their development. In particular, the maturation state of DCs is a critical determinant of their tolerogenic capacity. Based on their phenotypic and functional characteristics, DCs can be

identified at various stages of maturation, from immature through so-called “semi-mature” to mature. Hence, generally iDCs or “semi-mature” DCs promote tolerogenic responses, whereas mDCs promote immune responses [149]. Moreover, a broad range of molecules, including microbial components, tissue antigens and apoptotic cells, interact with specific receptors on DCs and program them to a tolerogenic state. Furthermore, anti-inflammatory cytokines (e.g. IL-10 and TGF- β) and immunosuppressive agents (e.g. vitamin A, vitamin D3 and retinoids), can also condition DCs to a tolerogenic state by promoting the expression of IL-10, indoleamine 2,3-dioxygenase (IDO) and TGF- β that are critical for the stimulation of Treg response, or up-modulating certain cell surface molecules such as immunoglobulin-like transcript 3/4 (ILT3/4), programmed death ligand-1/2 (PDL1/2), ICOS-L, B7.H, CD95L that promote T-cell anergy or deletion [149].

CHAPTER 3: AIM

LF is now recognized as a key element in mammalian immune system for its pivotal role in host defence against infection and excessive inflammation. In addition to its well-known antimicrobial properties [62-64], LF exhibits a variety of effects on the host immune system, ranging from inhibition of inflammation to promotion of both innate and adaptive immune responses [32,54,69]. However, the mechanisms underlying LF immunomodulatory properties have not been fully elucidated yet and growing evidence indicates that the capacity of this molecule to directly interact with APCs may play a critical role. Several studies have demonstrated that LF contributes to the regulation of inflammation and immunity by modulating important aspects of APC biology both at the functional level, such as migration and cell activation, and at the molecular level by affecting expression of soluble immune mediators, such as cytokines, chemokines and other effector molecules [32,54,69].

Among APCs, monocytes/M ϕ and DCs are of critical importance for the maintenance of tissue homeostasis and innate response to pathogens, as well as in linking innate to adaptive immune response. In particular, DCs play a key role in the immune system by virtue of their capacity to control, on the one hand, immune activation, by inducing the polarization and expansion of antigen specific T lymphocytes and, on the other hand, tolerance, by contributing to the expansion and differentiation of T cells with regulatory or suppressive properties [129,130,151] .

Although several studies have addressed the role of LF on monocytes/M ϕ , little is known about its activity on DCs. Accordingly, the aim of this work was to further define the LF immunomodulatory activity by focusing on its role in the process of DC generation. To reach this objective, iMD-DCs were generated *in vitro* by culturing human monocytes, purified from PBMC of healthy donors, in RPMI supplemented with

10% FBS containing GM-CSF and IL-4 in the presence or in the absence of bLF. Then, MD-DCs generated in the presence of bLF (bLF-MD-DCs) were analyzed for their phenotypical (surface markers typically expressed on iDCs and tolerogenic markers) and functional features (expression of activation markers, release of pro-inflammatory cytokines/chemokines and T cell activation and polarization ability upon TLRs stimulation). In addition, this study focused on the characterization of molecular mechanisms through which bLF modulates the DC-mediated immune functions. Hence, we analyzed the bLF ability to differently interact with DC precursor cells and already differentiated iMD-DCs by analysing, in these different cellular models, both the bLF-induced cytokines/chemokines release (IL-6 and CCL1) and bLF internalization ability, concomitantly with the analysis of receptors involved in these processes. Finally, we investigated the role of bLF-induced cytokines/chemokines release (IL-6 and CCL1) in the bLF-mediated inhibition of MD-DCs activation.

Overall, this work adds further evidence to the pivotal role of LF in the modulation of DCs biology, shedding light on the possible mechanisms involved in the immunomodulatory ability of LF.

Most of the results discussed in this thesis have been published in [152].

CHAPTER 4: MATERIALS AND METHODS

Cell separation and culture

Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood of healthy donors by Ficoll-Paque density centrifugation. CD14⁺ monocytes population was further purified by depleting the non-monocytic population by immunomagnetic bead selection (MACS monocyte isolation kit II from Miltenyi Biotec), according to the manufacturer's instructions. Immature MD-DCs were generated by culturing monocytes, seeded at 1×10^6 cells/ml for 5-6 days, in RPMI 1640 medium (Life Technologies) supplemented with 2 mM L-glutamine, 2 mM penicillin/streptomycin and 10% fetal bovine serum (FBS) (Hyclone), containing GM-CSF and IL-4 (50 ng/ml and 500 U/ml, respectively; kindly provided by Schering-Plough, Dardilly, France) in the presence or in the absence of bLF (100 μ g/ml). Freshly isolated monocytes were treated with bLF in the presence of differentiating factors soon after seeding, at day 2 and day 5 of culture, unless differently specified. In some experiments, on day 5, MD-DCs were stimulated with LPS (10 ng/ml) or polyinosinic:polycytidylic acid (poly(I-C)) (20 μ g/mL) for 24 hours. Cells were cultured at 37 °C, in a 5% CO₂ and 95% H₂O atmosphere.

Reagents

All culture reagents were purchased as endotoxin-free lots (Biowhittaker). LPS from Escherichia Coli (serotype EH100, Ra TLR grade, Alexis Biochemicals), and poly(I-C) by Sigma-Aldrich.

Highly purified bLF in lyophilized form was kindly provided by Morinaga Milk Industries Co., Ltd., (Tokyo, Japan). BLF was checked for purity, iron saturation and endotoxin content as previously described [84].

Monoclonal antibodies (Abs) against TLR4 (5 µg/ml; clone 15C1) and TLR2 (5 µg/ml; clone T 2.5) were kindly provided by Greg Elson. Monoclonal Abs against CD14 (5 µg/ml; clone 134620) and IgG1k isotype control Abs (5 µg/ml; clone 11711) were purchased from R&D. Monoclonal Ab against CD36 (5 µg/ml; clone FA6-152) was by GENETEX Inc.

MD-DC phenotype was characterized by using the following Abs: FITC-CD1a and PE-CD1a (clone HI149), FITC-CD14 and PE-CD14 (clone MWP9), FITC-CD40 (clone 5C3), FITC-CD80 (clone L307.4), FITC-CD86 (clone 2331(FUN-1)), FITC-CD83 (clone HB15e), FITC-HLA-DR (clone G46-6), FITC-HLA-ABC (clone G46-2.6), FITC-ILT3 (clone 293623, R&D), FITC-ILT4 (clone 287219, R&D), PE-CD274 (PD-L1, clone MIH1), PE-CD206 MR (clone 19.2), mouse purified anti-CD209 (DC-SIGN, DCN46) followed by FITC-goat-anti-mouse IgG (H+L) F(ab')₂ (PIERCE). Non-specific binding was checked by the respective isotype Abs FITC-IgG2a (G155-178), FITC-IgG1 and PE-IgG1 (clone MOPC-21), FITC-IgG2a (clone 20102, R&D), purified mouse IgG2bk (clone 27–35). Unless differently indicated, Abs were purchased from BD Biosciences.

Flow cytometric analysis

Phenotypic analysis of MD-DCs

The phenotypic analysis of surface differentiation and activation markers was performed in immature or LPS and poly(I-C) stimulated MD-DCs, generated in the presence or in the absence of bLF. About $3\text{--}5 \times 10^5$ cells were pre-incubated for 30 min

on ice with phosphate-buffered saline (PBS) containing 10% human AB serum to block nonspecific Ig binding. Then, cells were washed in Staining Buffer (SB), containing PBS, 10% FBS, 0.09% NaN₃, and incubated with the appropriate Ab for additional 30 min. After incubation, cells were washed twice in SB and fixed in PBS/4% paraformaldehyde (PFA). Finally, cells were acquired with FACS Calibur flow cytometer (BD Biosciences) and data analyzed by the FlowJo software (Tree Star, Inc.).

Antigen uptake assay

At day 5 of culture, MD-DCs, treated or not with bLF, were stimulated with LPS or poly(I-C). Twenty-four hours later, their ability for antigen capture was examined by flow cytometric analysis. Briefly, cells were washed twice with PBS, then 2×10^5 cells were incubated with 10 μ g of FITC-labeled dextran (DXT) (Molecular Probe) for 40 min at 37°C, or 0°C, to test unspecific binding. Cells were then fixed in PBS/4% PFA and analyzed by flow cytometry.

MD-DC/T cell co-cultures and Th profile

Allogenic CD4⁺ naïve T cells were isolated from PBMCs of healthy donors by using naïve CD4⁺ T cell isolation Kit II (Miltenyi Biotech) and co-cultured in RPMI 5% human AB pool serum with MD-DCs or bLF-MD-DCs primed with LPS (10 ng/ml) or poly(I-C) (20 μ g/ml), at DC/T ratio of 1:10, 1×10^6 T cells/well in 24-well plates. At day 5, supernatants were collected for cytokine determination while cells were extensively washed, re-suspended in fresh medium at 1×10^6 cell/ml and stimulated with ionomycin (2 μ g/ml) and PMA (50 ng/ml) (both from Sigma-Aldrich) for 5 h. Golgi Stop (BD Bioscience) was added during the last 3 h of culture following the manufacturer's instructions. After stimulation, CD4⁺ T cells were fixed, permeated using Cytofix/Cytoperm Plus™ (BD Bioscience) and intracellularly stained with

Fastimmune FITC-IFN- γ /PE-IL-4 cocktail (clones 25723.11 and 3010.211 respectively; BD Bioscience), and PE-CD154 (clone TRAP1; BD Bioscience). Cells were also stained with the respective isotype Abs, mouse FITC-IgG2a/PE-IgG1 (clones 639/640; BD Biosciences) and mouse PE-IgG1 (MOPC-21; BD Biosciences). In some experiments, allogenic CD4⁺ naïve T cells were primed with LPS-activated MD-DCs or bLF-MD-DCs at DC/T ratio of 1:10 for 7–10 days and successively re-stimulated with the original cryopreserved MD-DCs. Seven-to ten days after last stimulation, cells were processed for surface staining with FITC-CD25 (clone M-A251; BD Biosciences), then fixed and permeated for intracellular APC-FoxP3 (clone PCH101; eBioscience) staining and FACS analysis.

T cell proliferation assay

MD-DCs were co-cultured at different DC/T cell ratio, starting from 1:10, with peripheral blood lymphocytes (PBL), obtained from PBMCs depleted of CD14⁺ cells. After 5–10 days, cells were fixed and permeated using Cytofix/Cytoperm PlusTM (BD Bioscience), then stained with Ab to Ki67 and the relative isotype Ab, following the manufacturer's instructions (FITC Mouse Anti-Human Ki67 Set, BD Biosciences).

Otherwise, in some experiments MD-DCs were co-cultured at different DC/T cell ratio, starting from 1:10, with peripheral blood lymphocytes (PBL), obtained from PBMCs depleted of CD14⁺ cells and previously stained with CFSE. Ten millions PBL were labelled with CFSE (Sigma-Aldrich; 5 μ M, in 500 μ l final volume) in a 15 ml tube for 4 minutes at room temperature (RT). After incubation, the reaction was stopped by the addition to each tube of 10 ml ice-cold RPMI without serum, then cells were washed three times with PBS, suspended in warm RPMI supplemented with 5% human AB

serum and cultured with MD-DCs at 37°C in a 5% CO₂ incubator. After 5-10 days, cells were collected, washed with PBS and samples analyzed by flow cytometry.

Determination of cytokine and chemokine levels

Cytokine and chemokine levels in culture supernatants were measured by enzyme-linked immunosorbent assay (ELISA) kits for the following cytokines: TNF, IL-12 and IL-10 (sensitivity 32.5 pg/ml; homemade assay, Pierce Endogen), IL-6 (sensitivity 7.8 pg/ml; ELISA MAXTM Set, BioLegend), CCL2 and CCL1 (sensitivity 15.6 pg/ml; homemade assay, R&D System), IL-23 (sensitivity 31 pg/ml; eBioscience) and IL-2 (sensitivity 7.8 pg/ml; ELISA MAXTM Set, BioLegend).

Immunoblotting analysis

Immunoblotting analysis of STAT3, SOCS-3 and IDO was carried out in cells differentiated for 6 days in the presence or in the absence of bLF. Endogenously produced IL-6 was neutralized by anti-human-IL-6 (5 µg/ml, clone 6708.11; SIGMA) or anti-human-CCL1 (clone 35305; R&D System) or IgG1k isotype control (5µg/ml, clone 11711; R&D) Abs. Cells were pre-treated at day 0, day 3 and day 6 with anti-IL-6 or anti-CCL1 for 30 min, before bLF addition to the cultures. Six hours after the last bLF treatment, whole cell proteins were extracted as follow: cells were collected and washed three times with ice-cold PBS, then lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-Cl (pH 7.5), 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) containing a cocktail of protease (Roche) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail 1 and 3; Sigma-Aldrich). The protein concentration was determined using the Bio-Rad protein assay (Hercules, CA) according to the manufacturer's instructions. Twenty micrograms of lysate were boiled for 4 min in Laemmli sample

buffer, fractionated on 8% SDS-PAGE gel, and electroblotted to nitrocellulose filter (Protran BA 85, Schleicher & Schuell, Keene, NH). The following Abs were used for the immunoblots: anti-phospho-STAT3 (Y705; Cell Signaling Technology, diluted 1:1000), anti-STAT3 (BD Transduction Laboratories; diluted 1:2500), anti-SOCS-3 (Santa Cruz Biotechnology; diluted 1:500), anti-IDO (Upstate; diluted 1:500) and anti-Actin Ab-5 (BD Biosciences; diluted 1:5000) as gel loading control. Signals were revealed after incubation with anti-mouse or anti-rabbit Ig HRP secondary Abs followed by ECL detection reagent (Amersham).

Confocal laser-scanner microscopy analysis (CLSM)

Cells were fixed with 1% PFA for 15 min at RT, and permeabilized with Dulbecco's PBS (DPBS), containing Ca^{++} and Mg^{++} , 1% BSA and 0,1% Triton X-100 (Sigma-Aldrich Co.) for 30 min at RT. Then, cells were stained with a 1:50 dilution in DPBS/0.1% BSA of FITC-conjugated polyclonal rabbit anti-hLF Ab (DakoCytomation), cross-reactive to bLF, for 1 h at RT. As a negative control, the primary Ab was omitted. Cells were then extensively washed with DPBS and stained with the nuclear fluorescent probe TO-PRO-3 (1 mM; Molecular Probes) for 15 min at RT. After several rinses, cover lips were mounted in buffered glycerol (pH 9) and sealed with nail polish. Immunofluorescence imaging was performed using a Leica confocal microscope (Laser Scanning TCS SP2) equipped with Ar/ArKr and He/Ne lasers at X 40 magnification under an oil-immersion lens. A series of 12 optical sections with a step size of 1 μm through cells were acquired. Laser line was at 488 nm and 633 nm for FITC and TO-PRO-3 excitation, respectively. The percentage of positive cells was calculated by analyzing at least 350 cells for each experimental sample.

Statistical analysis

Statistical comparison between different experimental conditions was determined by the Student's *t* test (paired, two-tailed) by using SPSS software. Differences were considered significant when *p* values were < 0.05 (*), < 0.01 (**), < 0.001 (***)).

CHAPTER 5: RESULTS

Phenotypic properties of MD-DCs generated in the presence of bLF

The expression of a panel of surface antigens, typical of iDCs, was analyzed in monocytes stimulated to differentiate into classic MD-DCs in the absence or in the continuous presence of bLF. As shown in **Figure 9A**, bLF did not interfere with monocyte differentiation into MD-DCs since bLF exposed cells at day 6 of culture expressed CD1a, CD80, CD86 and CD40, as well as MHC class I (HLA-ABC) and II (HLA-DR) antigens, and barely detectable levels of CD83 and CD14 consistent with their differentiation into immature MD-DCs (iMD-DCs). However, a modest but reproducible increase in the expression of CD80, CD86 and HLA-DR, and to a higher extent of PD-L1 and ILT3 was observed in bLF generated MD-DCs (bLF-MD-DCs) with respect to control iMD-DCs. Conversely, ILT4 was expressed at comparable levels in control and bLF-MD-DCs. Furthermore, bLF influenced the dichotomy CD1a⁻/CD1a⁺ observed in *in vitro* generated MD-DCs [153,154]. The percentage of CD1a⁺ cells monitored in ten independent monocyte cultures substantially varied ($n = 10$; $* p < 0.05$) when cells were differentiated in the presence of bLF, with a preferential generation of CD1a⁻ cells (mean $18 \pm 6\%$, $n = 10$) with respect to control cultures (mean $8 \pm 3\%$, $n = 10$). Interestingly, the majority of CD1a⁻ MD-DCs also expressed high levels of HLA-DR and CD86, and some of them were positive for the activation marker CD83 (**Figure 9B**). In keeping with their immature phenotype, bLF-MD-DCs expressed high levels of DC-SIGN and MR, and exhibited a high capacity to uptake DXT (**Figure 9C**). Likewise, bLF-MD-DCs did not secrete effector cytokines typical of activated DCs, including IL-12, TNF, IL-23, IL-10 and CCL2 (see figure 10C).

Interestingly, bLF-MD-DCs produced IL-6 and CCL1 that were not found in control cultures (**Figure 9D**).

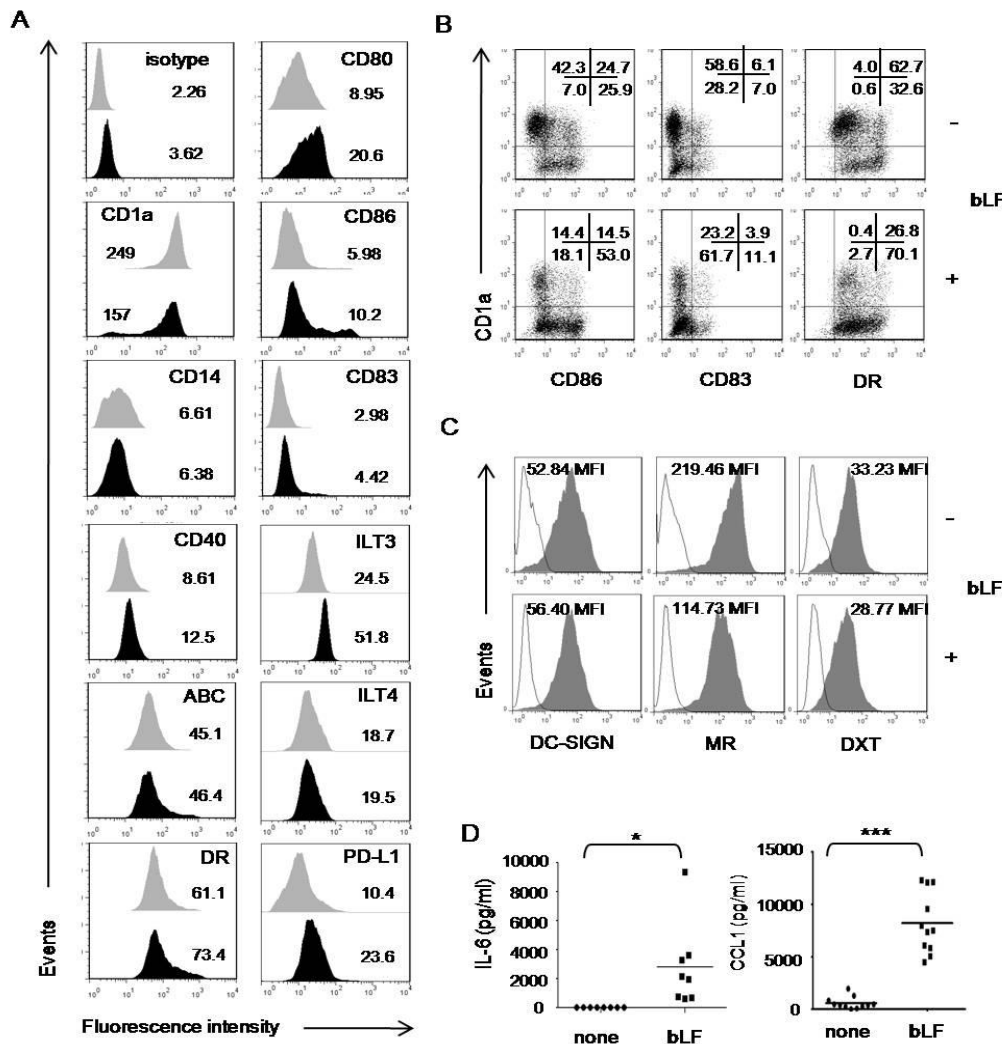


Figure 9. Phenotypic characterization of iMD-DCs generated in the presence of bLF. iMD-DCs were generated in the absence or in the continuous presence of bLF added to the culture at day 0, 2 and 5, concomitantly to the addition of GM-CSF and IL-4. At day 6, cells and culture supernatants were collected. (A) Cells were stained with the indicated Abs and analyzed by flow cytometry. The shaded and black areas represent the expression of phenotypic markers in control and bLF-treated cells, respectively. Numbers indicate median fluorescence intensity (MFI) values of markers analyzed on cells. One representative experiment out of 4 performed is shown. (B) FACS dot plots showing PE-CD1a *versus* FITC-CD86, -CD83 or -HLA-DR expression. Numbers indicate the percentage of cells included in each quadrant. One representative experiment out of 4 performed is shown. (C) Cells were stained with specific Abs to DC-SIGN, MR or FITC-conjugated DXT, and analyzed by flow cytometry. Open histograms represent the background staining of isotype-matched Abs for DC-SIGN and MR, or cells incubated with DXT at 0° C. MFI values are shown. One representative experiment out of 4 is shown. (D) IL-6 and CCL1 content in day 6 supernatants from bLF-treated or control cultures analyzed by ELISA. *p* values were calculated for IL-6 and CCL1 production in bLF-MD-DCs *versus* control iMD-DCs.

bLF-generated MD-DCs do not undergo phenotypic and functional maturation following TLRs stimulation

Next we evaluated whether bLF-MD-DCs could mature in response to TLR triggering. As shown in **Figure 10A**, up-modulation of CD80, HLA-DR and CD83, consistently observed in LPS stimulated control cultures, was markedly reduced in bLF-MD-DCs. As LFs of different origins can bind a variety of anionic biological molecules, including lipid A, with high affinity [32], we tested the effect of bLF on MD-DC maturation induced by other TLR agonists such as the TLR3 ligand poly(I-C) to exclude that the observed bLF-mediated inhibitory effect on DC activation was due to the capacity of this molecule to sequester LPS, thus neutralizing its biological activity. As shown in **Figure 10B**, poly(I-C) induced phenotypic changes were strongly reduced in bLF-MD-DCs as compared to control cultures. These results provide evidence that inhibition of MD-DC maturation is not merely related to bLF capacity to bind LPS but likely relies on direct effects of this molecule on its target cells. In keeping with the lack of phenotypic changes indicative of DC maturation, bLF-MD-DCs retained a high capacity to uptake DXT upon activation with both LPS and poly(I-C), consistent with an immature phenotype (**Figure 10C**). As expected, the high endocytic capacity exhibited by control MD-DCs at the immature state was markedly down-modulated upon maturation induction with LPS or poly(I-C) (**Figure 10C**). Likewise, bLF-MD-DCs treated with LPS and poly(I-C) failed to produce or produced remarkably less IL-12 (n = 6; * $p < 0.05$ for both stimuli), TNF (n = 11; *** $p < 0.001$ and ** $p < 0.002$ for LPS and poly(I-C), respectively), IL-23 (n = 14; * $p < 0.05$ for both stimuli) and CCL2 (n = 7; * $p < 0.05$ for both stimuli) than control MD-DCs stimulated under the same conditions (**Figure 10D**). In contrast, no significant differences were observed in the production of IL-10 that was up-modulated at a comparable extent in both control MD-

DC and bLF-MD-DC cultures stimulated with LPS or poly(I-C) ($n = 7$; $p = 0.298$ and $p = 0.228$, respectively) (**Figure 10D**). These results exclude that the bLF-mediated inhibitory effect on cytokines/chemokines relies on hyper-production of IL-10, a cytokine well-known as a negative regulator of IL-12 and other cytokines [155].

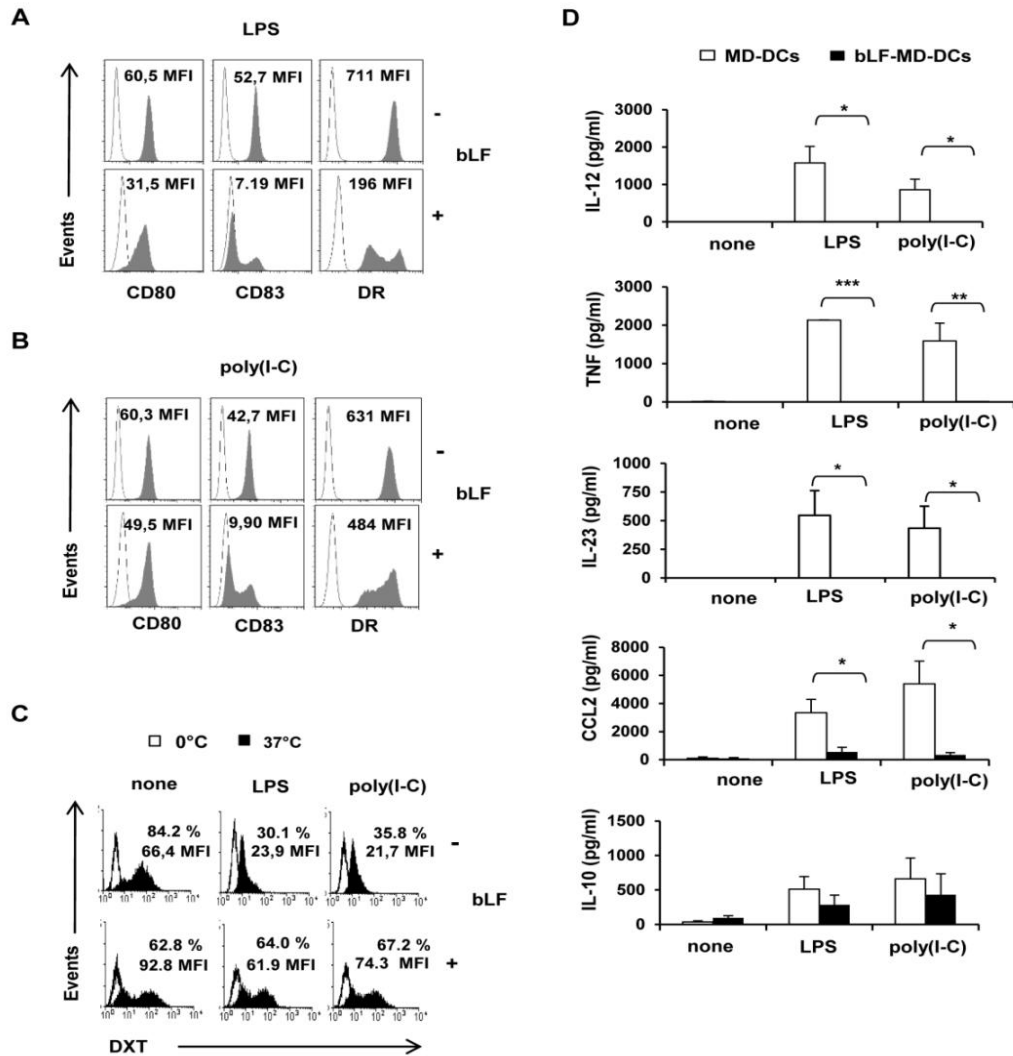


Figure 10. bLF-induced impairment of MD-DC maturation. iMD-DCs were generated as described in the legend to Figure 9. At day 5 of culture, cells were stimulated with LPS (10 ng/ml) or poly(I-C) (20 µg/ml). Twenty-four hours later, cells and culture supernatants were collected. (A and B) Cells were stained with the indicated Abs and analyzed by flow cytometry. Open and shaded areas represent staining with isotype Ab and indicated phenotypic markers, respectively. MFI values are reported. One representative experiment out of 4 is shown. (C) Control and bLF-MD-DCs were stained with DXT and analyzed by flow cytometry. Open and black histograms represent staining with DXT at 0°C or 37°C, respectively. The percentage of positive cells and MFI values are shown. One representative experiment out of 4 is shown. (D) Cytokine/chemokine contents in culture supernatants. Mean \pm SE of 6 to 14 independent experiments is shown. p values were calculated comparing results from bLF-MD-DCs versus MD-DCs.

bLF generated MD-DCs exhibit an impaired capacity to induce T cell activation

Cells generated in the presence of bLF were further characterized for their functional properties. The capacity to induce T cell activation was examined in allogenic mixed lymphocyte reactions (MLRs). After stimulation with TLR agonists, bLF-MD-DCs turned out to be weak activators of T cell proliferation, as demonstrated by the drastically reduced number of lymphocytes expressing Ki67, an intracellular antigen associated with DNA replication, with respect to control activated cells (**Figure 11A**). In keeping with these results, bLF-MD-DCs failed to prime naïve allogenic CD4⁺ T lymphocytes towards the expected Th1 polarization both in terms of percentage of IFN- γ expressing cells and the MFI for the IFN- γ positive cells (**Figure 11B**). Accordingly, IFN- γ production was not detected in the supernatant of bLF-MD-DC/T lymphocyte co-cultures with respect to control activated MD-DCs (data not shown). However, bLF-mediated impairment in IFN- γ production did not favour the expression of IL-4, since the low frequency of IL-4 producing cells was comparable in all experimental conditions (**Figure 11B**). Likewise, no IL-10 secretion was detected in the co-culture medium of both bLF exposed and control cultures (n = 5; < 32.5 pg/ml for each experimental point). A deeper characterization of T lymphocytes in MLRs unravelled a very low intracellular expression of the T lymphocyte activation marker CD154, comparable to that observed in iMD-DCs, in CD4⁺ T cells co-cultured with bLF-MD-DCs activated with TLR agonists with respect to T lymphocytes primed by activated control MD-DCs (**Figure 11C**). In keeping with the functional unresponsiveness of bLF-MD-DC primed T lymphocytes, a marked reduction in IL-2 content was found in LPS activated bLF-MD-DC/T lymphocyte co-culture supernatants (n = 7; * $p < 0.05$) with respect to those collected from control activated MD-DC/T lymphocyte co-

cultures ($n = 5$; *** $p < 0.001$) (**Figure 11D**). To exclude the possibility that the hyporesponsiveness induced by bLF-MD-DCs could be due to the preferential expansion of Treg cells, we characterized the phenotype of expanded $CD4^+$ T cells. However, no major differences were observed in the percentage of $FoxP3^+/CD25^{high}$ T cells in MLRs with MD-DCs generated or not in the presence of bLF (**Figure 11E**).

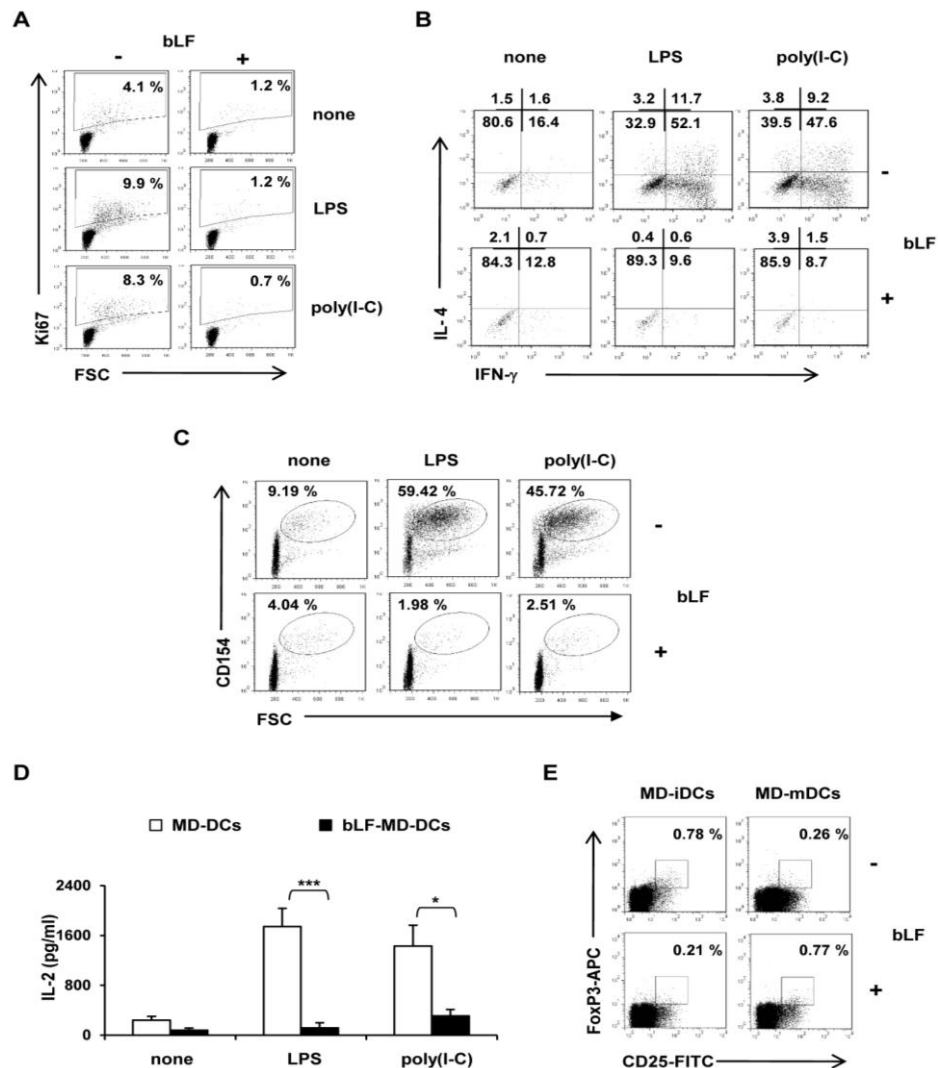


Figure 11. bLF inhibition of MD-DC-mediated T cell responses. iMD-DCs were generated as described in the legend to Figure 9. Control or bLF-MD-DCs were stimulated with LPS or poly(I-C) for 24 h and then co-cultured with (A) allogenic PBL at different DC/PBL ratio (1:100 is shown) or (B-E) allogenic $CD4^+$ naïve T cells at ratio DC/T of 1:10. (A) At day 9 of co-culture, proliferating T cells were intracellularly stained with Ki-67. FACS analysis was performed on lymphocyte population according to FSC/SSC parameters. 20,000 events were acquired per sample. Numbers indicate the percentage of positive cells. (B and C) At day 5 of co-culture, cells were stimulated with PMA (50 ng/ml) and ionomycin (1 μ g/ml), then intracellularly stained with the indicated Abs. Numbers indicate the percentage of lymphocytes included in each quadrant. (D) Day 5 supernatants of co-culture analyzed by ELISA for

IL-2 content. Figure shows mean \pm SE of 5 to 7 independent experiments. *p* values were evaluated comparing results from bLF-MD-DCs *versus* MD-DCs co-cultures. (E) CD4⁺ naïve T cells were primed with control or LPS-primed MD-DCs or bLF-MD-DCs and further re-stimulated with the original MD-DCs before staining with the indicated Abs and FACS analysis. A representative FACS profile is shown as dot plots of FITC-CD25 *versus* APC-FoxP3. The quadrant gates were set according to the negative isotype control Abs in the respective experimental conditions. Numbers indicating the percentage of cells included in each quadrant are shown.

bLF promotes the expression of tolerogenic markers in MD-DCs

To further elucidate the mechanisms by which bLF stimulation of monocytes generates DCs with an impaired capacity to undergo activation and immunosuppressive potential, the expression of tolerogenic markers was examined in bLF-MD-DCs. As described above in Figure 9A, bLF-MD-DCs express a higher level of PD-L1 and ILT3 with respect to control iMD-DCs. The expression of PD-L1 and ILT3 have been linked with the negative regulation of DCs activation and with their ability to induce tolerance [156-158]. Furthermore, enhanced suppressor of cytokine signaling-3 (SOCS-3) expression in murine DCs has been reported to block the IL-12/IL-23 signaling in these cells and to drive them toward a tolerogenic phenotype promoting Th2 responses both *in vitro* and *in vivo* [159]. Likewise, IDO activity in DCs has been suggested to impair T cell responses by altering the microenvironment at the DC/T cell interface [160]. Moreover, signal transducer and activator of transcription 3 (STAT3) activation has been linked to the induction of DCs with a tolerogenic phenotype [161-163]. To further characterize the expression of tolerogenic markers in bLF-MD-DCs, cell lysates prepared from both control iMD-DCs and bLF-MD-DCs were analyzed for the presence of SOCS-3, IDO and tyrosine phosphorylated STAT3 (STAT3-pY705). As shown in **Figure 12**, while STAT3-pY705 and IDO expression was not detected in control iMD-DCs, both these proteins were up-modulated in bLF-MD-DCs. Conversely, SOCS-3 was expressed in control iMD-DCs. However, when MD-DCs

were generated in the presence of bLF, a clear-cut up-modulation of SOCS-3 was observed.

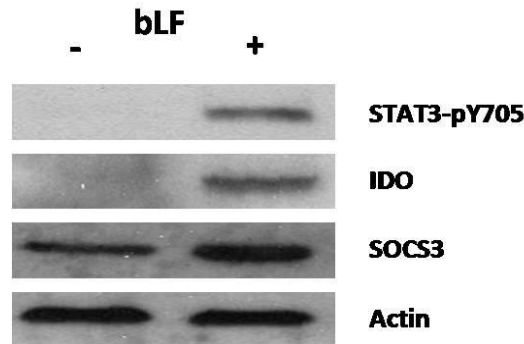


Figure 12. Expression of STAT3-pY705, SOCS-3 and IDO in bLF-MD-DCs. iMD-DCs were generated as described in the legend to Figure 9. At day 6 of culture, cells were lysated and proteins extracted. Immunoblotting analysis for the indicated proteins in control and bLF-MD-DCs is shown. Samples were resolved in 10 % SDS-PAGE gels. One representative experiment out of 5 is shown.

bLF differently interacts with DC precursor cells and differentiated iMD-DCs

Previous studies have demonstrated that exposure of iMD-DCs to recombinant hLF results in their functional activation and promotes Th1 responses [89,95]. However, in our study, cells were treated with bLF at different time of culture, immediately after monocytes isolation and every three days until sample processing, thus allowing bLF to exert differential effects on the two different cell targets. The achieved results suggest that stimulation with bLF during DC generation could suppress the development of functional DCs by differently interacting with differentiated iMD-DCs and their monocyte precursors. To explore this hypothesis, we assessed the effect of a single treatment with bLF of freshly isolated monocytes (day 0) or iMD-DCs (day 5) on the production of IL-6 and CCL1. As shown in **Figure 13A**, high levels of IL-6 were found in monocyte cultures treated with bLF ($n = 13$; $** p < 0.01$) while this cytokine was not

secreted when bLF was added to iMD-DCs ($n = 6$; $p = 0.363$). IL-6 production entirely occurred within the first 18 h after cell seeding. In fact, when the culture medium of bLF-treated monocytes was replaced with fresh medium 18 h post stimulation and cells were cultured for additional 5 days, no IL-6 was detected in the supernatant at the end of culture (**Figure 13B**). Differently from IL-6 production, bLF induced significant levels of CCL1 in both cell types treated at day 0 or day 5, although CCL1 production was significantly higher in cultures stimulated at day 0 ($n = 22$; *** $p < 0.0001$), with respect to the treatment at day 5 ($n = 22$; * $p < 0.05$) (**Figure 13C**). Interestingly, cells continued to produce CCL1 even upon medium replacement 18 h post bLF treatment (**Figure 13D**), suggesting that CCL1 production could be a result of permanent changes induced in bLF-treated DC precursor cells.

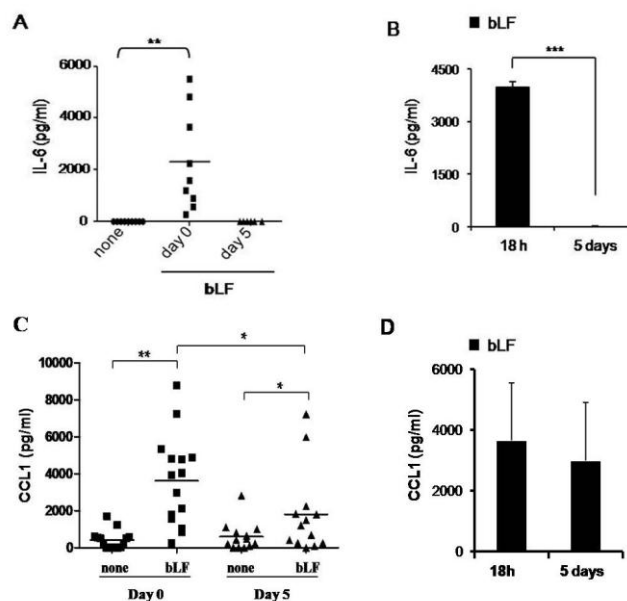


Figure 13. bLF differentially interacts with DC precursor cells and differentiated iMD-DCs. (A and C) Cells were treated once with bLF soon after seeding (day 0) or at day 5 of culture in the presence of GM-CSF and IL-4. Controls were left untreated (none). p values were calculated comparing results from bLF-treated *versus* control cells and day 0 *versus* day 5 bLF-treated cells. (A) At day 6, supernatants were assessed for IL-6 content. The results of 13 independent experiments are shown. (C) Eighteen hours after bLF treatment, supernatants were assessed for CCL1 content. The results of 22 independent experiments are shown. (B and D) Cells were treated with bLF for 18 h, then the culture medium was replaced with fresh medium containing GM-CSF and IL-4, and cells cultured for additional 5 days. IL-6 and CCL1 content in the 18 h and 5-days conditioned medium was assessed by ELISA. Mean \pm SE of (B) 5 and (D) 4 independent experiments is shown. p values were calculated comparing results from 5-days *versus* 18h conditioned medium.

bLF is internalized by DC precursor cells but not iMD-DCs and accumulates into the nucleus

To explore the possibility that a different interaction of bLF with DC precursor cells and iMD-DCs underlies the distinct effects mediated by this molecule, bLF uptake and internalization were investigated by confocal microscopy in freshly isolated monocytes and iMD-DCs. Time-course experiments revealed that bLF was rapidly internalized in freshly isolated monocytes, and its sub-cellular distribution was dependent on the time point examined. As shown in **Figure 14**, bLF was distributed in spotted dots in the cytoplasm of DC precursor cells already after 10 minutes of treatment (panels A, C, D), accumulated in the perinuclear area after 1 h (panels E, G, H), and entered the nucleus at 3 h of treatment, as demonstrated by merged green and blue fluorescence (panels K and L). Conversely, iMD-DCs failed to internalize bLF and very few cells exhibiting some bLF cytoplasmic staining were detected at the later time point (panels V, X, Y).

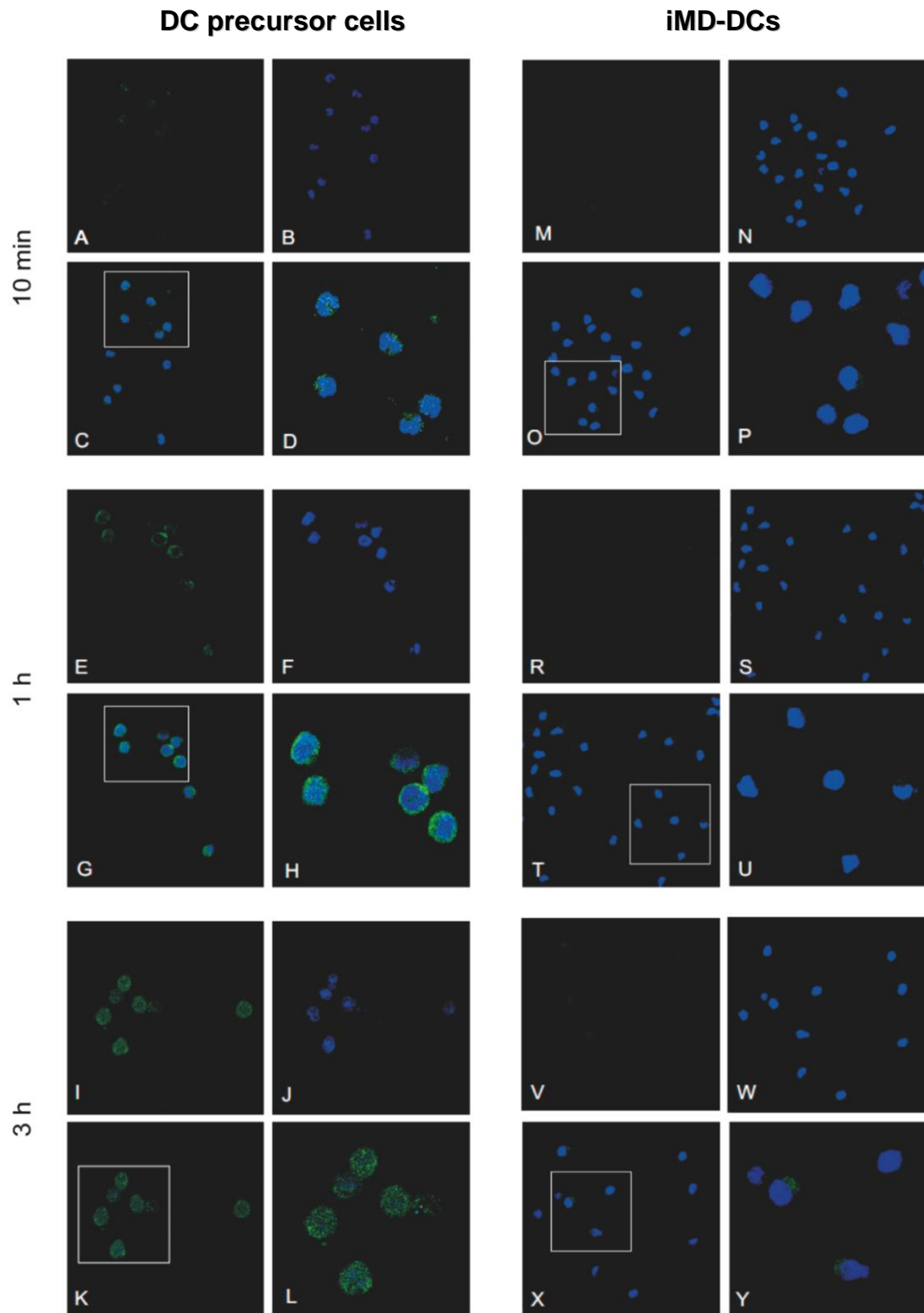


Figure 14. Differential bLF internalization in DC precursor cells and iMD-DCs. CLSM images of cell-associated bLF in freshly isolated monocytes and MD-DCs treated once with bLF soon after seeding or after 5 days of culture, respectively, at the indicated time-points after treatment. Images taken at level of the nuclear section of one representative experiment out of 3 performed are shown. Single green and blue fluorescences represent bLF (panels A, E, I, M, R, V) and nuclei (panels B, F, J, N, S, W), respectively. Panels C, G, K, O, T, X, and their respective 2 X magnification (panels D, H, L, P, U, Y) show merged green and blue fluorescence.

To precisely define the differentiation stage in which changes in the capability of individual cells to respond to bLF occur, progression of monocyte to MD-DC differentiation was monitored in parallel with bLF internalization and IL-6 secretion. To assess and quantify progression, percentage of CD14⁺ and CD1a⁺ cells, and CD1a/CD14 MFI ratio were used as qualitative and quantitative measure of MD-DC differentiation, respectively. As shown in **Figure 15A**, monocyte to MD-DC differentiation progresses through intermediate stages reflected by specific up-modulation of CD1a and down-regulation of CD14. Day 0 monocytes were essentially all CD1a⁻ and CD14⁺. At day 1, and more markedly at day 2, cells began to express CD1a while decreasing CD14 expression. By day 3, most cells have down-regulated CD14 and acquired the expression of CD1a. By day 4, the majority of cells were fully differentiated CD1a⁺/CD14⁻ MD-DCs. Likewise, CD1a/CD14 MFI ratio steadily increased during differentiation and showed clustering of the numerical values at each stage of differentiation (**Figure 15B**), thus providing a reliable indicator of differentiation progression. According to these metrics, a full MD-DC phenotype is acquired between day 3 and 4 of culture in the presence of GM-CSF and IL-4. Concomitant analysis of bLF uptake and internalization revealed that, at day 0 and 1 of culture, most cells internalize bLF, which localizes into the nucleus (**Figure 15C**). Reduction in the percentage of cells internalizing bLF was already observed at day 2, increased at day 3 with only half of the cells positively stained for bLF, which mostly localized into the cytoplasm. The capacity to internalize bLF further decreased with differentiation progression and, at day 4 and 5 of culture, most cells did not exhibit any intracellular bLF. In keeping with these results, reduction in IL-6 secretion was already observed at day 2 of culture, while this cytokine was no longer produced in response to bLF in more differentiated cells at day 4 and 5 of culture (**Figure 15D**). These results

suggest an intimate relationship between differentiation progression and capacity to internalize bLF within the nucleus. Furthermore, bLF nuclear internalization appears to be an important requisite for bLF-induced IL-6 expression.

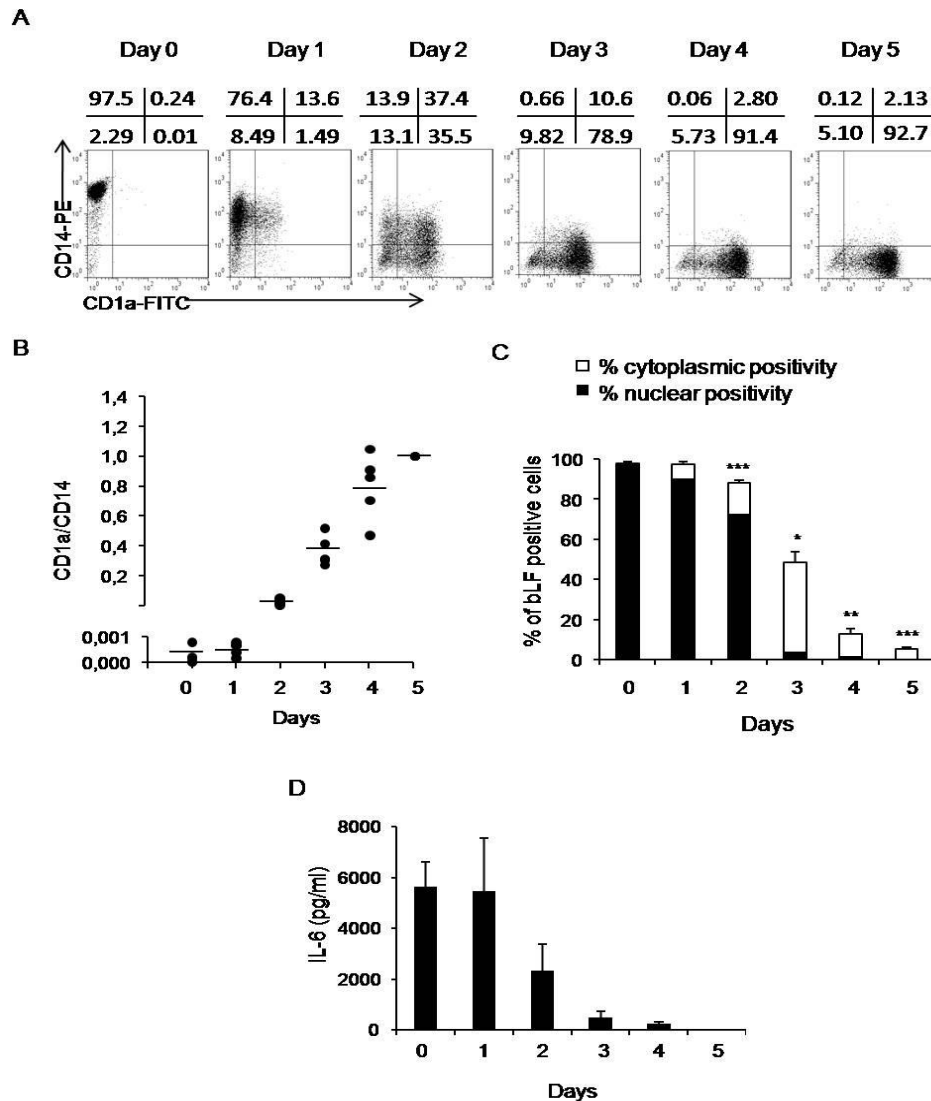


Figure 15. bLF internalization during monocyte to iMD-DC differentiation progression. Monocytes were stimulated to differentiate into iMD-DCs in the presence of GM-CSF and IL-4. (A) Flow cytometric analysis showing cell-surface phenotype during MD-DC differentiation. Monocytes and iMD-DCs can be distinguished by their CD14 and CD1a expression profiles. Data from one representative donor out of 5 are shown. (B) Quantification of CD1a/CD14 expression ratios in 5 independent donors. Ratios were calculated using MFI of CD1a/ MFI of CD14 and normalized to the day 5 ratio of each donor, which was set at 1.0. (C) Monocytes were exposed to bLF soon after seeding (day 0) or at day 1, 2, 3, 4 or 5 of culture in the presence of GM-CSF and IL-4. Three hours later, bLF internalization was assessed by CLSM. Figure shows the mean \pm SE of bLF positive cells in 3 independent experiments. The percentage of positive cells was calculated by analyzing at least 350 cells for each experimental point. The nuclear or cytoplasmic positivity is also indicated. *p* values were calculated comparing the percentage of bLF positive cells at day 1, 2, 3, 4, and 5 *versus* day 0. (D) At each time points, IL-6 produced after a 18 h treatment with bLF was assessed by ELISA. Mean \pm SE of 3 independent experiments is shown.

Role of TLR2 and TLR4 and their co-receptors in bLF-induced IL-6 and CCL1 production

LFs bind to a variety of cell determinants with different grade of specificity, including molecules involved in pathogen recognition [32,54]. Experiments were then designed to define the role of CD14, TLR2 and TLR4, both in bLF entry and in bLF-mediated IL-6 production. Although blocking each of these receptors failed to affect bLF internalization in monocytes (data not shown), blocking CD14 ($n = 7$; $** p < 0.01$) and TLR2 ($n = 8$; $*** p < 0.001$) with specific Abs strongly reduced the capacity of bLF to induce IL-6 production (**Figure 16A**). However, only a partial but significant reduction of IL-6 secretion was achieved when Abs against TLR4 ($n = 7$; $* p < 0.05$) were added to the cultures. As expected, isotype control Abs did not show any effect. According to these results, the effect of neutralizing Abs specific for TLR4, TLR2 and their co-receptors CD14 and CD36 was evaluated on bLF-mediated CCL1 production in both freshly isolated monocytes (day 0) and iMD-DCs (day 5) culture supernatants collected 18 h post bLF treatment. As shown in **Figure 16B and C**, blocking CD36 significantly reduced the bLF-mediated CCL1 production in day 0 ($n = 12$; $** p = 0.01$) but not in day 5 treated cells ($n = 14$; $p = 0.537$). However, significant inhibition of CCL1 release was neither observed at day 0 nor at day 5 bLF-treated cells when TLR4 or CD14 triggering was inhibited (**Figure 16B and C**). Surprisingly, neutralization of TLR2 significantly potentiated the bLF-induced CCL1 release in day 0 treated cells ($n = 12$; $*** p = 0.001$) (**Figure 16B**), whereas chemokine production was markedly inhibited in iMD-DCs ($n = 12$; $*** p = 0.001$) (**Figure 16C**). To understand the biological meaning of these results, the expression of CD36, CD14, TLR2 and TLR4 was analyzed in both freshly isolated monocytes and iMD-DCs. According to literature [136], freshly isolated monocytes expressed higher levels of all markers analyzed on their surface with respect

to iMD-DCs (**Figure 16D**). However, although the very low expression of TLR2 on iMD-DCs, our results on CCL1 production revealed that this receptor is strongly involved in the release of this chemokine (Figure 16C). These results strongly suggest that bLF could interact with different molecular/cellular determinants, likely activating specific pathways in dependence of the cell differentiation stage.

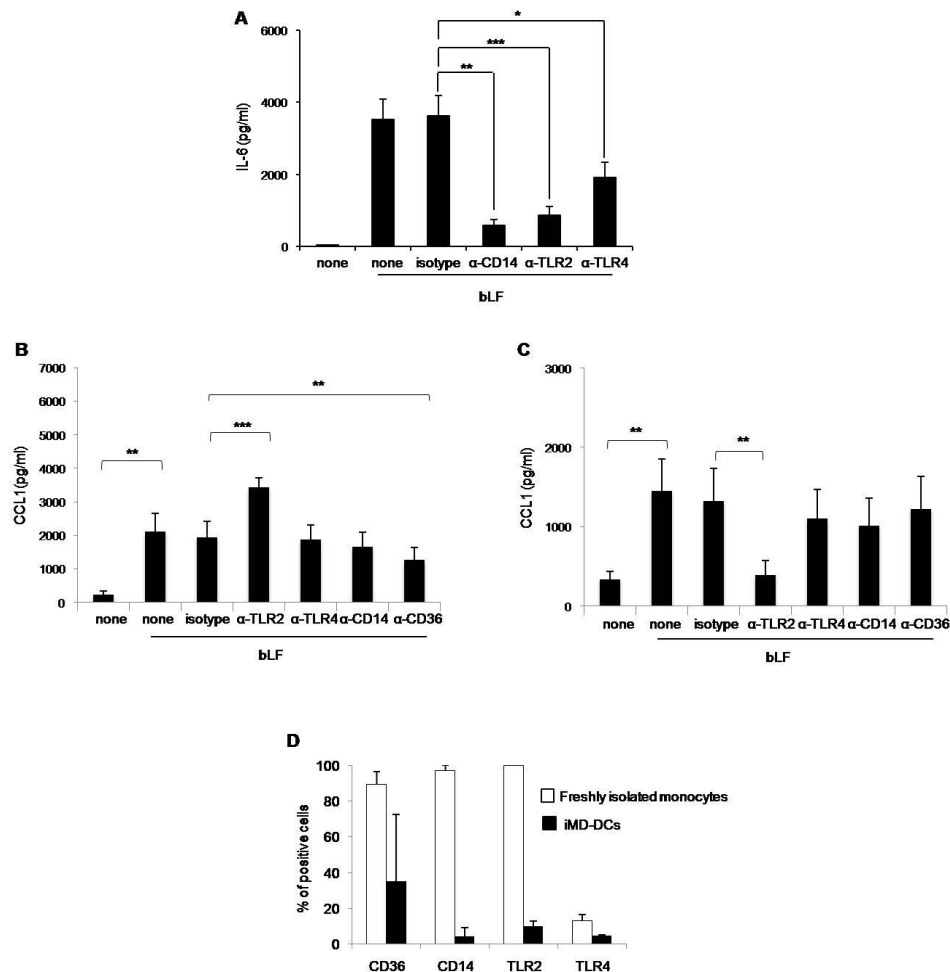


Figure 16. Role of TLR2, TLR4, CD14 and CD36 in bLF-induced IL-6 and CCL1 production. (A) Freshly isolated monocytes were pre-treated for 30 minutes with neutralizing anti-CD14, anti-TLR2, anti-TLR4 or isotype Abs prior to bLF addition and then cultured for 6 days. Culture supernatants were collected and then assessed for IL-6 content by ELISA. Figure shows the mean \pm SE of 4 independent experiments. (B) Freshly isolated monocytes or (C) iMD-DCs were pre-treated for 30 minutes with neutralizing anti-CD14, anti-CD36, anti-TLR2, anti-TLR4 or isotype Abs prior to bLF addition and then cultured for 18 h. Culture supernatants were then collected and assessed for CCL1 content by ELISA. Figure shows the mean \pm SE of (B) 12 and (C) 14 independent experiments. (A-C) *p* values were calculated comparing the results from anti-CD14, anti-TLR2, anti-TLR4 or anti-CD36 versus isotype control treatment in supernatans of bLF treated cells. (D) Expression of indicated surface markers in freshly isolated monocytes or iMD-DCs analyzed by FACS. Mean \pm SD of 2 to 11 independent experiment is shown.

Role of IL-6 in bLF-induced inhibition of MD-DC activation

Previous studies demonstrated that IL-6 plays a major role in maintaining DCs at an immature state both *in vivo* and *in vitro* [164]. Importantly, STAT3 activation by IL-6 is required for the IL-6 mediated suppression of DC maturation *in vivo* [165]. Furthermore, STAT3 activation has been linked to the induction of DCs with a tolerogenic phenotype [161-163]. To address the issue of whether IL-6/STAT3 signalling could play a role in the inhibitory effect of bLF on DC activation, cell lysates prepared from control iMD-DCs and bLF-MD-DCs were analyzed for the presence of tyrosine phosphorylated STAT3. As shown in **Figure 17A** and already described above in Figure 12, while STAT3-pY705 was not detected in control iMD-DCs, bLF-MD-DCs exhibited high levels of the phosphorylated form. STAT3 activation strongly depended on IL-6 since blocking the biological activity of this cytokine with specific neutralizing Abs markedly reduced the levels of STAT3-pY705. In contrast, STAT3 activation levels did not change in bLF-MD-DC cultures treated with the isotype control Ab (**Figure 17A**). Despite the fact that STAT3 activation strongly relies on IL-6, blocking the biological activity of this cytokine did not rescue the capacity of bLF-MD-DCs to undergo maturation as assessed by CD83 expression, TNF and IL-12 secretion (**Figure 17B**). However, replacement of culture medium 18 h after bLF addition, thus depleting bLF-induced soluble factors and residual bLF, partially restored the capacity of bLF-MD-DCs to respond to LPS as assessed by restoration of LPS-induced CD83 up-modulation, and partial rescue of TNF, but not IL-12 production (**Figure 17C**). Conversely, bLF addition to day 5 iMD-DCs did not block LPS-induced CD83 up-modulation while completely abrogated IL-12 and TNF secretion when the culture medium was not replaced. As expected, replacement of culture medium 18 h after bLF addition did not interfere with MD-DC capacity to up-modulate CD83 and

secrete TNF in response to LPS. Conversely, IL-12 production was not rescued even after medium replacement. Thus, bLF interaction with monocytes may affect very early stages of their differentiation into DCs that, at least under certain circumstances, translate into permanent changes of activation related parameters. These changes, although not apparently involving IL-6, may at least in part rely on soluble factors released upon bLF addition.

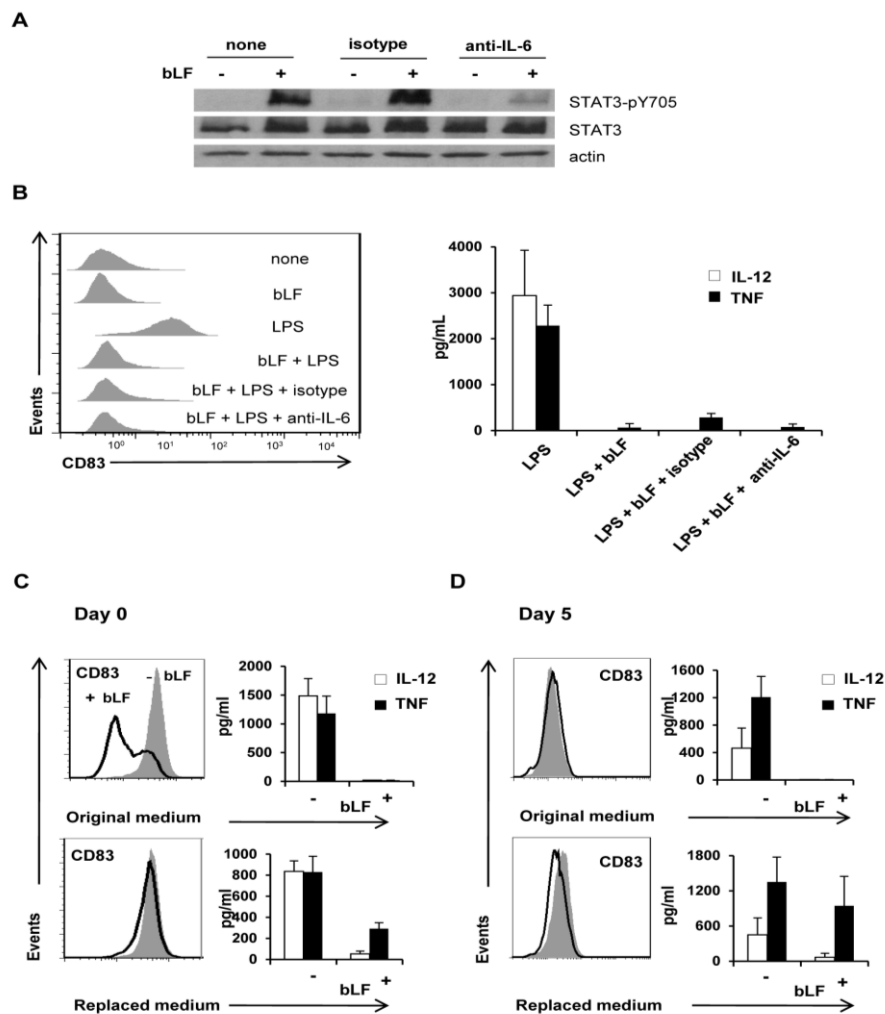


Figure 17. Role of IL-6 in bLF-induced inhibition of MD-DC activation. (A) Immunoblotting analysis for the indicated proteins in both control and bLF-exposed iMD-DCs. Monocytes were plated in complete medium containing GM-CSF and IL-4. Soon after seeding, cells were pre-treated for 30 minutes with neutralizing anti-IL-6 or isotype Abs or left untreated, then exposed to bLF. Treatments were repeated at day 3 and 6 of culture, and proteins were extracted 6 h after the last treatment. Samples were resolved in 10 % SDS-PAGE gel. One representative experiment out of 6 is shown. (B) Monocytes were cultured as indicated in panel A. At day 6 cells were stimulated or not with LPS for 24 h, then stained with anti-CD83 or isotype Abs while culture supernatants were assessed for cytokines content.

One representative experiment out of 4 is shown for CD83 expression. IL-12 and TNF secretion is shown as mean \pm SE of 3 independent experiments. (C and D) Soon after seeding or at day 5 of culture, cells were treated with bLF for 18 h, and then medium was or not replaced with fresh medium (replaced medium and original medium, respectively). At day 5 cells were stimulated with LPS for 24 h. The expression of CD83 and IL-12 and TNF production in MD-DCs, cultured without or in the continuous presence of bLF (original medium) or exposed to bLF for 18 h (replaced medium), was assessed by FACS analysis and ELISA, respectively. Open and dashed areas represent CD83 expression in cells exposed or not to bLF, respectively. One representative experiment out of 3 performed is shown.

Role of CCL1 in bLF-induced inhibition of MD-DC activation

To further characterize the factors involved in the bLF-induced inhibition of MD-DC activation, we addressed the role of CCL1 released by bLF-MD-DCs. As bLF treatment induces a long-lasting STAT3 phosphorylation in MD-DC cultures (Figure 12) and CCL1 expression has been reported to be indispensable for toxin-induced RhoA activation of STAT3 in Hek293 cell line [166], we hypothesized that this phenomenon could be linked, in addition to IL-6, in some way also to CCL1 expression. However, exogenous CCL1 did not induce STAT3 activation in iMD-DCs (**Figure 18A**). Accordingly, neutralization of CCL1 did not abolish the bLF-mediated STAT3 phosphorylation in bLF-MD-DCs (**Figure 18A**), although endogenously produced CCL1 was almost completely neutralized (**Figure 18B**). The autocrine role of CCL1 on MD-DCs immunostimulatory activities was further investigated in MLR experiments performed with bLF-MD-DCs under conditions in which the biological activity of CCL1 was neutralized by specific Abs. At day 6 of culture, cells were stimulated with LPS for 24 h and then CD83 surface expression, IL-12 production and immunostimulatory capacity were assessed (**Figure 18C-E**). As expected, unlike fully mature LPS-stimulated MD-DCs, bLF-MD-DCs failed to either up-modulate CD83, produce IL-12 or activate T cell responses. However, neutralization of bLF-induced CCL1 did not rescue the immunostimulatory capacity to levels comparable to control LPS-stimulated MD-DCs since these cells retained the typical features of LPS-

stimulated bLF-MD-DCs. Specifically, even upon blocking the biological activity of CCL1, bLF-MD-DCs failed to up-modulate CD83 expression and IL-12 production and to induce T cells proliferation (**Figure 18C-E**).

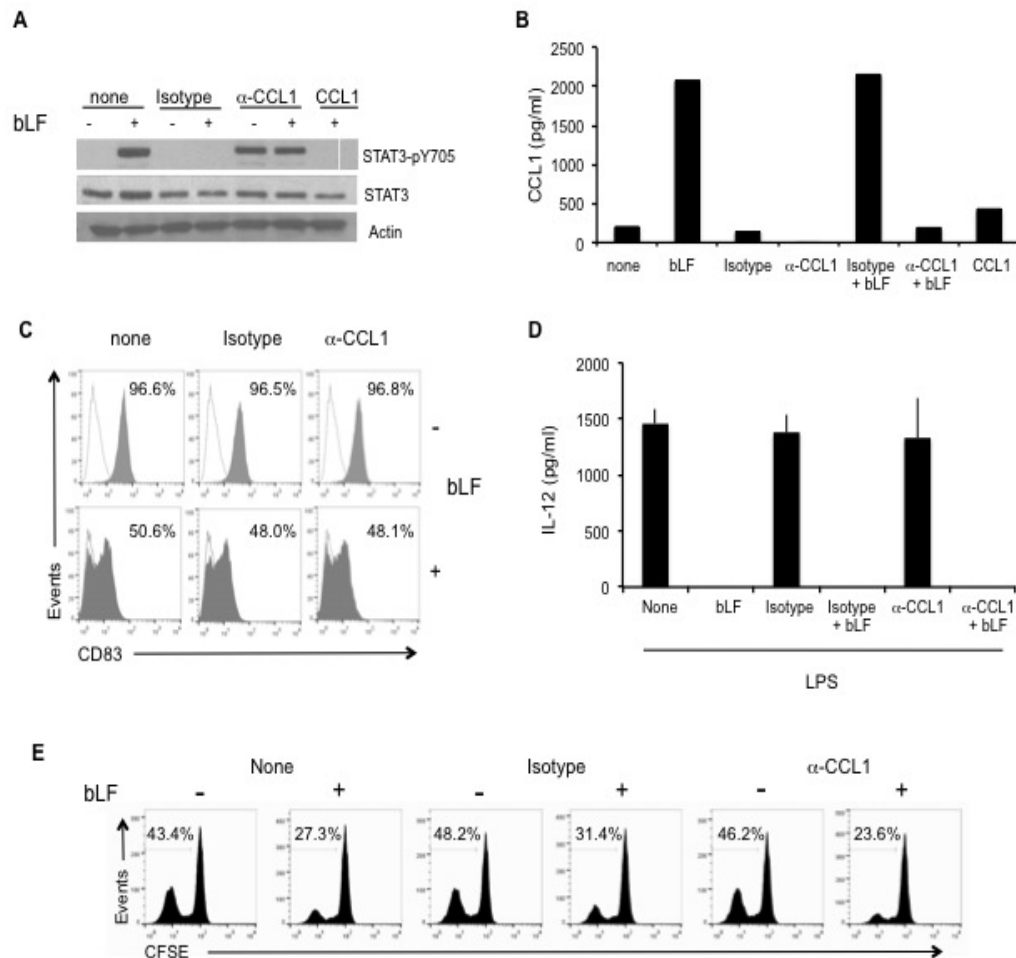


Figure 18. Role of CCL1 in bLF-induced inhibition of MD-DC activation. (A-E) Monocytes were plated in complete medium containing GM-CSF and IL-4. Soon after seeding, cells were pre-treated for 30 minutes with neutralizing anti-CCL1 or isotype Abs or left untreated, then exposed to bLF or not. (A and B) Six hours after the last treatment, cells and culture supernatants were collected. (A) Cells were lysated and proteins extracted. Immunoblotting analysis for the indicated proteins in control and bLF-exposed iMD-DCs. Samples were resolved in 10 % SDS-PAGE gel. One representative experiment out of 2 is shown. (B) CCL1 content in culture supernatants analyzed by ELISA. One representative experiment out of 2 is shown. (C-E) At day 6, cells were stimulated with LPS for 24 h. Then cells and supernatants were collected (C) Cells were stained with anti-CD83 or isotype Abs. One representative experiment out of 6 is shown for CD83 expression. (D) IL-12 production is shown as mean \pm SD of 2 independent experiments. (E) Cells were co-cultured with allogenic PBL, previously stained with CFSE, at different DC/PBL ratio (1:100 is shown). At day 5 of co-culture, proliferating T lymphocytes were assessed by FACS analysis. One representative experiment out of 3 is shown.

CHAPTER 6: DISCUSSION

In this study, we report that human monocytes differentiated into DCs in the continuous presence of bLF exhibit most of the features of iDCs but, upon TLRs stimulation, do not acquire the phenotypic and functional properties of mDCs as assessed by impaired up-modulation of activation related molecules and cytokines/chemokines, and retention of high endocytic activity. Accordingly, bLF-MD-DCs exhibit a reduced capacity to promote the expansion of IFN- γ producing Th1 cells. However, bLF neither favours the expression of IL-4 nor modulates IL-10 production, suggesting that this molecule might control the extent of Th1 polarization rather than *per se* promoting a shift towards Th2 responses. Phenotypic and functional characterization of T lymphocytes, primed by TLR agonist activated bLF-MD-DCs, reveals a less activated phenotype, as assessed by a reduced expression of CD154, and a markedly diminished ability to produce IFN- γ and IL-2, suggesting a state of anergy [167]. In keeping with this assumption, functional unresponsiveness of T lymphocytes has been characterized as a profound inability of CD4⁺ T cells to produce IL-2 *in vitro* [168]. Moreover, DCs generated in tolerogenic environments are capable to induce anergy in memory T cells and to skew cytokine polarization toward low IFN- γ /high IL-10 profile of naïve T cells [169]. Interestingly, anergy induction in memory T cells does not rely on the expansion of CD25^{high} Treg cells, and is partially reversed by IL-2. Conversely, the addition of exogenous IL-12 during DC/T cell priming prevents anergy induction in memory T cells and cytokine polarization in naïve T cells. Accordingly, we did not detect a preferential expansion of CD4⁺/CD25^{high} Treg cells in T cell cultures primed by bLF-MD-DCs.

Previous studies reported that TLRs stimulation during the period of DC generation interferes with and deviates DC differentiation [170-173]. In this regard, it could be argued that minimal amounts of LPS bound to bLF might have a role in the generation

of tolerogenic-like DCs we described. However, experimental evidence argues against this conclusion. Specifically, we observed that blocking LPS activity by polymixin B does not completely abrogate bLF capacity to induce IL-6 (data not shown). In addition, we clearly demonstrated that the maturation arrest of bLF-MD-DCs does not merely depend on the capacity of this molecule to sequester LPS [32,174], as similar results have been achieved when poly(I-C) was used. Finally, bLF-MD-DCs do not show the differentiation block described for the MD-DCs generated in the presence of low amounts of LPS [170,172]. However, our results indicate that bLF has the capacity to modulate the CD1a⁺/CD1a⁻ ratio since a modest but significant reduction in the number of CD1a⁺ DCs has been detected in the presence of bLF. Monocytes can give rise to two populations of myeloid DCs differing in CD1a expression [153,154]. Interestingly, activated CD1a⁻ MD-DCs produce low levels of IL-12 but up-modulate IL-10 secretion, exhibit a scarce capability to induce IFN- γ production and naïve CD4⁺ Th1 polarization [153,154], and can direct differentiation of Th0/Th2 cells [175]. Although a detailed characterization of the functional properties of bLF-induced CD1a⁻ cells has not been performed, the capacity of bLF to modulate the CD1a⁺/CD1a⁻ ratio suggests a role for this compound in the *in vivo*-relevant pathway of monocyte differentiation into DCs.

DCs have a pivotal role in both priming of adaptive immune responses and induction of self-tolerance. This latter function is mediated by specialized subsets of DCs, named tolerogenic DCs, as well as by DC activated or differentiated in the presence of specific biological or chemical agents [151]. They all share the ability to negatively regulate T cell responses, yet their phenotypes, cytokine profiles and thus their mode of action are divergent [151]. In this regard, it has been reported that expression of PD-L1, mainly described as a negative regulatory molecule, is linked with the ability of DCs to induce tolerance [156,157]. Likewise, the inhibitory receptor ILT3 has been shown to

negatively regulate DCs activation [158]. Interestingly, both molecules are up-modulated in bLF-MD-DCs suggesting that they may play a role in the negative regulation of T cell function. Accordingly, bLF-MD-DCs show an enhanced expression of phospho-tyr-STAT3, SOCS-3 and IDO, all molecules expressed in DCs endowed with tolerogenic features [149,159-163].

Among the factors described to drive the generation of tolerogenic DCs is IL-6 [164]. This cytokine is now considered as an important mediator of the immune response especially by directly acting on CD4⁺ T cells and determining their effector functions [164,176]. Furthermore, IL-6 promotes monocyte differentiation into tolerogenic DCs unable to produce TNF and IP-10, to induce allogenic T cell proliferation, and to express CCR7 [177]. Likewise, circulating DCs isolated from multiple myeloma patients exhibit an impaired capacity for T cell stimulation that is partly caused by IL-6-mediated inhibition of DC development [178]. However, it remains an open question how IL-6 orchestrates all these different functions. The contribution of other factors will probably represent a likely explanation. Studies performed in mouse models have enlighten a major role for IL-6/STAT3 signalling pathway in maintaining tolerance *in vivo* [165,179]. In our study, we show that bLF-exposed monocytes transiently produce high amounts of IL-6 soon after treatment, and this cytokine is responsible for the hyper-activation of STAT3 observed in these cells. However, blocking the biological activity of this cytokine neither rescues LPS-induced up-modulation of CD83, TNF and IL-12 nor inhibits, but rather up-modulates, the expression of tolerance related molecules, hyper-expressed in bLF-MD-DCs, such as SOCS-3 and IDO (data not shown). Our results that IL-6 neutralization is not sufficient to counteract the tolerogenic-like phenotype of bLF-MD-DCs argue against a role of this cytokine in the bLF-mediated effects. However, the observation that bLF induces IL-6 secretion and

downstream STAT3 activation in DC precursor cells, but not in iMD-DCs that conversely respond to bLF by up-modulating their immunostimulatory potential [89,95], suggests that other not yet identified aspects of DC functional activation could rely on IL-6. Hence, our results suggest that the bLF-mediated inhibition of DCs activation may at least in part rely on soluble factors released upon bLF treatment, other than IL-6. Moreover, we show that, in contrast to IL-6, both DC precursor cells and iMD-DCs produce CCL1 upon bLF treatment, even though with differences in the protein amount. Indeed, this production seems to be the result of permanent changes induced by bLF in DC precursor cells as they continue to release CCL1 in the medium even after its replacement 18 h post-treatment. Unlike the majority of chemokines, CCL1 has been shown to have only one high-affinity mammalian receptor, CCR8, which is preferentially expressed in polarized Th2 cells, Treg cells, monocytes and DCs [180]. Although CCL1 was the first among a long series of CC chemokine to be discovered [181,182], CCL1-CCR8 is one of the least understood chemokine axis. *In vivo* experimental evidence indicates a skin restricted CCL1 expression, whereas *in vitro* CCL1 may be released by a variety of cells, including activated monocytes, lymphocytes, endothelial cells and mast cells [180]. In particular, in human monocytes CCL1 production seems to be finely regulated since a double signal, involving FcγR engagement and costimulation by signals (IL-1β and LPS), is required for its optimal production [183]. However, a recent study showed that single TLR stimulation induces CCL1 production in human monocytes [184]. Accordingly, we show that bLF can induce *per se* relevant levels of CCL1 in the absence of other stimulations. CCL1 plays a crucial role in the regulation of the immunosuppressive functions and trafficking of Tregs [185,186]. However, its role in the monocyte differentiation into DCs has not been investigated yet. Despite recent evidence on the role of CCL1 in the activation of

STAT3-dependent signaling pathways in Hek293 cell line [166], we show that this chemokine is not involved in the bLF-mediated STAT3 activation in bLF-MD-DCs. In addition, the bLF-mediated inhibition of MD-DCs activation is not reversed by blocking the biological activity of this chemokine since it does not rescue the LPS-mediated up-modulation of CD83 and IL-12 production in bLF-MD-DCs as well as their ability to induce T cell proliferation.

Evidence indicates that different immortalized cell lines, in particular Caco-2 human small intestinal cell line and macrophage-like THP-1 cells, can internalize LF which localizes in their cytoplasm and nucleus [87,187-190]. However, LF internalization by primary cells has not been investigated yet. Hence, in this study we report for the first time that bLF is rapidly internalized by freshly isolated monocytes, but not iMD-DCs, and reaches the nucleus. Interestingly, an intimate relationship between differentiation progression and capacity of bLF to reach the nucleus was also found. The nuclear localization of LF suggests that this molecule may be involved in the transcriptional regulation of genes ultimately controlling monocyte differentiation. In this regard, co-transfection experiments in which a LF expression vector was used together with a vector carrying a reporter gene linked to the GM-CSF promoter revealed that LF reduces the activity of the GM-CSF promoter [191]. Consistent with these results, we found that bLF nuclear localization correlates with bLF capacity to stimulate IL-6 expression. The different ability of monocytes to interact with bLF with respect to iMD-DCs may provide, at least in part, an explanation for the opposite effects, anti-inflammatory versus immunostimulatory [89,95], observed in these cell types.

The mechanism of LF entry is still unknown, but is thought to occur via a LFRs. LF is a cationic protein capable to bind, with different grade of specificity, a variety of cellular determinants, including bacterial components, strongly anionic molecules, CD14, and

PPRs including CLRs and TLR4 [32,54]. Although the relevance of these receptors in triggering bLF effects in human primary monocytes and MD-DCs is unknown, it is of interest that major differences in the expression of at least some of these receptors (e.g. CD14 and DC-SIGN) have been reported in monocytes *versus* iMD-DCs. In particular, CD14 and CD36 take part in receptor complexes that play a crucial role in governing inflammation such as TLR4 [192,193] and TLR2 [194-196] pathways. In this regard, our study demonstrates that although not involved in bLF uptake by monocytes, TLR2 and TLR4 and their co-receptors CD14 and CD36, play a role in bLF-induced signalling leading to both IL-6 and CCL1 production. Since their involvement occurs at different extent depending on the differentiation stage of cells, our results strongly suggest that bLF could interact with different molecular/cellular determinants, possibly activating specific pathways in differentiation dependent manner.

Overall these results shed light on the mechanisms underlying bLF anti-inflammatory activity, highlighting the importance of monocytes as a preferential target for this molecule, and providing further evidence for its potential therapeutic application to inflammatory diseases.

CHAPTER 7: REFERENCES

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