Universidad de Navarra

Facultad de Ciencias

Tráfico de células dendríticas a través de endotelio linfático en condiciones de inflamación.

Dendritic cell traffic across lymphatic endothelium under inflammatory conditions.

Álvaro Teijeira Sánchez

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Dendritic cell traffic across lymphatic endothelium under inflammatory conditions.

Memoria presentada por D./Dª Álvaro Teijeira Sánchez para aspirar al grado de Doctor por la Universidad de Navarra

El presente trabajo ha sido realizado bajo mi dirección en el Departamento de Bioquímica de la Universidad de Navarra y en el Departamento de Oncología del Centro de Investigación Médica Aplicada y autorizo su presentación ante el Tribunal que lo ha de juzgar.

Pamplona,...... de .... de 20

Dra Ana Rouzaut Subirá   Dr Ignacio Melero Bermejo

A mis Abuelos: Javier y Eduardo

A Karmele

"Be it known that, waiving all argument,

I take the good old fashioned ground

that the whale is a fish,

and call upon holy Jonah to back me."

De Ismael en Moby-Dick de Herman Melville

AGRADECIMIENTOS/AKNOWLEDGMENTS

Intentando quizás justificar la cita de la página anterior podríamos decir que hay dos Melville literarios: el grandioso escritor de la novela Moby Dick, lleno de ambición y de motivaciones. Un Melville radiante como su libro, probablemente la "Gran Novela Americana". Por otro lado está el escritor del no menos genial "Bartleby, el escribiente": el escritor de la derrota, del sinsentido, del fracaso.

Cuatro años trabajando en Ciencia no son muchos, pero es tiempo suficiente para conocer a estos dos Melville bien a fondo. Por un lado hay pequeños fracasos, desesperanzas, dudas, y por otro hay resultados, satisfacciones, alegrías. Hay un Moby Dick luminoso en la experiencia de una Tesis pero también mil pequeños Bartlebys que lastran o quizás, impriman carácter, a los años de formación que implica este trabajo. En cualquier caso, aunque esta Tesis no es, ciertamente, la ballena blanca de la inmunología, ha sido mi particular primer viaje en un barco ballenero buscando quizás, capturas menos ambiciosas, pero con una clara vocación arponera.

El barco que zarpó hace cuatro años, ya ha terminado su viaje, impulsado por muchos vientos y agitado por pequeñas tormentas. Si el barco de esta Tesis no ha naufragado, si de alguna forma hemos conseguido cazar algún despistado leviatán, ha sido, sin duda, gracias a una gran tripulación. En primer lugar gracias a dos capitanes intrépidos y apasionados: mis directores, Ana y Nacho, grandes conocedores del inmenso mar de la biología y sus misteriosas profundidades. Gracias a una tripulación de los mejores compañeros, con los arpones siempre listos y afilados: Saray, Rafa, Lili, Xabi, Marta, Erik; también Asís, Iván, Arantza, Aizea, Juancho, Carlos. Gracias a un montón de marineros que mantienen, sin descanso, el rumbo de la nave: Mantenimiento, Biblioteca, Morfología, Imagen, Secretarías, bedeles, dirección… Gracias también a otros barcos, que nos han acompañado en esta expedición: Al Departamento de Bioquímica que me ha dado la posibilidad de impartir docencia y al Dr Martínez de Irujo por acompañarme y enseñarme a querer a los alumnos. *To Dr Salvatore Valitutti and his wonderful team for teaching me so many things in Toulousse.*

Por último dar gracias a aquellos que se han quedado en tierra, atentos siempre a los progresos de la expedición. Gracias al apoyo de mi familia, al buen humor de mis amigos, a la alta-cocina de mis compañeros de piso. Gracias sobre todo a Karmele, por ser mi Queequeg particular.

Sin todos vosotros no habría sido posible obtener estas poquitas gotas de preciado aceite que, espero, pueda iluminar a otros científicos en su búsqueda particular de la gran ballena blanca. Ojalá el siguiente barco que me recoja lleve semejante tripulación.

INDEX

ABBREVIATIONS

A

APC- Antigen presenting cell

B

BCL-2- B cell lymphoma 2

BM- Bone marrow

C

CDP- Common DC precursor

CFA- Complete Freund Adjuvant

CHS- Contact hyper-sensibility

CLEVER-1- Common lymphatic endothelial and vascular endothelial receptor 1

CTL- Cytotoxic T lymphocyte

CTLA-4-Cytotoxic T lymphocyte antigen 4

D

DC- Dendritic cell

DIC- Differential interphase contrast

DN- Double negative

E

EC- Endothelial cell

ECM- Extracellular matrix

eROS- Endothelial Reactive Oxygen Species

EZRM- Ezrin moesin family proteins

F

FLT-3- Fms like Tyrosine Kinase

G

GEF- Guanine Nucleotide Exchanging Factors

GMCSF- Granulocyte macrophage Colony Stimulating Factor

H

HEV- High endothelial venule

HUVEC- Human umbilical vein endothelial cell

I

ICAM- Intercellular adhesion molecule

IFN- Interferon

IL-1β- Interleukin 1 beta

IM- Intercellular matrix

ITG- Integrin

J

JAM-A –Junctional adhesion molecule A

L

LBRC- Lateral border recycling components

LC- Langerhans cells

Lck- Lymphocyte specific protein kinase

LE- Lymphatic endothelium

LEC- Lymphatic endothelial cell

LFA-1- Lymphocyte function associated antigen 1

LN- Lymph node

LPS- Lipopolysaccharide

LYVE-1- Lymphatic vessel endothelial hyaluron receptor 1

M

mAb- Monoclonal antibody

MAC-1- Macrophage 1 antigen

MAPK- Mitogen-associated-phosphorylated-kinase

MCP-1- Monocyte chemotactic protein

MHC- Major histocomapatibility complex

MIP1-α- Macrophage inflammatory protein 1 alpha

MMP- Matrix Metalloprotease

MR- Mannose receptor

N

NFκB- Nuclear factor kappa- B

NK- Natural killer

NOD- Nucleotide Oligomerization Domain

P

PARPs- Pathogen associated related patterns

PBMC- Peripheral blood mononuclear cells

PD-L1- Programmed cell death 1 ligand

PLD-1- Phospholipase D1

PMA- Phorphol myristate acetate

Prox-1- Prospero homeobox domain 1

Ptx- Pertussis toxin

S

S1P- Sphingosine 1 phosphate

S1PR- Sphingosine 1 phosphate receptor

SPHK- Sphingosine kinase

T

TCR-T cell receptor

TEM- Transendothelial migration

TGF-β- Transforming growth factor beta

TIMP- Tissue inhibitor of metalloprotease

TLR – Toll-like receptor

TNFRSF9- Tumor necrosis factor receptor super family member 9

TNFα- Tumor necrosis factor alpha

TRAF2- TNF receptor associated factor 2

V

VCAM- Vascular cell adhesion molecule

VEGFR-3- Vascular endothelial growth factor receptor 3

VLA-4- Very-late antigen 4

INTRODUCTION

1- MORPHOLOGICAL AND FUNCTIONAL CHARACTERISTICS OF LYMPHATIC ENDOTHELIUM.

1.1 Introduction to the biology of lymphatic system.

The lymphatic vessels form a vascular network independent of blood vasculature that is responsible for the normal maintenance of fluid balance in tissue. Besides, it is devoted to the transport of fat from the gut and of immune cells from tissues to the lymph nodes. Up to 50% of the protein that extravasates from blood vessels cannot be directly reabsorbed by these capillaries and has to be taken back to blood through the lymphatic network (Pepper and Skobe, 2003).

This vascular system was first documented by ancient Greeks. Hipocrates described lymph as "white blood". But, surprisingly, the research on the molecular structures and physiology of this circulatory system has been hampered due to the lack of appropriate specific markers.

Relatively recently, a number of molecules have been described that allowed us to specifically distinguish lymphatic vessels from blood vessels. These are: the tyrosin kinase receptor VEGFR-3 (Kaipainen et al., 1995), the membrane glycoprotein Podoplanin (Breiteneder-Geleff et al., 1999), the hyaluronan receptor LYVE-1 (Banerji et al., 1999), and the transcription factor Prox-1 that specifically determines lymphatic endothelial lineage during embryonic development (Wigle and Oliver, 1999). Furthermore, the lymphatic endothelium does not express the transcription factor PAL-E, normally expressed on blood vasculature (Sleeman et al., 2001). These molecular tools have made possible the isolation and culture of primary lymphatic endothelial cells (LEC) and their labeling for microscopy-based analysis of these vessels (Hirakawa et al., 2003; Podgrabinska et al., 2002). Additionally, some cell lines of mouse lymphatic endothelial cells have been established (Ando et al., 2005; Sironi et al., 2006).

The anatomical structure of the lymphatic vessels has been compared to a tree composed of a wide trunk, which drains fluid back into the blood. This trunk branches in smaller vessels, that in turn branch into thinner ramifications, that finally terminate

into blind-ended capillaries (Skobe and Detmar, 2000). Therefore, the lymphatic network constitutes a one-direction system of vessels that initiates in blind-ended capillaries. These blind-ended capillaries drain fluid into vessels of increased caliper until the thoracic ducts, where lymph is poured into the blood vascular system through subclavian veins.

In order to accomplish its draining function, the lymphatic capillaries form an intricate network inside the tissue, in close contact with blood vessels, but without anastomose with them. The main surface of these capillaries is deprived of perycites and myocites that can be found in wider vessels. Myocite's contraction helps these vessels to pump lymph, which enters in wider lymphatic vessels, known as collecting vessels with increased amounts of smooth muscle cells. These smooth muscle covered surfaces originate small areas, that act as pumps located between two bulb-like sections of the lymphatic vessel (Randolph et al., 2005). Besides, in order to avoid retrograde lymph flow, small valves are located on larger lymphatic vessels. The collecting vessels finally drain fluid to thoracic ducts that are directly connected to both left and right subclavian veins.

The lymphatic system forms part of the immune system, since lymph nodes are integrated in the lymphatic network. The lymph nodes are part of the secondary lymphoid tissues where immune responses are started and shaped. Lymph nodes are highly organized and dynamic organs grossly divided into a fibrous capsula, subcapsular sinus, a cortex, and a central medulla. The cortex harbors in its most external part the B cell follicles, and has an inter-follicular part rich in T cells. The medulla is traversed by vessel-like structures called sinusoids that separate, medulla cords enriched in macrophages and reticular cells (Mak and Saunders, 2006). Lymph enters into the lymph node through several afferent collecting lymphatic vessels that drain fluid into the trabecular and medullary sinuses that converge in the hillium where lymph leaves the node through the efferent lymphatic vessel (Fig 1) (Belz and Heath, 1995). Furthermore, there are also

Figure 1. Schematic representation of the lymph node. Adapted from von Andrian and Mempel (2003)

blood vascular vessels inside the node, coated by an endothelial layer functionally specialized in leukocyte migration. These structures known as High Endothelial Venules (HEV) are critical for the formation of lymphoid tissue (Girard and Springer, 1995).

The lymph nodes are the tissue scenario where immune responses are orchestrated. Antigen presenting cells (APC) present cognate antigen to specific T and B lymphocytes. Antigens and cell debris may arrive from tissue to the lymph nodes directly via the afferent lymph (Sixt et al., 2005) or ferried by dendritic cells travelling also via afferent lymphatics.

1.2 Morphological characteristics of the lymphatic endothelium

The morphological differences that discriminate lymphatic capillaries from their blood counterparts have been reviewed by Mihaela Skobe (Skobe and Detmar, 2000). In summary, the lymphatic vessels present an irregular lumen that is wider than that of blood capillaries. The mean diameter of blood capillaries is up to 22 μm diameter, whereas that of lymphatic vessels can rise up to 60 μm. Besides, the endothelial cells that line the lymphatic capillaries present an extremely thin cytoplasm and are covered by a poorly developed or even absent basal membrane. This along with the lack of pericytes, results in vessels that are more labile than blood capillaries (Fig 2).
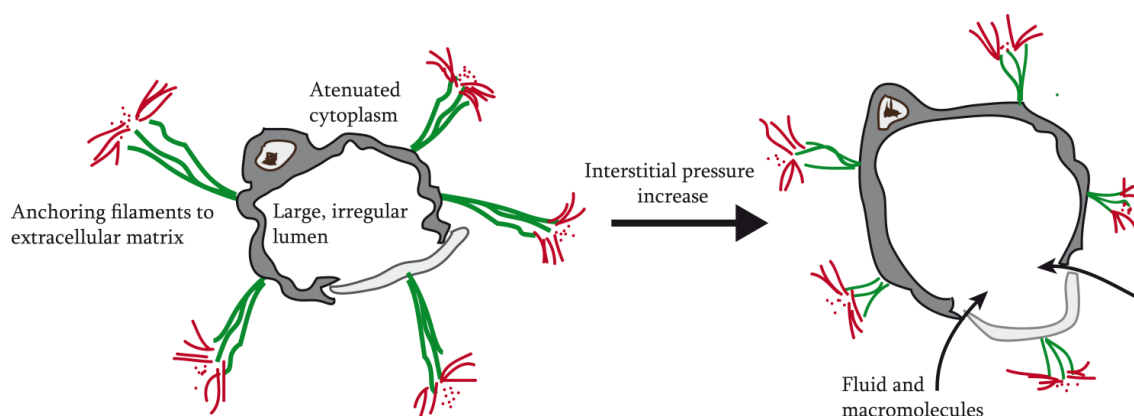
Figure 2. Schematic representation of a lymphatic vessel displaying its specific morphological characteristics, both under steady steate and under interstitial pressure. Adapted from (Skobe and Detmar, 2000)

The lymphatic endothelial cells (LEC) are fastened to the extracellular matrix (ECM) by specialized anchoring filaments which contain elastic fibers. As shown in figure 2, increments of pressure in the tissue promote the traction of EC by the anchored filaments that are pulled by the fibers of the ECM. This event leads to the opening of small gaps, formed between specialized overlapping cell-cell junctions that allow the entrance of the fluid attracted to the negative pressure of the vessel. The filling of the lymphatic vessel induces fiber relaxation and the closure of the gaps when the pressure returns to normal values. This mechanism originates small unidirectional propels of fluid in the capillaries that are directed exclusively by the interstitial pressure. However, wider lymphatic vessels present smooth muscle cells that give contractility and pushes fluid along the vessel by peristaltism.
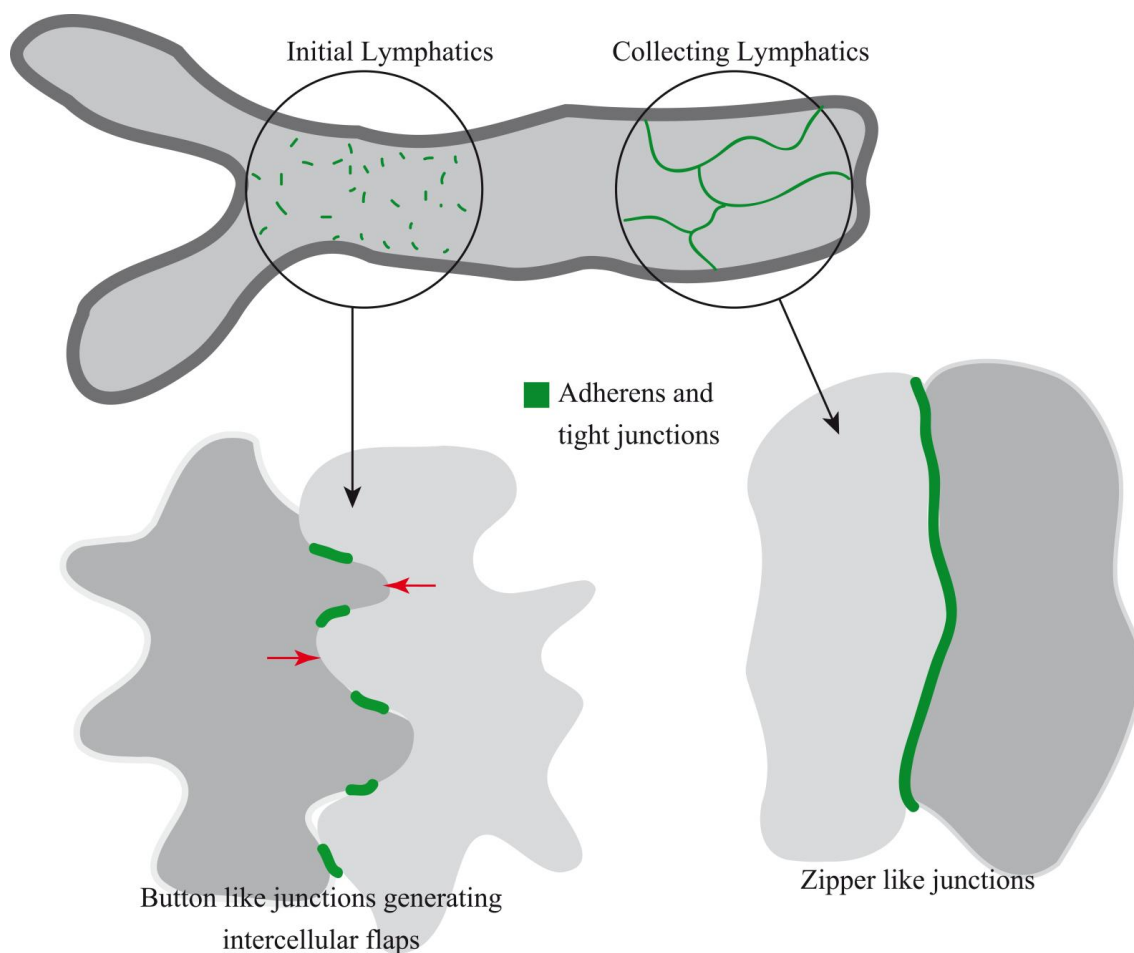
Figure 3. Scheme of the microanatomy and LEC disposition of a lymphatic capillary. In figure A an schematic representation of a lymphatic vessel is shown exhibiting button like junctions on capillaries but not on wider collecting vessels which exhibit zipper- like junctions. B shows a detailed view of the shape of LEC and the distribution of button-like junctions as represented on C and D for two different cells with membrane flaps between each other. Baluk et al. (2007).

The anatomical dissimilarities between lymphatic and blood capillaries are highly related to their different physiology and become more evident when these vessels are analyzed at the subcellular level (Baluk et al., 2007). LEC present an oak-tree-leaf morphology in which the overlapping flaps of cytoplasm interdigitate between different endothelial cells (Fig 3). These flaps present discrete regions in which tight intercellular junctions enriched in JAM a and VE-cadherin are formed in a button-like disposition that leaves entire sections of the remaining intercellular junctions bound by loose adhesive forces (Fig 3). This is in sharp contrast with the zipper-like distribution of tight junctions on the inter-endothelial contacts of blood capillaries. The presence of these button-like structures in the lymphatic capillaries supports the specialized clearance of the excess of fluid by the lymphatic vessels and the passive transport of macromolecules via these flaps (Fig 3D). These membrane zones deprived of tight junctions also provide an entrance route for leukocytes into lymphatic vessels.

Besides the afore-mentioned, other morphological studies reported the existence of different specialized structures that would allow cell migration across the endothelial capillaries via small microchannels formed across the lymphatic endothelial layer that directly connect the tissue with the lumen of the vessel in a continuous way (Azzali, 2007a; Azzali, 2007b).

All the above mentioned anatomical and molecular differences between blood endothelium and the lymphatic endothelium may be important not only for fluid and macromolecule transport, but also for the migration of leukocytes to the lymph nodes. Indeed, flux interference on lymph vessels also impairs leukocyte migration to the lymph nodes (Liao et al., 2011).

1.3 Lymphatic vessels under inflammation

Inflammation is a physiological response to pathogens or mechanical harm in order to remove and/or combat the damaging agent. This local vascular response often starts by the secretion of soluble mediators such as prostaglandins, TNFα or IL-1β among others (Wilting et al., 2009). The main features that characterize an inflammatory response are: vasodilatation and arrival of leukocyte (infiltrate) and fluid (exudate) to the tissue (Wilting et al., 2009). These changes in the tissue

microenviroment promote immune responses to control potential pathogens. LEC, as many other cell types, can sense inflammatory cytokines through a wide range of cytokine receptors and respond to inflammation (Alitalo, 2011). These pro-inflammatory stimuli can be delivered locally or reach the lymphatic vessels from distant sites. For example, it has been described how mast cells are able to secrete TNFα-containing microparticles, upon inflammation. These particles may either deliver high local concentrations of this cytokine to the nearest lymphatic capillary or be transported inside the lymphatic vessels to reach the lymph nodes were they also elicit inflammatory functions (Kunder et al., 2011; Kunder et al., 2009).

Early studies performed on TNFα-treated human LEC showed that these vessels have a similar transcriptomic response to inflammation compared to blood vascular endothelium. This response is characterized by the increased expression of chemokines such as CCL21 and several surface adhesion molecules (Johnson et al., 2006; Johnson and Jackson, 2010). Recently, a similar transcriptomic result was observed using models of Contact Hipersensibilty (CHS) in lymphatic vessels from inflamed ears in mice (Vigl et al., 2011).

Furthermore, a very similar response to that observed upon TNFα treatment of LEC can be observed when LEC are treated with TLR ligands such as POLY I:C and LPS, showing the ability of LEC to promote a direct response of the lymphatic endothelium to microorganisms (Pegu et al., 2008).

On the contrary, when complete Freund adjuvant (CFA) injection was used as a model of inflammation this inflammatory response was not as effectively induced (Vigl et al., 2011), indicating that the response to inflammation of the lymphatic vessels depends on the original stimuli that causes it.

Under inflammation, hydrostatic tissue pressure that is locally increased promoted a clear upregulation of CCL21. Furthermore, CCL21 was dramatically reduced under very low flow conditions corresponding to induced lymphedema in mice (Miteva et al., 2010). Interestingly, lymphatic endothelium activation on inflamed tissue is also able to control inflammation by increasing the flow and reducing interstitial edema (Huggenberger et al., 2011).

Lymphatic endothelial cells also respond to inflammation by increasing proliferation and lymphangiogenesis (Flister et al., 2010). This is accompanied by lowering their permeability through the replacement of the button-like intercellular junctions characteristic of mature lymphatic capillaries by continuous zipper-like tight junctions (Kajiya et al., 2012, Yao et al., 2012).

In conclusion, lymphatic vessels have shown to have a dynamic, complex and varied response to inflammation, which includes increased promotion of leukocyte migration as a result of the expression of chemotactic factors and adhesion molecules on LEC surface. Such a response helps leukocyte transit through lymph and to lymph nodes and improved clearance of fluid excess.

2- DENDRITIC CELLS AND THEIR MIGRATORY CAPACITIES

2.1 Introduction to the biology of DCs, the Langerhans Cell paradigm.

Dendritic cells are a leukocyte population firstly identified by Steinmann and colleagues in early seventies (Steinman and Cohn, 1973; Steinman et al., 1983). These cells showed different from macrophages since they can present antigen and activate resting T lymphocytes.

The identification of DCs was centered into the discovery of a cell type of the skin known as Langerhans cell (LC). These cells were identified as a probable neural cell type due to its morphology.

The main function of DC is to process antigen and present it to other cells of the immune system. In fact, these cells constitute a very potent subset of APC, due to their high expression of co-stimulatory molecules such as CD80 and CD86 on their extracellular membrane, their high efficiency in antigen processing and the concomitant production of cytokines (Banchereau and Steinman, 1998).
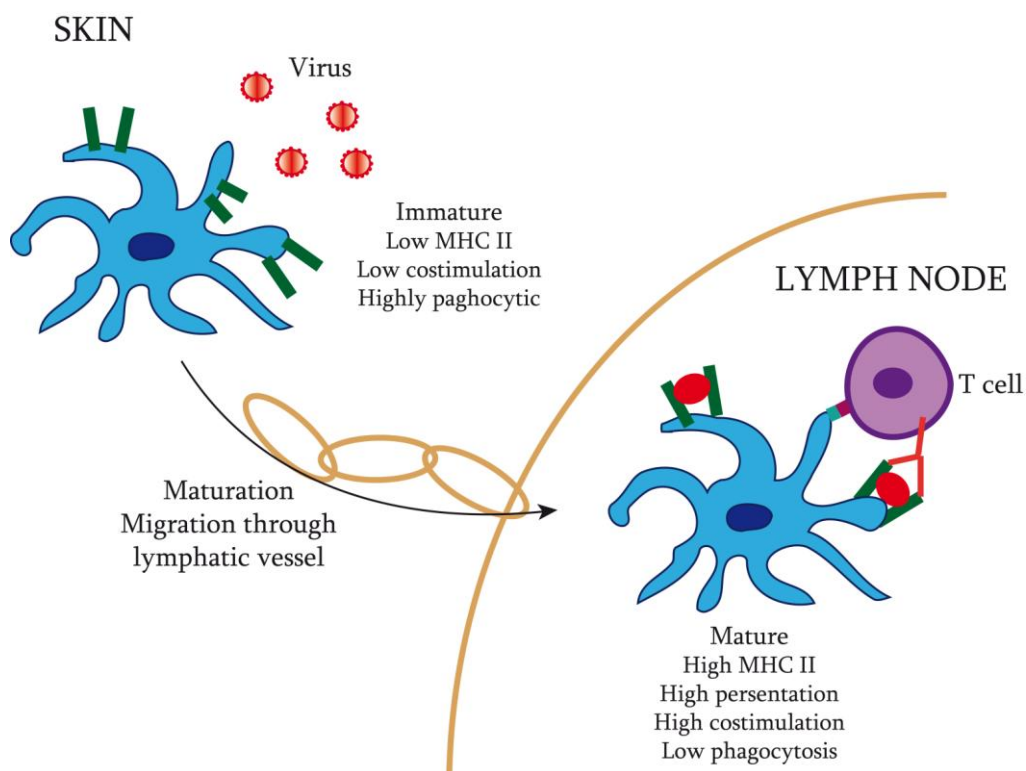
Figure 4. Schematic picture representing the Langerhans cell paradigm of DC migration. Adapted from Heath and Carbone (2009)

DC process and present the antigen on their surface bound to MHC molecules. The MHC-bound antigen is recognized by T cell specific receptors (TCR). Antigen recognition by T lymphocytes, promotes the specific functions of these cells against pathogens, such as increased proliferation, activation of cytotoxic machinery, or the activation of other neighboring leukocytes (Mak and Saunders, 2006). The accessibility of skin LC made this particular DC subtype a good model to investigate DC biology. All the early research in DC biology was performed using LCs and lead to the development of what has been named the "Langerhans cell paradigm".

As shown in figure 4, the paradigm tries to reflect the behavior of DCs on peripheral tissues and their response against a pathogen invasion. DCs are usually found in the peripheral tissue, in a steady-state known as immature DC. Immature DCs are phenotypically characterized by a low surface expression of MHC class II and co-stimulatory molecules, while they exhibit very efficient phagocytosis functions. Nevertheless, DC have an enormous ability to respond to a great number of pathogens. This is due to the expression on their surface of pathogen associated molecular pattern receptors (PARPs) such as Toll-like receptors (TLRs) (Iwasaki and Medzhitov, 2004), nucleotide-binding oligomerization domain (NOD) proteins (Meylan et al., 2006), RIG-I-like receptors (Meylan et al., 2006) and C-type lectin receptors (Figdor et al., 2002).

Therefore, after a pathogen is detected by binding to one of the above-mentioned receptors, DCs change their repertoire of chemokine receptors and enhance their migration towards chemokine gradients established in the damaged tissue. During the migration process, DCs mature and upregulate the surface expression of both MHC-Class II and I molecules and co-stimulatory molecules such as CD80 receptors that promote T cell activation in the lymph nodes. The microbial signals can cooperate in this maturation by acting on DC receptors.

2.2 The Langerhans cell paradigm extended. Functional and migratory complexities of dendritic cell network.

A number of recent publications have introduced interesting complexities in this model, as different DC subsets are identified, and as a consequence, a variety of specialized functions are attributed to them (Sporri and Reis e Sousa, 2005; Villadangos

and Heath, 2005; Villadangos and Schnorrer, 2007). DCs can be also isolated from a number of tissues going from blood to the majority of epithelium-associated tissues. The most comprehensive DC classification divides DC into **plasmacitoid DC (pDC), conventional (cDC)**, and **monocyte derived DCs (moDC)**. This classification is valid for both human and mouse species (Table 1) (Heath and Carbone, 2009).

Plasmacitoid DCs (pDC) can be derived from Myeloid, lymphoid or a common Pre-DC precursor dependent on the tissue that they are colonizing (Ardavin, 2003). pDC do not exhibit the dendrite-rich morphology characteristic of skin DCs. pDC are specialized in the secretion of type I IFN, after viral infections (Perussia et al., 1985).

Conventional DCs (cDC) derive from a bone marrow specialized precursor called common DC progenitor (CDP) which evolves into a pre-DC progenitor that can be found in blood, although at low frequency (Belz and Nutt, 2012). These cells present dendrite-like membrane extensions. **cDCs** as well, can be subsequently classified into *migratory* and *lymphoid* subtypes. *Migratory* cDCs can be found under steady state conditions in lymph nodes and in some peripheral tissues such as epidermis (Langerhans cells), lung and intestine. Different subpopulations of *migratory cDC* can be defined depending on the organ that colonize and the surface markers they exhibit. For example, the skin is colonized by several different subsets of migratory cDCs: such as Langerhans cells, and at least two populations of cDCs. Meanwhile, dermis exhibit two different populations: a majoritarianone composed of dermal DCs, and a minoritarian population of cells that express Langerin. Besides, *migratory cDCs* exhibit the expression of chemokine receptors such as CCR7 which guide them into the lymph nodes (Segura et al., 2012). *Lymphoid cDCs,* however, are found in most lymphoid tissues, including spleen and lymph nodes, although they can also be found in blood in relative low numbers. As happened with *migratory cDC,* many *lymphoid cDCs* subpopulations can be identified attending the expression of different membrane markers (Table 1). The arrival of these different subsets to the lymph nodes is not completely defined. Since *lymphoid DCs* have been detected in blood, they are thought to enter to the lymph nodes through an hematogenous route. In fact, this has been demonstrated for pDCs (Diacovo et al., 2005), but the entry route for other important DC subsets, such as $CD8_\alpha$ (that participate in antigen crosspresentation (Dudziak et al., 2007)), is yet to be described (Randolph et al., 2008).

Lastly, **monocyte derived DC (moDCs)** are originated from a monocytic common precursor known as CMP. These cells also exhibit a characteristic dendritic morphology. Interestingly, moDCs cannot be found in the lymph nodes or in blood under steady-state conditions. However, they can be found in the lymph nodes under inflammation. Monocytes migrate into a number of tissues and differentiate to moDC under inflammatory stimuli (Domínguez and Ardavín, 2010). Once transformed into inflammatory DC, they migrate from these peripheral tissues to lymph nodes (Belz and Nutt, 2012). Due to its easy accessibility and fairly high numbers in blood, moDC are easily derived from their blood precursors and used as a model for human DC studies. Besides, *ex-vivo* differentiated moDCs are widely used in protocols of adoptive transfer in animal models and in clinical assays (Gilboa, 2007). Most of the studies based on this models point to lymphatic vessels as their entrance route to the lymph nodes.

A

Subset	B220	EPCAM	CD11b	CD205	Langerin	DCSIGN	Antigen presentation	Migratory phenotype
pDC	+	-	-	-	-	+	low	Lymphoid
CD8α+	-	-	+	+	-	-	Crosspresentation	Lymphoid
CD4+	-	-	+	+	-	-	CLASSII	Lymphoid
DN	-	-	+	-	-	-	CLASSII	Lymphoid
CD11b (cDCs including dermal DCs	-	-	+	+	-	ND	CLASSII	Migratory
CD103+ including Lung, intestin, dermal Lang+ DC	-	*	*	*	*	-	Crosspresentation (CLASSII?)	Migratory
LC	-	+	-	+	+	-	Tolerance (CLASSII?)	Migratory
Monocyte derived	-	-	+	-	-	+	Mixed	Mixed

B

Subset	BDCA4	DEC205	CLEC9A	MR	Langerin	DCSIGN	Antigen presentation	Migratory
pDC	+	-	-	-	-	-		Lymphoid
CD16+	-	+	-	-	-	-		Lymphoid
BDCA1	-	+	-	+/-	-	-		Lymphoid
BDCA3	-	-	+	-	+/-	-	Crosspresentation	Lymphoid
CD1a+ (cDC including Dermal DC)	-	-	-	+	-	-		Migratory
LC	-	+	-	-	+	-		Migratory
moDC	-	-	-	+	-	+	-	Mixed

Table 1. Summary of both mice (A) and human (B) DC subsets under steady state conditions. Specific markers, migratory phenotypes and specificities for antigen presentation are shown in each table heading. Regarding migratory phenotype the word mixed specifies that both hematogenous and lymphatic routes have been suggested for moDCs.* Each tissue origin exhibit different combination of these markers. +/- Subpopulations of these subsets do express the marker.

All these subsets of DCs have been identified mainly under steady state conditions. The changes in DCs populations in the lymph nodes under inflammation have not been deeply explored, with the exception of some studies that demonstrated increased numbers of tissue-resident DCs under inflammation (Jakubzick et al., 2008).

As shown above, the increased knowledge of DC origin and biology has definitely overcome the Langerhans cell paradigm, as new complexities emerged. For example, Langerhans cells had been described to be not only immunostimulatory but also act as immunomodulatory in some circumstances such as hapten induced inflammation (Kissenpfennig et al., 2005). Besides, lymph node resident DCs have also been described to cross-present peripheral antigens to T lymphocytes in the lymph nodes. These antigens may reach the nodes inside migratory DCs that merely as cargo cells (Allan et al., 2006).

Therefore, the nature of the pathogen or stimuli that promotes DC response, the peculiarities of the tissue where the response starts, along with the subsets of DCs mobilized as well as other immune phenomena, may shape the phenotype and function of a specific DC population, in a far more complex way than firstly envisioned. Thus, understanding the migration of DCs may contribute to clarify the phenomena and to point at key steps susceptible of intervention, in order to modulate the immune response against different pathogens.

3- DENDRITIC CELL MIGRATION ACROSS LYMPHATIC VESSELS

3.1. The essential role of lymphatic chemokines in driving DC migration.

DCs express a broad range of chemokine receptors that guide and help them during their migration and maturation. Immature DC express chemokine receptors related to their extravasation from blood to damaged tissue (Alvarez et al., 2008). Some examples of these receptors and their ligands are CXCR3-CXCL10 (Flier et al., 2001), CCR4-CCL17, CCR4-CCL22 (Katou et al., 2001), CCR2-CCL2 (Dieu-Nosjean et al., 2000) among others.

Once DC mature by sensing pro-inflammatory cytokines or/and pathogen denoting moieties, they change their repertoire of chemokine receptors. In these situations the axis CCR7/CCL21, CXCR4/CCL12 and SP1PR/S1P plays a determinant role.

CCL21/CCL19-CCR7 chemokine-chemokine receptor axis is a determinant component of DC migration towards the lymph nodes during steady-state conditions (Schumann et al., 2010) and under inflammation. CCR7 receptor is expressed on DC surface at low levels under steady-state conditions, but upon DC maturation its expression significantly increases (Sallusto et al., 1998). In fact, studies performed in CCR7 knock-out mice have demonstrated that this receptor is necessary for DC migration from tissue to the lymph nodes. This has been illustrated in experiments using both adoptive transferred DCs and endogenous skin dermal DCs in animal experiments performed both, under inflammation and steady state conditions (Martin-Fontecha et al., 2003; Ohl et al., 2004).

The CCR7 ligands, CCL21 and CCL19 are highly homolog proteins that are different only in 40 Aa located in its C terminus (de Paz et al., 2007; Hirose et al., 2002; Patel et al., 2001). CCL19 is a soluble ligand produced by dendritic cells (Ngo et al., 1998), among other cell types, and it is present in tissue in a soluble form. CCL21 is encoded by two different genes (Vassileva et al., 1999), that produce two CCL21 variants known as CCL21Leu and CCL21Ser. CCL21Leu is expressed by lymphatic capillaries and CCL21Ser is mainly expressed in the lymph nodes (Randolph et al., 2005). To date no functional differences have been reported between these two forms of

CCL21. CCL19 is a soluble cytokine, while CCL21 appears mostly bound to heparan residues on endothelial cells and to the ECM (Gunn et al., 1998), promoting what has been called haptotactic migration (Bao et al., 2010; Schumann et al., 2010)

Interestingly, it has been shown that the *in vivo* truncation of CCL21 C- terminal amino acids, that are involved binding to ECM, produces a soluble form of CCL21 and severely impairs lymphocyte intravasation through HEV (Stein et al., 2000).

CCL21 is produced by lymphatic vessels under steady-state conditions (Kriehuber et al., 2001) and is further increased upon inflammation (Johnson and Jackson, 2010; Martin-Fontecha et al., 2009; Vigl et al., 2011). Under inflammation DCs increase CCR7 expression, fostering their migration towards CCL21 gradients produced by inflamed lymphatic vessels. Interestingly, CCL21 is distributed and exposed in a dotted pattern on lymphatic vessels close to the intercellular flaps (Johnson and Jackson, 2010; Tal et al., 2011). DCs have been found directly in contact with this CCL21 patches located in the gaps of the endothelial basal membrane. Finally, DCs lacking CCR7 do not dock to CCL21 patches nor crawl into lymphatic vessels (Tal et al., 2011).

The CXCR4/CXCL12 chemokine-receptor axis is also upregulated upon DC maturation (Sallusto et al., 1998). CXCL12 is expressed by lymphatic vessels upon inflammation. The implications of CXCL12 in DC migration have been demonstrated in *in vivo* experiments where the inhibition of CXCR4 impaired DC migration in animal models of FITC and CHS-induced inflammation (Kabashima et al., 2007). Interestingly, CXCL12, has also been proposed to enhance survival and to promote maturation on DC (Kabashima et al., 2007), suggesting other functions that may be added to its chemotactic properties.

The function of Sphingosine-1-P (S1P) and of its receptors in immunity has been explored in depth. S1P is a sphingolipid, mainly generated by phosphorylation of sphingosine by two kinases (Sphingosine kinases 1 and 2). S1P signals intracellularly, and can also be secreted and act in a paracrine fashion. There exist at least five different G-protein coupled receptors ($S1PR_{1-5}$) able to bind S1P and induce intracellular signaling (Maceyka et al., 2009). First reports on the intervention of S1P in DC migration addressed its implication in lymphocyte egress from the lymph nodes (Matloubian et al., 2004; Pappu et al., 2007). Besides, S1P have been demonstrated to

direct mature dendritic cell migration in a number of studies using bone marrow derived DCs (Czeloth et al., 2005), skin DCs (Gollmann et al., 2008), and many other DC subsets (Rathinasamy et al., 2010). Treatment of DCs with FTY720, a potent drug that induces internalization of some S1PRs (Graler and Goetzl, 2004), block DC migration across LEC and to lymph nodes in many models (Czeloth et al., 2005). On the contrary, lack of expression of CD69, which is known to sequestrate S1PRs in lymphocytes (Bankovich et al., 2010; Shiow et al., 2006), enhances the effects of S1P on DC migration (Lamana et al., 2011). Lymphatic vessels had been described to express Sphingosine kinases (SPHKs) and secrete S1P (Pham et al., 2010), which has been found to be augmented upon tissue inflammation (Ledgerwood et al., 2008).

3.2 DC migration across lymphatic vessels under non-inflammatory conditions.

DC migration under steady-state conditions (Merad et al., 2002) and inflammation (Martin-Fontecha et al., 2009) has been a subject of intense research during the past decade. From these studies it has become clear that DC show important differences in terms of the mechanism and molecules governing cell movement under these conditions. DC migration in non-inflammatory conditions has been elegantly described in two studies performed by the group lead by M. Sixt. In their first piece of work, DCs migration across tissue and lymphatic vessels is explored using integrin deficient DC. Surprisingly, both interstitial migration and transendothelial migration were integrin independent. (Lammermann et al., 2008). In their second work, the same authors described by confocal microscopy the preferential entry of DCs to the lymphatic vessels by traversing "portals" formed in the discontinuous basal membrane, followed by fast squeezing through the valve-like gaps observed in the microanatomy of the lymphatic capillaries (Pflicke and Sixt, 2009). These data suggested an integrin and metalloprotease independent migration of DCs under steady state (Fig 5).
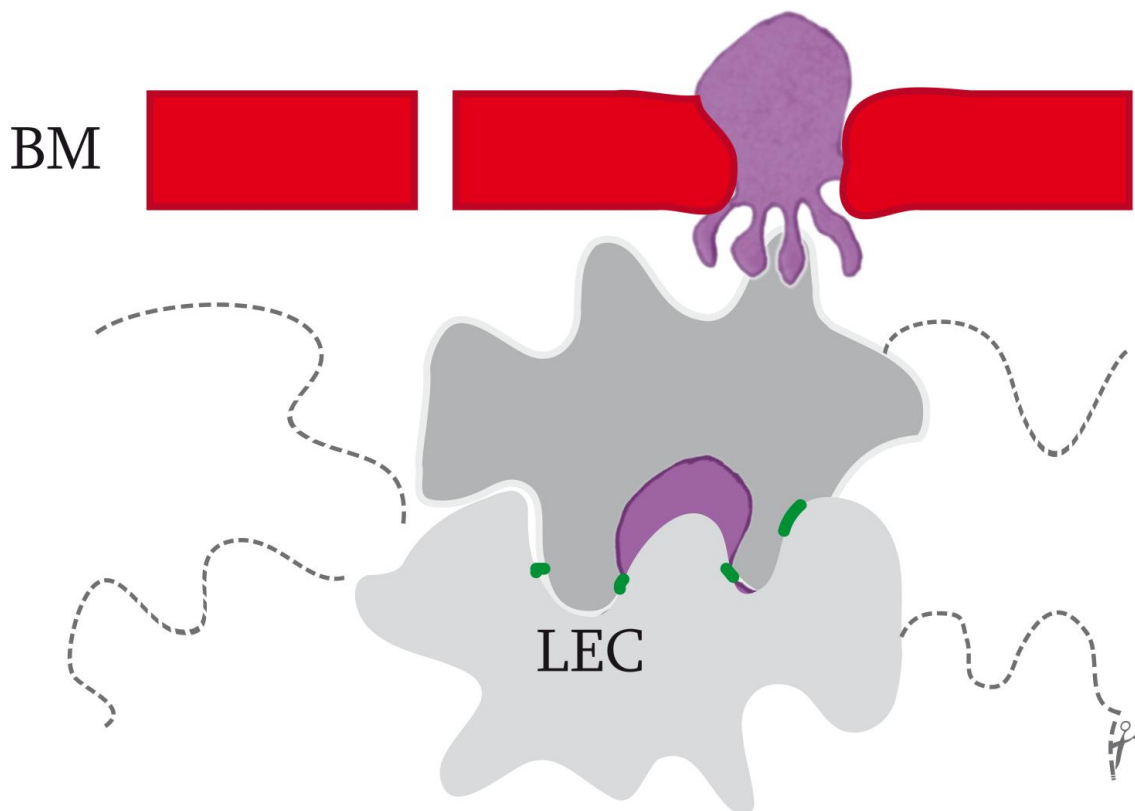
Figure 5. Schematic representation of DC migration across lymphatic vessels. Dendritic cells (purple) migrate across LEC in an integrin independent manner. Firstly, they avoid the basal membrane of the capillaries traversing through existing discontinuities in its structure. Subsequently DC cross the endothelial layer squeezing through the intercellular flaps. Adapted from Pflicke and Sixt (2009).

3.3 Surface molecules involved on DC migration into lymphatic vessels.

A number of surface molecules have been identified due to their implication in DC transmigration across LEC. The great majority of them have been described to be present under inflammation, reinforcing the existence of different mechanisms at work in non-inflammatory versus inflammatory conditions. The main integrin receptors involved in leukocyte trafficking across blood endothelium are ICAM/CD54 and VCAM/CD106 surface receptors (Diacovo et al., 1996). Knowledge on the role of these receptors in DC traffic across lymphatic vessels is nonetheless relatively recent. Studies of DC transendothelial migration across TNFα treated LEC demonstrated that ICAM and β-integrin blockade dramatically reduced DC transendothelial migration and DC arriving to lymph nodes. (Johnson and Jackson, 2010). This study was probably the

clearest evidence on the differential mechanisms in DC migration to lymph nodes under inflammation versus non-inflammatory conditions. This study was supported by later studies, implying also the leukocyte integrin Mac-1 (Podgrabinska et al., 2009).

Other integrin receptors have been implicated in DC migration across lymphatic vessels. One of these is PECAM/CD31 a molecule expressed on the surface of almost all endothelial cell types. Its participation in leukocyte transmigration across blood vessels has been known for long (Nourshargh et al., 2010). Lymphatic vessels also express CD31 (Hirakawa et al., 2003), albeit at lower densities than their blood counterparts. This molecule is distributed mainly in endothelial lateral cell-cell interactions (Johnson et al., 2006). Interestingly, recent studies showed, that blocking of CD31 and CD99 in CXCL12 treated LEC was able to reduce DC transmigration both i*n vitro* and *ex-vivo* (Torzicky et al., 2012).

L1-CAM, another adhesion molecule belonging to the immunoglobulin superfamily, has been involved in DC adhesion to LEC. Although its relevance in this process is far from being clear. L1-CAM is a membrane protein widely described in nervous system (Maness and Schachner, 2007) and is known to mediate both homophilic (Wei and Ryu, 2012) and heterophilic interactions with a number of integrins (Felding-Habermann et al., 1997). L1-CAM receptor is also expressed by many leukocytes (Pancook et al., 1997), on LEC, and on inflamed lymphatic vessels (Maddaluno et al., 2009). Recent studies showed the participation of L1-CAM in DC adhesion and transmigration across LEC, presumably by homophilic binding (Maddaluno et al., 2009).

Other surface molecules with different chemical nature have been described in leukocyte adhesion to lymphatic vessels. For example, Clever-1/Stabilin is a molecule which has been involved on *in-vitro* (Salmi et al., 2004) and *in vivo* (Karikoski et al., 2009) migration of lymphocytes. Besides, the mannose receptor known to participate in the endocytic functions of both macrophages and some DC subsets (Gazi and Martinez-Pomares, 2009) has been also proposed to participate in these events. Lymphocytes transferred to mice lacking the expression of the mannose receptor failed to arrive to lymph nodes. Although mannose receptor expression has been described on lymphatic vessels (Hirakawa et al., 2003; Irjala et al., 2001), works of Marttila-Ichihara and co-workers only explored the adhesion of lymphocytes to lymphatic sinuses in lymph

nodes (Marttila-Ichihara et al., 2008). Junctional adhesion molecules (JAMs) have also been widely explored as responsible for leukocyte transmigration across vascular endothelium. The expression of these proteins have also been reported on lymphatic vessels and LEC in culture (Ueki et al., 2008). JAM-A participates in the button-like structures that constitute the inter-endothelial junctions of the lymphatic endothelium (Baluk et al., 2007). Interestingly, knocking down JAM-A expression in mice increased trafficking to lymph nodes in CHS models (Cera et al., 2004). This effect, although consistent, needs to be investigated in more detail.

Overall, the molecular interplay that results in leukocyte transmigration across LEC has not been studied with the same detail as it has been done in the blood vessels.

4- ROLE OF INTEGRINS ON LEUKOCYTE TRANSENDOTHELIAL MIGRATION ACROSS BLOOD VESSELS

4.1 Sequential role of selectins and integrins in diapedesis.

The process of extravasation of leukocytes to inflamed tissues from blood vessels was first documented in 19th century. More than 150 years later, the discovery of selectins and integrins and their implication in this process led to the proposal of a three step model of leukocyte extravasation, including rolling, tethering and endothelial arrest (Butcher, 1991). These steps are illustrated in figure 6.
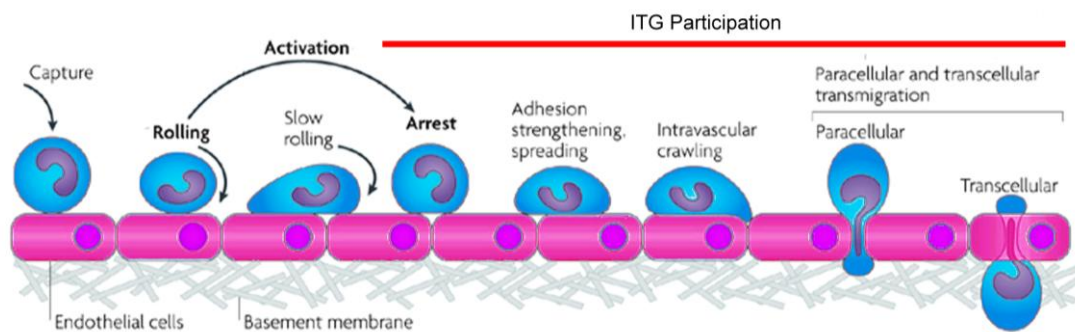
Figure 6. Representation of the sequential steps of leukocyte adhesion and transendothelial migration across blood inflamed endothelium. Adapted from Ley et al. (2007).

Briefly, upon cytokine activation of the vessel, blood leukocytes are captured on the luminal side surface of the vascular capillary first, by selectins that are specifically expressed on the endothelial surface. Once leukocytes tether to the endothelial layer, they roll over the endothelial surface following flow direction and guided by chemokine gradients located on the surface of the endothelium. Signaling from these chemokine receptors in turn activate integrins on the surface of the leukocytes. Integrin activation mediates firm arrest on the endothelial surface by interacting with the correspondent ligands present on inflamed endothelium. Once arrested, as the adhesive force increases, the leukocyte flattens on the endothelial surface and reduces its silhouette exposed to blood flow. At this moment leukocytes crawl on the endothelium in search for a particular favorable spot for transendothelial migration (TEM). Finally, TEM may occur

through two different mechanisms: either leukocytes cross the endothelial cytoplasm, in what has been called transcellular diapedesis (Ley et al., 2007; Muller, 2011) or they traffic along the cell-cell contacts between two adjacent endothelial cells (paracellular transmigration) (Muller, 2011).

Integrins expressed on the surface of leukocytes as well as their counter-receptors on the endothelial cell do participate in several of these steps (Nourshargh et al., 2010).

Integrins are a large family of 24 surface molecules formed as heterodimers of α and β subunits that are non-covalently associated. However, surface expression needs α and β subunits pairing. At least 18 different α subunits and 8 different β subunits have been described. Many integrin dimers are promiscuous and also many ligands can join different integrins (Avraamides et al., 2008). These surface proteins allow cell interactions with components of the extracellular matrix and/or with cell surface receptors that belong to the immunoglobulin superfamily. The so-called "leukocyte integrins" that are mainly β2 heterodimers can mediate adhesion between different leukocytes (Zhang and Wang, 2012). However, different leukocyte subsets may exhibit important differences in the use of adhesion molecules (Ley et al., 2007).

Under inflammation, ICAM and VCAM integrin receptors are upregulated and clustered in microdomains on the surface of endothelial cells along with other molecules such as tetraspanins (mostly CD9, and CD151) (Barreiro et al., 2005; Barreiro et al., 2008). The formation of membrane domains enriched in integrin receptors was firstly described in endothelial microvilli projections that were highly enriched on ICAM-1 and VCAM. These microvilli surrounded leukocytes and served as cell docking places on the inflamed endothelium. (Barreiro et al., 2002). Besides, these locations were also enriched on proteins that functionally bridge surface adhesion molecules with the leukocyte actin cytoskeleton such as moesin and ezrin (Barreiro et al., 2002).

These endothelial microvilli-like structures were initially related to the capture of the leukocytes under flow. Carman and co-workers demonstrated how these structures participate not only in leukocyte capture but also in initial TEM (Carman et al., 2003). This group showed how the microvilli-endothelial structures enriched in ICAM-1 and VCAM could be found around leukocytes undergoing TEM and renamed

these endothelial membrane projections as "transmigratory cups". They also showed interesting rearrangements of integrins, particularly Lymphocyte function associated antigen 1 (LFA-1), when leukocytes were eliciting the formation of these "transmigatory cups" (Carman and Springer, 2004). Nonetheless, it was not until the discovery of ICAM/VCAM nanoplatforms that integrins were definitely implicated in the formation of these projections (Barreiro et al., 2008). ICAM and VCAM were showed to be joined by tetraspanins under inflammation and only the blockade of both ICAM and VCAM integrin ligands inhibited the formation of these structures. To avoid confusing terms that are still under debate, we will generally use the term "microvilli-like projections" in this thesis work to refer to these structures as described in blood vasculature.

Following integrin ligation, leukocytes acquire a more flattened morphology presenting a 2D migration that polarizes the cell into a leading edge and a rear-end uropod (Ridley et al., 2003). Besides, lymphocytes have been reported to protrude podosome-like structures into endothelial cytoplasm. This has been interpreted as a first step for transcellular diapedesis (Carman et al., 2007) or as a way of sensing chemokines located in intracellular vesicles in the endothelial cell that drive leukocyte active transmigration (Shulman et al., 2012).

4.2. Signaling cascades triggered upon integrin-mediated leukocyte adhesion to endothelial surfaces.

The adhesion of leukocytes to the endothelial cells under flow, cytokine exposition or inflammation, promotes biochemical signals on both the leukocytes and the endothelium that facilitate leukocytes extravasation into the inflamed tissues.

In leukocyte transmigration across endothelial layers, the integrins VLA-4 ($\alpha4\beta1$) and LFA-1 ($\alpha L\beta2$) and their respective counter receptors VCAM (CD106) and ICAM-1 (CD54) on the endothelial side, play a most prominent role. Integrin binding to their ligands is highly regulated in terms of the affinity (the ability of each single receptor to interact with its ligand), and of avidity (that describes the combined strength of multiple bond interactions). Changes in integrin affinity are associated with three different conformations of the β chain. This issue has been studied in depth for the

integrin LFA-1 that mediates DC adhesion to endothelial cells. LFA-1 is one of the three integrins that leukocytes express in form of dimmers of the β2 integrin (CD18) and different α chains. The α chain in LFA-1 is an $α_L$ subunit also known as CD11a. $β_2$ monomers can also be found on leukocytes forming dimmers with $α_X$ (named also CD11c) and $α_M$ (CD11b). LFA-1 can be found in leukocytes in three different affinity conformations. The first one is the low affinity form, in which the integrin chain is folded over, resembling a closed penknife. This low affinity isoform is characteristic of non-stimulated leukocytes. The second conformation is the so called intermediate affinity isoform, in which the integrin is partially open. Finally, when the integrin is completely extended, leukocytes exhibit LFA-1 in a high affinity conformation (Figure 7). High and intermediate affinity conformations appear only upon leukocyte stimulation. Indeed, in a population of activated leukocytes all the three different integrin conformations co-exist, in a very dynamic way depending on the environmental stimuli (Springer and Dustin, 2012).

In fact, many stimuli drive integrin activation and increased affinity such as PMA (Hogg et al., 2011). Between the physiological stimulus, chemokines are known to induce integrin active conformations prior to the adhesion of lymphocytes to ICAM-1 expressed on inflamed endothelium (Salas et al., 2006; Shamri et al., 2005). Besides, it seems that shear force and preliminary union to ICAM-1 on the endothelial surface is also able to induce active conformations in a quick way mediated by cytoskeleton tracking forces (Alon and Dustin, 2007; Chen et al., 2010) (Fig 7B). Additionally, although TCR signaling has been shown to induce integrin active conformations, subsequent ICAM-1 engagement is needed to preserve them.

Only high affinity conformations allow adhesion stabilization and efficient force transduction towards cytoskeleton components such as kindling and talin. (Alon and Shulman, 2011). Integrin cytoplasmic tails allow both an inside-out and outside-in signaling that in many ways trigger the high affinity state of the integrin.

Once stable integrin- integrin-ligand binding occurs, outside–in signaling causes the reinforcement of the adhesion complex and changes the cytoskeletal dynamics. Besides, chemokine signaling seems to induce long lasting high affinity conformations of integrins (Feigelson et al., 2010). For example, CCL21, is able to induce the high affinity conformation of LFA-1 on DC (Eich et al., 2011; Johnson and Jackson, 2010).

The cytoplasmic signaling induced after integrin activation has been explored recently. As shown in figure 7C Rap1 (Katagiri et al., 2003; Shimonaka et al., 2003), PLD1(Bolomini-Vittori et al., 2009), VAV1(Garcia-Bernal et al., 2009) and probably Kindlin 3 (Malinin et al., 2009) are the main molecular elements involved in the inside-out activation of the integrin. Outside-in signaling after ICAM-1 ligation can also promote high affinity conformations.
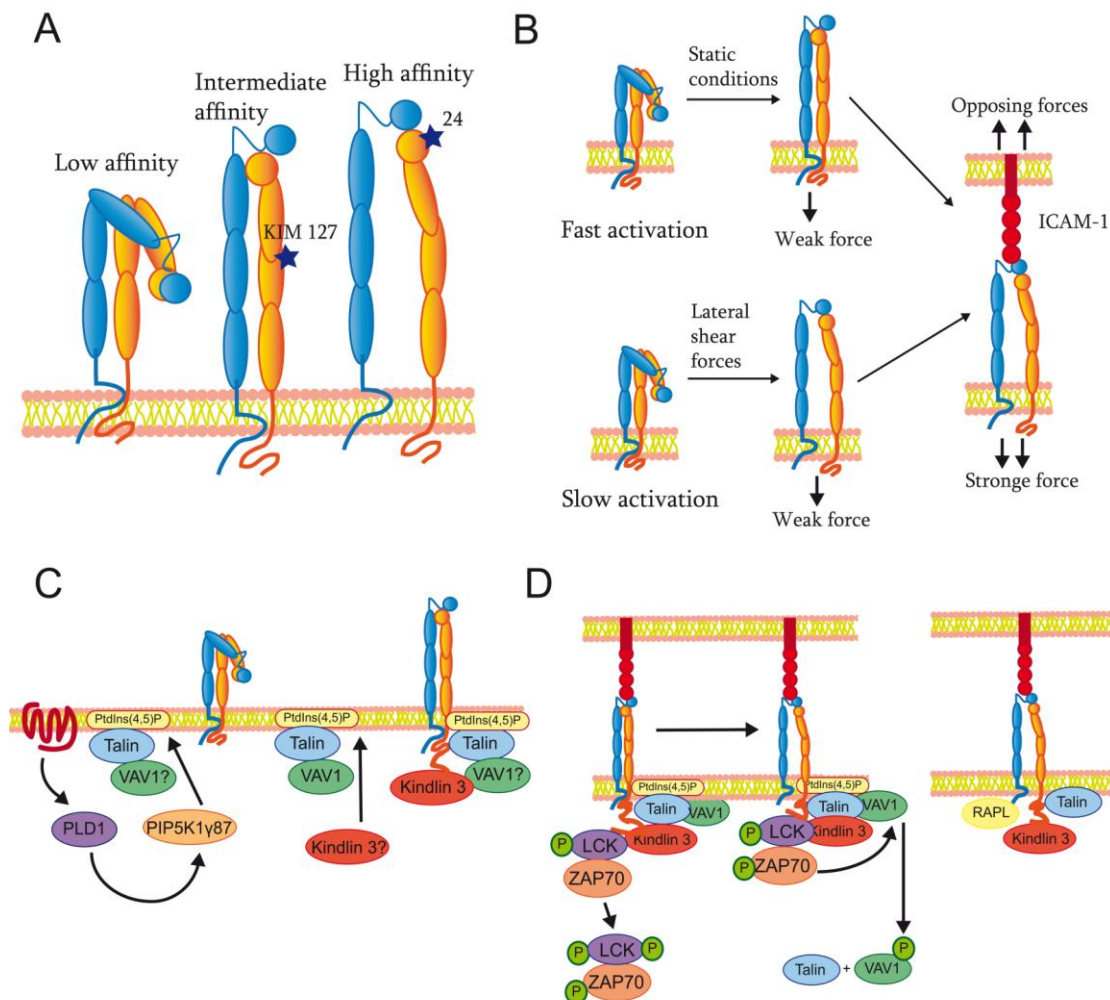
Figure 7. LFA-1 integrin conformation and signaling. (A) Schematic representation of the αL and β2 integrin chains of LFA-1 heterodimer. Starts represent the area of epitope recognition for both KIM127 and 24 monoclonal antibodies. (B) Different activation status of LFA-1 integrin regarding on the stimuli used. (C) Chemokine-driven inside-out signaling involved in the formation of high affinity LFA-1 epitopes. (D) Ligand driven outside-in signaling after integrin LFA-1 activation. Adapted from (Hogg et al., 2011).

In addition to affinity regulation, the avidity of the integrins can also be modulated to enhance the binding of their ligands on the endothelial surface. Avidity is mainly regulated in leukocytes by changes in the distribution of the integrin. LFA-1 has been shown to be included in lipid rafts, and therefore can be rapidly mobilized along the plasma membrane (Leitinger and Hogg, 2002). The formation of microclusters of active LFA-1 has been demonstrated to appear when lymphocytes crawl over endothelial cells and seems to be important for this kind of motility (Shulman et al., 2009; Smith et al., 2005). In fact, chemokines trigger integrin clustering and this has been demonstrated to be important at least with regard to adhesion to endothelial cells expressing low densities of integrin ligands (Constantin et al., 2000). Nonetheless, macro clustering of the molecules has been elegantly showed to occur only after cognate ligation of integrin ligand (Kim et al., 2004). However, integrin redistribution seems to be less important than the affinity state of the integrin for leukocyte adhesion to endothelial cells (Kim et al., 2004).

Signaling events downstream of ICAM on the endothelium involve multiple small GTPases. On the one side, integrin ligand mediated signaling enhances cytoskeleton contractility. On the other side, adhesion molecule triggering induces changes in the inter-endothelial junctions to increase endothelial permeability for leukocytes which transmigrate following a paracellular route, as shown in figure 8 (Cernuda-Morollon and Ridley, 2006). These signaling events also induce mobilization of PECAM and other adhesion molecules in small vesicles from lateral borders of the endothelial cells towards sites of diapedesis. This event takes place in a kinesin and microtubule dependent fashion, both for para- and transcellular TEM (Mamdouh et al., 2003; Mamdouh et al., 2008; Mamdouh et al., 2009).
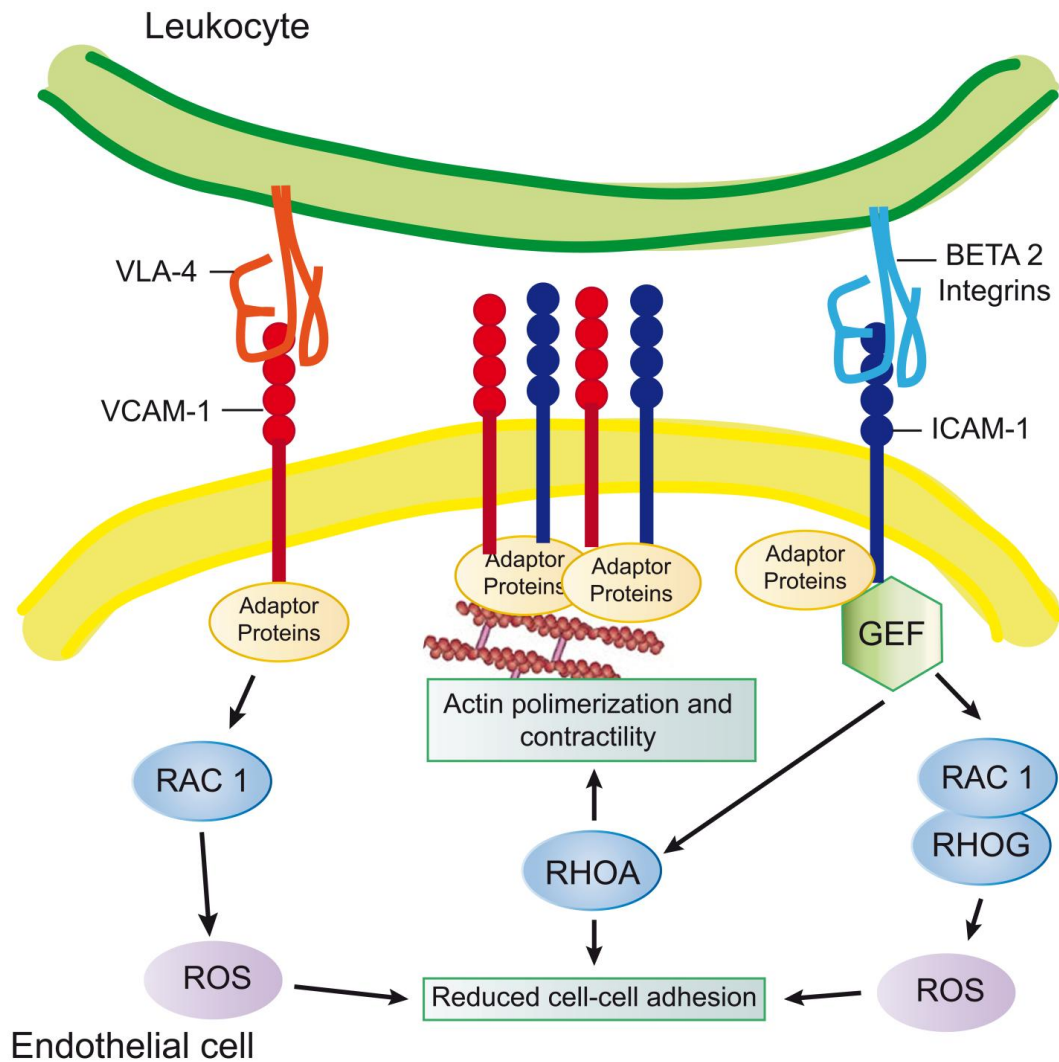
Figure 8. Schematic representation signaling cascades activated by integrin ligation on endothelial cells. Adhesion molecules have been reported to induce signaling through small GTPases. Signalling transduction of these molecules drives to augment ROS and eNOS activity that, in turn increases vascular permeability. Rho family of proteins also mediates increments in the contractility of the cytoskeleton. Adapted from (Nourshargh et al., 2010)

5- DC MIGRATION IMPACT IN IMMUNOTHERAPY: CD137 IMMUNOSTIMULATORY MONOCLONAL ANTIBODIES AND DC VACCINATION

5.1 Dendritic cells in immunotherapy

As mentioned above, since monocytes can be easily differentiated *in-vitro* to DCs by stimulation with IL4 and GMCSF, has made of moDCs one of the most convenient models to study DC biology, especially in human. In fact, therapeutic vaccines are being developed using this cell population that are assayed in clinical trials in many hospitals. Therefore, understanding the specifics of artificially generated moDC migration is a key step for developing better strategies leading to the optimization of these therapies
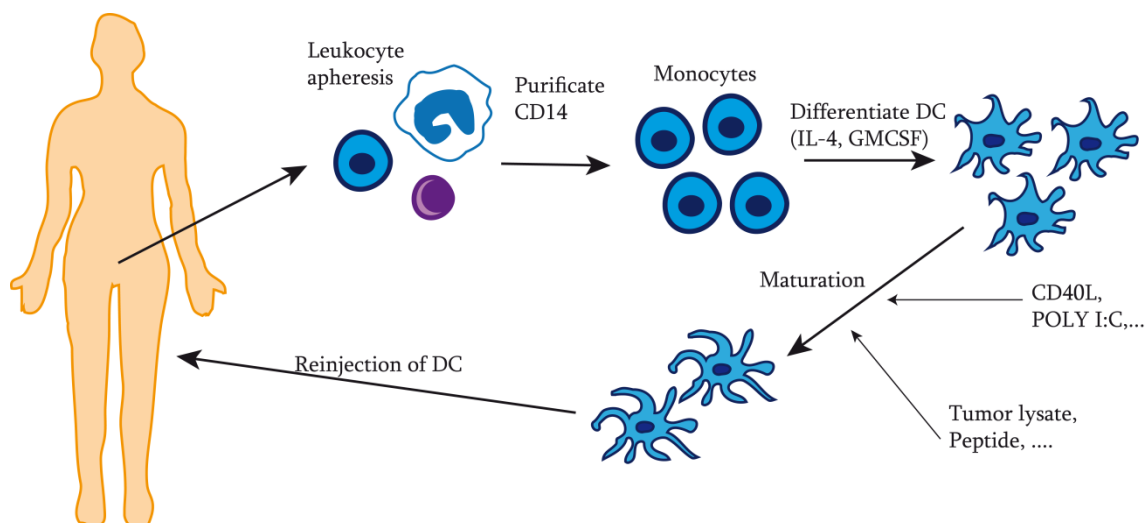
Figure 9. Schematic representation of the generation of antitumoral DC based vaccines from blood monocytes. Adapted from (Berzofsky et al., 2004)

As shown in figure 9, DC vaccines are based on obtaining CD14$^+$ monocyte precursors from the blood of patients. DC maturation can be instigated by adding a number of cytokines. Once DC have matured, they are exposed to tumor antigens. There exist different strategies to provide tumor antigen to mature DC. These include: adding apoptotic tumoral cells, tumor lysates or gene therapy. Defined protein antigens or synthetic peptides can be used to load antigen-presenting MHC molecules. After that, mature antigen-loaded DCs can be re-injected into the patient in order to induce specific immune responses against tumors (Palucka and Banchereau, 2012).

Different strategies are being implemented in order to improve DC-based vaccines including the use of defined DC subsets or expansion of CD34 precursors by exposure to FLT-3 ligands (Banchereau et al., 2001; Fong et al., 2001; Mackensen et al., 2000). Other strategies rely on the activation of specific DC subsets already present in the organism by antigen targeted delivery to DCs specific receptors. In these approaches tumor antigens are conjugated to specific ligands or specific antibodies that are exposed by surface molecules on tissue resident DCs such as for example, lectin receptors (Tacken and Figdor, 2011).

Up to date, an important number of clinical studies using DC vaccines have been performed, especially against cancer (Palucka and Banchereau, 2012). The majority of these studies used DC derived from CD14$^+$ precursors by GMCSF and IL-4 treatment. Up to now these studies have obtained some objective clinical responses (Geiger et al., 2001; Nestle et al., 1998; Thurner et al., 1999) and in some cases of tumor relapse, they presented incremented long-term survival (Palucka et al., 2006; Salcedo et al., 2006). Despite being promising in preclinical models, these clinical trials have also shown some disappointing outcomes. For example, DC-based clinical trials against melanoma have not been as effective as originally expected (Schadendorf et al., 2006). One of the main causes for these is the relative low numbers of DC reaching the lymph nodes after subcutaneous injection, around 4% of the injected DC (Gilboa, 2007). This problem can be partially derived from the maturation stage of DCs (De Vries et al., 2003a; de Vries et al., 2003b) and should be overcome by the application of different maturation protocols. However, deeper understanding of the migration events from the intradermal injection is needed to improve these therapeutical approaches.

5.2 IFN-DC as possible improvement for DC vaccination procedures.

As mentioned above, enhancing the migratory capacities of DC is very important to improve the therapeutic effects of these treatments. The incorporation of different cytokines to the development of DCs from monocytes can be a way to achieve a more migratory phenotype on DC.

IFN was discovered in 1957 by Isaacs and Linderman (Isaacs and Lindenmann, 1957) as a soluble protein factor which was able to interfere with viral replication. Type

I IFNs (IFNs α and β) are the most abundant members of this family. IFNα family of interferons (13 subtypes in human) are encoded by separate genes while all the different IFN type I species are encoded by a single gene. IFNα is a pleiotropic cytokine secreted by almost every cell type upon viral infection, being pDC the highest IFNα-producing cell upon viremia. IFNα mediates direct effects on T lymphocytes and NK cells. Besides, it is a potent antiviral molecule that has also a number of other functions which include the blocking of proliferation or the enhancement of cytotoxicity on T-cells (Pestka et al., 2004). IFNα also participates in direct anti-effector responses such as blocking viral and tumor proliferation (Hervas-Stubbs et al., 2011). IFNα also indirectly mediates a number of effects as the secretion of second wave cytokines that regulate other immune cell functions as IL-15 (Nguyen et al., 2002).

Regarding DC function, IFNα is involved in T cell crosspriming in response to viral pathogens (Le Bon et al., 2003) and can also modulate, DC differentiation, maturation, and migration. It has been described how IFNα can act as a stimulus that promotes the differentiation towards a moDC called IFN-DC. These DC exhibit a phenotype of partially mature DCs, while showing a strong capability to induce a primary human antibody response and CTL expansion. This has been observed in IFN-DC pulsed with antigen and injected into humanized severe combined immunodeficiency (SCID) mice (Lapenta et al., 2003; Parlato et al., 2001). These IFN-DCs exhibit a more migratory phenotype acquired because of the upregulation of CCR5 and CCR7 (Parlato et al., 2001). Other effects have been described for IL4 DCs treated short term *in vitro* with IFNα, showing upregulation of costimulatory and MHC molecules among others (Gallucci et al., 1999; Ito et al., 2001).

5.3. CD137 in immunotherapy.

Monoclonal antibodies (mAb) are emerging as potent immunomodulatory agents that help patients to control cancer disease. Co-stimulatory molecules such as CD137 and co-inhibitory ones as CTLA-4, or PD-L1 have shown to be interesting targets for mAb-based therapies. Among them, CD137 is considered a very promising target as it is only expressed after leukocyte activation.

CD137, also known as 4-1BB or TNFRSF9, is a membrane glycoprotein of the TNFR family expressed in most immune cells, including activated T cells, NK cells (Melero et al., 1998), monocytes (Kienzle and von Kempis, 2000), DCs (Heinisch et al., 2000) and neutrophils (Heinisch et al., 2001). Human CD137 was firstly identified on activated T lymphocytes and originally named ILA (Induced by Lymphocyte Activation). To date, one cognate ligand has been identified in mouse and human that is termed CD137L (4-1BBL). This ligand is mostly expressed on APCs (Alderson et al., 1994; Pollok et al., 1994) although a soluble form of the ligand has also been described (Salih et al., 2001). CD137 has been classified as a co-stimulatory molecule that receives the signals from APCs in the immune synapse (Watts, 2005). Ligation of this molecule on T lymphocytes promotes a number of signaling events that involve TRAF2-mediated nuclear translocation of NFκB (Arch and Thompson, 1998; Jang et al., 1998), and also p38 MAPK activation (Cannons et al., 2000), and increased IFNγ secretion (Croft, 2009). Other CD137 signaling events promote enhanced viability of T cells by modulating the expression and activity of members of the BCL superfamily including downregulation of Bim and upregulation of BCL-XL (Lee et al., 2002; Sabbagh et al., 2008).

Treatment of mice bearing transplanted tumors with agonistic antibodies resulted on a reproducible anti-tumor effect (Melero et al., 1997). Since then, these models have been used to elucidate the molecular mechanism mediating its anti-tumor effect such as the enhancement of effector functions of cytotoxic CD8 T cells (May et al., 2002; Wilcox et al., 2004). Anti-CD137 agonistic antibodies are currently in a number of clinical trials, both as a single agent and in combination with other therapies (Ascierto et al., 2010). Phase II trials using agonistic antibodies against CD137 (NCT00612664) in melanoma showed signs of clinical activity.

There are scarce studies addressing the role of CD137 in endothelial cells. It has been described its expression in tumor vessels (Broll et al., 2001). Other authors observed CD137 in inflamed atherosclerotic lesions, where ligation of the molecule promoted chemo-attraction and adhesion of leukocytes by upregulating molecules such as MCP-1, ICAM and VCAM (Jeon et al., 2010). CD137 expression has been described in *in vitro* inflamed Human Umbilical Vascular Endothelial Cells (HUVEC) (Drenkard et al., 2007). Results recently published in our group showed how CD137 ligation by

mAb on tumor endothelium promoted increased lymphocyte accumulation on tumors dependent on ICAM-1 and VCAM upregulation (Palazon et al., 2011).

The importance of pathway is highlighted by recent results in adoptive T cell therapy with T lymphocytes provided with artificial CD137 costimulation that exhibit interesting antitumoral results (Kalos et al., 2011). However, the effects of CD137 on DCs or endothelium need still to be addressed (Wilcox et al., 2002).

CD137 manipulation will help to better design of the treatments, find the limitations of its use and discover its possible uses against other pathologies or its combination with existing therapies.

OBJECTIVES

1. To study the effects of IFNα in DC migration across lymphatic endothelium.

2. To describe some of the mechanisms that underlay the participation of integrins and integrin receptors in DC transendothelial migration across inflamed lymphatic vessels.

3. To analyze the expression of CD137 on lymphatic vessels under inflammation and its role in DC migration.

RESULTS

1st MANUSCRIPT:

Dendritic cells adhere to and transmigrate across lymphatic endothelium in response to IFN-α.

2nd MANUSCRIPT

ICAM-1 mediates dendritic cell adhesion and transmigration across

inflamed lymphatic endothelium.

Submited to Blood

3th MANUSCRIPT

CD137 on inflamed lymphatic endothelial cells enhances CCL21-guided migration of dendritic cells

DISCUSSION

This thesis compiles three related projects focused on the interplay of DC and LEC. The interest in investigating the immuno-modulatory capabilities of DC has incremented enormously, since Steinmann and co-workers firstly grasped their prominent role in promoting T lymphocyte responses (Steinman and Cohn, 1973; Steinman et al., 1983). Besides, the relatively easy culture protocol for differentiating DC from the circulating blood monocytes, enabled the establishment of pre-clinical protocols to study the efficacy of DC vaccination in mice. These protocols have been rapidly adapted for their use in clinical trials (Palucka and Banchereau, 2012). However, DC vaccination has not been as successful as predicted. One of the main limitations in DC vaccination-based therapies emanates from the relatively low numbers of DC reaching the lymph nodes, never higher than 4% of the total injected DC (De Vries et al., 2003a; Feijoo et al., 2005). The most used route of DC administration to the patients is by intradermal injection (Palucka and Banchereau, 2012). Following this procedure, DC migration towards the lymph nodes occurs mainly through the lymphatic vessels (Martin-Fontecha et al., 2009). Therefore, exploring the molecular dialogue between DC and the lymphatic endothelium in this process is particularly interesting to define mechanisms that would increment DC transit towards the lymph nodes. As DC migration through lymphatic vessels is not a route exclusively followed by adoptively transferred moDC, other DC-based therapies can also be improved through research on this specific step of migration. Therapies based on direct antigen delivery into the tissue in order to activate tissue-resident DC may be an example, since these DC ought to migrate to lymphoid tissue.

In the present work, we have identified at least two mechanisms that may improve DC migration across lymphatic vessels: the first one consists in differentiating DC in the presence of type I IFN and the second one consists on the agonistic activation of inflammation-induced receptors on the surface of the lymphatic endothelium, in order to increment its adhesive properties. Additionally, we have characterized some of the molecular structures that participate in DC transmigration across lymphatic endothelial cells under inflammation. We chose to explore leukocyte migration under inflammatory conditions, because several pre-clinical models have demonstrated that inflammatory pre-conditioning of the injection site increments the arrival of DC towards the lymph nodes. (Martin-Fontecha et al., 2003; Tripp et al., 2010).

Interestingly, as already mentioned in the introduction, under steady state conditions DC transendothelial migration has been reported to be an integrin-independent process (Lammermann et al., 2008). In addition, our results and those from others (Johnson et al., 2006) suggest that under inflammation this situation changes with an important involvement of integrins and their ligands. These statements are sustained in our work by demonstrating the incremented expression of the integrins with the concomitant exposure of their active conformational epitopes under inflammatory conditions. These increments correlated with increased transmigration across LEC. These observations have been made both, using IFN-DC, or conventionally differentiated DC (IL-4-DC). Importantly, blocking integrins in *in vitro* and *in vivo* approaches impaired TEM and DC arrival to the lymph nodes. We have added confocal image studies in which the participation of integrin ligands is demonstrated in 3D structures that clearly resemble those described by the groups of Sanchez-Madrid (Barreiro et al., 2002) and Carman (Carman and Springer, 2004). Such structures are clearly involved in leukocyte transmigration across blood capillaries. In our hands, integrin ligands guide DC that are following para- and transcellular TEM under inflammatory conditions. These highly specialized structures are dependent on their interaction with active-$\beta 2$ integrins and are able to induce strong phosphorylated Tyrosine signaling in LEC upon DC contact. Interestingly, we report that CCL21 participates in the formation of these structures. We also provide means to increase CCL21 expression by inflamed LEC through the agonistic activation of CD137 receptor, which is unexpectedly exposed on the surface of inflamed LEC. Finally, we present the first evidences on the appearance of integrin-ligand enriched structures in *ex vivo* tissue samples obtained from human and mouse skin.

1- EXPERIMENTAL MODELS IN LYMPHATIC RESEARCH

The research on the biology of lymphatics is relatively recent and therefore, some issues remain to be discovered. For instance, cell transit across this endothelium under inflammation is clearly understudied (Pepper and Skobe, 2003). Some limitations have to be taken into consideration when working with this vascular system:

1-One of them emanates from the difficulty of LEC purification and culture. Although primary cultures are already commercially available, *in vitro* LEC culture is still laborious, cells are not obtained in elevated numbers and, as occurs with other primary cultures, cell phenotype changes after cell passage. In fact, analyses of the LEC transcriptome in culture presents over-expression of genes involved in cell survival and energetic metabolism (Wick et al., 2007). However, important characteristics as for example, inflammation-induced secretion of CCL21, are diminished (Johnson and Jackson, 2010). Therefore, it is needed to work with early passages of these cells in order to maintain cell phenotype.

2- In our hands it is important to work in cell microenvironments that resemble its natural niche by growing LEC on ECM proteins such as fibronectin and collagen mixtures, in order to fully investigate LEC heterotypic interactions. Although it has been reported that LEC monolayers show no polarity (Johnson et al., 2006), lymphocytes have been demonstrated to shown polarized migration across mice LEC (Ledgerwood et al., 2008). Therefore, basal to apical models must be implemented to study DC TEM through LEC. We have set up models of reverse transmigration in Boyden chambers which allows us for quantitative measurements and microscopical imaging of DCs undergoing LEC migration. In this work, we have developed two *in vitro* models to resemble apical-to-basal, and basal-to-apical cell migration, and observed for instance that integrin ligands are clustered around an adhered leukocyte regardless of the direction they follow. More interestingly, we have also cultured LEC monolayers inside collagen gels to perform assays that resemble the disposition of lymphatic vessels on the matrix. These models allow us to investigate DC/LEC interaction in a more physiological setting, but we still face some of the limitations stated for *in vitro* studies with LEC such as the absence of the capillary structures and other stromal cells.

Additional *in vivo* experimental systems are important to unravel the mechanisms that underlay lymphatic vessel biology. In this sense, interesting models are implemented to study DC transit in *in vivo* animal settings such as FITC skin painting or footpad injection of DC. We have chosen footpad injection to corroborate *in vivo* the data obtained *in vitro*. In addition, direct imaging of fixed or alive whole mounted skin (from mouse ear skin explants) has permitted us the direct visualization of lymphatic vessels networks and the binding of DC to their surface (Tripp et al., 2008). These experiments worked unexpectedly in spite of constrains of whole tissue incubations under culture conditions. This model can also be used for investigating other dynamic processes of leukocyte migration (Lammermann et al., 2008; Pflicke and Sixt, 2009). Intravital microscopy of vessels has also been achieved (Tal et al., 2011) and can be an interesting tool for the study of lymphatics. The recent development of new knock-in mice models with fluorescent proteins selectively expressed on lymphatic vessels may also be useful for this kind of imaging approaches (Choi et al., 2011; Martinez-Corral et al., 2012). In any case it is important to note that, as we observed in this work for CD137 expression on lymphatic endothelium, there are some differences between mice and human species. For these reason complementary human models as for example, *ex vivo* explanted tissue culture, may help to reach definitive results. Although these *ex vivo* models have important limitations, in our hands short term culture of skin is possible and early events on DC migration such as adhesion to lymphatic vessels can be observed while maintaining the tissue architecture.

2- DC TRANSENDOTHELIAL MIGRATION ACROSS LYMPHATIC VESSELS UPON INFLAMMATION

2.1 Differential mechanisms that underlay DC migration under inflammatory conditions.

Under physiological conditions, a low but continuous traffic of tissue dendritic cells towards lymph nodes exists. These DC are the responsible of the maintenance of peripheral tolerance to self-antigens (Vitali et al., 2012) and enter the lymphatic vessels through a mechanism in which integrins do not participate (Lammermann et al., 2008). Once an inflammatory insult occurs, the lymphatic capillaries respond by incrementing the transcription and expression of cytokines and adhesion molecules (Johnson et al., 2006; Vigl et al., 2011). This complex response of the lymphatic endothelium to inflammation is heavily related to the massive traffic of DC towards the lymph nodes under inflammation, and the onset of immunomodulatory functions. In fact, there are evidences for higher ICAM-1 and VCAM expression in inflamed vessels as well as of impaired DC arrival to lymph nodes when antibodies blocking integrins or their corresponding ligands are used (Johnson et al., 2006; Podgrabinska et al., 2009). Accordingly, we observed decreased DC recovery in the lymph nodes when pre-incubating DC with anti-LFA-1 blocking antibodies under inflammatory conditions in mouse experiments. We also observed inhibition of DC adhesion and transmigration across monolayers of LEC in *in vitro* experiments performed under inflammatory conditions. All these lines of evidence support the idea that under inflammation integrin-dependent mechanisms come to aid in order to regulate DC transit towards the lymph nodes. We support this on the following evidences.

> **I.** We and other have demonstrated incremented CCL21 secretion by inflamed lymphatic vessels. These increments are accompanied by increased chemokine receptor expression. CCL21 depots presented on the endothelial surface would increase leukocyte crawling (Tal et al., 2011). As a result, faster mobility of DCs in inflamed tissue towards the lymph nodes has been reported (Tal et al., 2011). Besides, it has been described how CCR7

occupation promotes LFA-1 high affinity conformations 1 (Eich et al., 2011; Johnson and Jackson, 2010) resulting in increased ICAM-1 mediated binding and endothelial microvilli projection.

II. Under non-inflammatory conditions DC migration is independent of MMP activity as described by Sixt and colleagues (Lammermann et al., 2008) In fact, there is no directional MMP activity and secretion without integrin engagement. In contrast, other published results sustained that the lack of MMP9 and MMP2 impairs the migration of mature LC and dermal DC in *ex vivo* experiments performed under steady state conditions (Ratzinger et al., 2002). Consistently, maturation of DC is accompanied by the inhibition of TIMP (tissue inhibitor of metalloproteinases) (Darmanin et al., 2007) and by the upregulation of MMP9. These results mean that DC migration requires MMP, depending on their maturation status. Most probably tissue architecture, and ECM density may also condition the need of MMPs. Regarding to the specific entrance of DC to the lymphatic vessels, the status of the basal membrane under inflammatory conditions with regard to base-line has not been addressed yet, but would constitute an interesting area of research.

III. Lymphatic endothelial cells express on their surface low but detectable amounts of ICAM-1 under normal conditions and almost negligible VCAM. But, under inflammation, the intensity of both integrin receptors on LEC surface increases dramatically. In fact, as we and others have demonstrated, blockade of these molecules (Johnson et al., 2006) correlates with impaired migration to lymph nodes. We have observed that ligation of adhesion molecules; mainly by β2 integrin promotes supra-molecular adhesion structures that help DC crawling and migration across LEC. In fact these structures do not participate in DC transit under non-inflammatory conditions. More interestingly, we could not detect any event of transcellular migration across non-inflamed LEC monolayers and all the observed transcellular TEM observed across inflamed LEC occurred through ICAM-1 enriched 3D structures. Besides, integrin blockade impaired lateral DC crawling on LEC monolayers. Therefore, integrin ligands aid DC

adhesion and lateral migration of DCs as described for vascular endothelium (Carman et al., 2003)

It is not yet explored if these key changes can be extrapolated to the lymphatic vessels from other tissues and under every inflammatory condition. Of note, it should be really interesting to analyze this process in inflamed skin as it is the most common model on the study of DC migration. In terms of DC therapy, morphology of the lymphatic vessels that are present in tumors would also be interesting to address the type of migration that DC adopt in the malignant tissue. If this inflammation-driven phenomenon could also be observed in most tissues, this would mean that DC migration across lymphatic vessels could be radically different from the migration observed under steady-state conditions and support our findings.

2.2 Microvilli projections enriched in integrin ligands and their function in adhesion and TEM across lymphatic vessels

In 2002 Sanchez-Madrid and coworkers identified special structures enriched in inflammation induced adhesion molecules (ICAM and VCAM) that surrounded leukocytes undergoing adhesion to endothelial monolayers (Barreiro et al., 2002). These structures had a characteristic spike shape and were enriched in a number of EZRM (Ezrin-moesin-radixin moieties) proteins. This finger-shaped morphology and the increased expression of integrin ligands were suggestive of a role in leukocyte capture under flow. Shortly afterwards, the role of these membrane structures was broadened by results published by Carman and colleagues (Carman et al., 2003). In their work these authors demonstrated the participation of the microvilli-like membrane protrusions in the preparation of leukocyte TEM and, importantly, that these structures were also formed in the absence of flow. We have described the same kind of structures formed on the surface of LEC in the absence of flow. As already stated, DC migration towards the lymphatic vessels occurs, contrarily to what happens in blood vessels, from the tissue side towards the lymphatic lumen. In this situation, DC migrate in 3D environment in a chemokine-guided way until they encounter the endothelial wall. To our surprise we found that lymphatic endothelial cells protruded 3D finger-shaped microvilli-like structures into ECM made of laminin and collagen. Therefore, the function of these structures is not just capturing leucocytes that are under flow, but

somehow they sense leukocyte surface, guide them towards endothelial passage and usher them across the endothelial layer. This last issue was especially clear in those experiments in which LFA-1 integrin was blocked. We observed that DC indeed adhered to LEC surface but hesitated in their lateral movements and failed in cell transmigration. In fact, we have also observed that microvilli projections are very dynamic structures that assemble and disassemble as DC crawl in an integrin dependent manner, as proposed by Barreiro and co-workers for blood endothelial cells (Barreiro et al., 2005). Interestingly, all the transcellular TEM events observed were preceded by the formation of these microvilli protrusions and showed ICAM-1 enrichments accompanying DC while crossing the endothelial body.

Up to date there is no *in vivo* evidence on the formation of these tridimensional structures neither in blood or lymphatic vessel. Electron microscopy studies of neutrophil migration across blood vessels demonstrated the presence of endothelial flaps that surrounded neutrophils before TEM (figure 1). These flaps turned into a dome
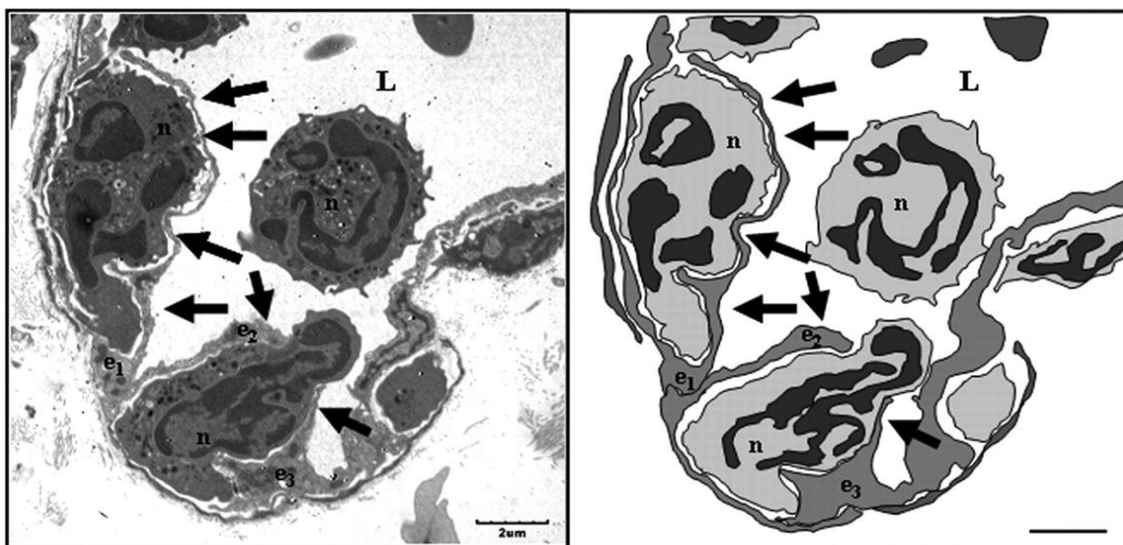
Figure 1. Transmision electronic microscopy microphotograph of neutrophils adhered to avenular wall and correspondent drawing of the same microphotograph. L indicates the lumen of the vessel, n indicates neutrophils and e1, e2 and e3 indicate the cytoplasm of three different endothelial cells. Flaps of the endothelial cells embrace neutrophils, at different extension suggesting different time points of the same process. These structures have been identified as dome structures, at least, enriched on PECAM and dependent on PSGL-1 protein on the endothelium. From Petri et al. (2011).

as diapedesis progressed, suggesting that these endothelial extensions could form a "pressurized hatch" to enable neutrophil diapedesis (Petri et al., 2011; Phillipson et al., 2008). But this mechanism, however, is not acting on every event of diapedesis, since

transendothelial migration of neutrophils which do not need to form these endothelial domes has been reported *in vivo* (Woodfin et al., 2011).

In this work we present evidence for the formation of zones enriched in integrin ligands on LEC that surround adhered DC and protrude out of the endothelial plane. *Ex vivo* inflamed tissue samples obtained from the skin of mice and humans strongly indicates that these structures are not a culture artifact. This is to our notice the first evidence of these structures *in vivo*. Despite of the technical limitations, the structures observed *in vivo* are very similar to the ones that we observed in our *in vitro* setups. No ICAM-1 enrichment was observed if DC were not interacting with vessels and the formation of these structures could be inhibited by blocking LFA-1 on DC. The appearance of these structures correlates with integrin polarization towards LEC surface and cannot be observed under basal conditions. Further research in the detailed composition of these supramolecular structures is warranted. The connections to the cytoskeleton, to secretory granules and to intercellular communication promises to be a fertile area of work. Moreover visualization of these microvilli-like structures in living mice by two-photon microscopy would be of great interest taking advantage of mice whose lymphatic vessels express fluorescent proteins.

3- IFNα AND CD137 AS PROMOTERS OF DC MIGRATION

3.1 CD137 functions on LEC

We and others (Johnson et al., 2006) have shown that ICAM-1 is expressed on LEC under inflammation, and that these integrin receptors are directly implicated on the active promotion of DC migration across them via an integrin-adhesion molecule mechanism.

Besides, other LEC functions related to immunity have been shown under inflammation. ICAM-1 does not only promote TEM, but also can modulate DC functions following Mac-1 ligation. Interestingly, lymphatic endothelium can block maturation of DCs by this mechanism, but only under inflammation induced by non-infectious agents (Podgrabinska et al., 2009). This discovery suggests a role in preventing autoimmunity in contexts of non-microbial inflammation. Interestingly, we have observed phosphorylated Tyr and phosphorylated Src on the contact area of DC with adhesion molecules forming microvilli like projections. These signaling events may regulate other functions on DC such as migration or maturation. Therefore, it could be interesting to explore the bidirectional immunomodulatory effects of lymphatic endothelium and DC under different inflammatory conditions.

To our knowledge, there are no previous reports on leukocyte-LEC interactions that result in immunity enhancement. A possible modulation of DC function may be transduced from the outside by the integrins upon ligation of adhesion molecules (Ramgolam et al., 2010). The effect of this stimulus may depend on the general context in which inflammation is occurring, on the duration of this exposition or on the concentration of the pro-inflammatory agent.

In this sense we provide evidence for increased expression of CD137 by the lymphatic vessels under inflammation. CD137 facilitates DC chemotactic attraction and transit across LEC monolayers. The unique ligand of this molecule, CD137L is expressed on DC. Therefore the interaction between LEC and DC may promote CD137/CD137L interactions. We have shown that the natural ligand is able to induce

CD137 triggering and increased chemoattraction for myeloid cell. Therefore, in a way, it is expected that upon DC interaction with LEC, CD137/CD137L would increase chemoattraction of other DCs in a CCL21 dependent mechanism. Furthermore, reverse signaling via CD137L, at least in moDC, has been demonstrated to enhance migratory capacities (towards CCL21 and CXCL12). CD137L crosslinking on myeloid cells also increases co-stimulatory functions for T cells (Lippert et al., 2008). Therefore, CD137 ligation could be a mechanism to enhance migration of DC towards lymph nodes by increasing CCR7 and CXCR4 on DC and the secretion of CCL21 on the endothelial side. Interestingly, CD137 needs an important and established inflammation to be clearly expressed on LEC (i.e: 48 hour with at least 50 ng/ml TNFα usually) and other inflammatory cues cannot modulate it with the same effectiveness.

Interestingly, as we have shown, CD137 can be pharmacologically activated by agonistic mAb. Treatment with these antibodies promoted increased DC migration across LEC both in a VCAM and CCL21 dependent manner. Therefore, CD137 ligation may promote DC migration when the antibody is given as an immunotherapeutic agent. As we have also shown, CD137 agonistic antibodies promote lymphocyte entrance in tumors by upregulating integrin ligands. Therefore, CD137 activators that are being used for the treatment of tumors in clinical and pre-clinical models, may also favor the traffic of leukocytes to the tumor milieu in addition to the activation of CD8 responses (Melero et al., 2008).

3.2 IFNα Treatment for the enhancement of DC migratory capacities

In the search of better immunotherapy strategies against tumors using vaccines, different protocols for preparing DCs from the patients are being assessed. Tumor microenvironment is complex and tends to provide many immunoregulatory mechanisms that weaken immune responses. As the most adequate immune responses against tumors should resemble the ones performed against viral infections many strategies try to induce an antiviral-like response against tumors. For example, treatment with POLY I:C of tumors would mimic the presence of virus and induce responses that attack tumors as if they were infected cells (Okada, 2009).

Another cytokine that promotes anti-viral responses is IFNα. In our group we have successfully used IFNα in the treatment of tumors in preclinical models (Dubrot et al., 2011). IFNα is also capable of inducing differentiation of monocytes into DCs (Parlato et al., 2001) and to provide them with increased antiviral capabilities. Additionally, it has been stated that effective antiviral responses rely on a rapid access of DCs to LN (Le Bon et al., 2003; Montoya et al., 2002).

Direct effects of IFNα/β on DC are well described (Lapenta et al., 2003; Le Bon and Tough, 2008; Parlato et al., 2001; Santini et al., 2003; Santodonato et al., 2003). In vitro differentiation of monocytes in the presence of IFNα and GM-CSF renders DC that strongly stimulate antiviral lymphocytes *in vitro* and *in vivo* (Lapenta et al., 2003; Santini et al., 2003)

In our work, we showed for the first time that type I IFNs also change the adhesion and migratory activities of DC in their interplay with lymphatic endothelium. The adhesion and transmigration effects were much more evident when lymphatic endothelial monolayers had been pre-exposed to TNFα to mimic lymphatic vessels from inflamed tissue (Johnson et al., 2006; Johnson and Jackson, 2008). Previous studies performed on human cultured lymphatic endothelium clearly showed that TNFα selectively induced ICAM-1 and VCAM (Johnson et al., 2006).

We have observed that the differentiation of DC in the presence of type I IFN, augments LFA-1 expression levels on DC and promotes the appearance of an epitope on the integrin chain that indicates its active molecular conformation (Cabanas and Hogg, 1993). Moreover, interference with LFA-1 with specific mAb decreased the arrival of mouse IFN-DC injected in the footpad to draining LN.

The importance of our findings with IFN-DC arises from the fact that IL-4-DC are the most widely used DC in immunotherapy (Melief, 2008), although the appropriateness of this particular cytokine combination for differentiation of DC has been questioned (Palucka et al., 2006). In addition, when IL-4-DC are intradermally injected into humans, they show limited migration to LN (de Vries et al., 2005a). IFN type I inclusion in clinical-grade cultures has been proposed for DC production and maturation (Mailliard et al., 2004; Santodonato et al., 2003), due to the fact that IFNα (i) enhances crosspriming, (ii) improves the expansion of CTL and (iii) favours DC

adhesion/migration properties. Therefore, IFNα could be artificially provided upon local release of antigens to improve vaccines (Bracci et al., 2008; Gallucci et al., 1999; Sangro, 2005). Our findings suggest the inclusion of these type of treatments in DC maturation protocols could be interesting. We are currently including IFNα in the maturation protocol of DCs for cancer vaccination in clinical trials as previous results showed that migration could be also enhanced in patients (Alfaro et al., 2011). Additionally, other groups have also proposed similar strategies, as the pretreatment of DC with CCL21 (Eich et al., 2011).

4- DC MIGRATION MODEL AND IMPLICATIONS FOR IMMUNOTHERAPY

On a whole, from the data obtained in this work in conjunction with previous data obtained from a number of different groups we propose a sequential model of DC migration across lymphatic vessels under inflammation. Although many of the processes and molecules involved in this mechanism are in need of further investigation. We suggest that, under inflammation, increased CCL21 produced by LEC may massively chemoattract CCR7 expressing mature DCs (Martin-Fontecha et al., 2003). CCR7 engagement during interstitial migration and more importantly the activation of CCR7 upon DC docking to CCL21 enriched deposits on LEC, should activate the leukocyte integrins (Eich et al., 2011; Schumann et al., 2010; Tal et al., 2011). Activated integrins, especially LFA-1 would then direct the formation of adhesion molecule-rich microvilli projections (Barreiro et al., 2005; Carman and Springer, 2004). These ICAM-1 and VCAM enriched structures would dynamically lead DCs to sites of diapedesis, proactively favoring TEM. This route is slower than the fast squeezing through endothelial flaps described under non-inflammatory conditions, but is envisioned as very efficient in promoting transendothelial migration. Intimate contact between LEC and DC permits subsequent signaling. Upon DC-LEC interactions, CD137 would be induced on LEC and if ligated would increase CCL21 secretion and hence further DC chemotaxis (Figure 2).

We also propose two immunotherapeutic strategies that could boost DC vaccination by the increasing of DC migration (Figure 3).

(I) We think that the inclusion of clinical grade IFNα in DC differentiation protocols would enhance migratory capacities.

(II) We advocate for combinations of DC vaccines with CD137 agonists will not only better CTL responses in tumors but also increase the number of DCs trafficking to/from lymph nodes and the number of lymphocytes that recirculates from tumors.
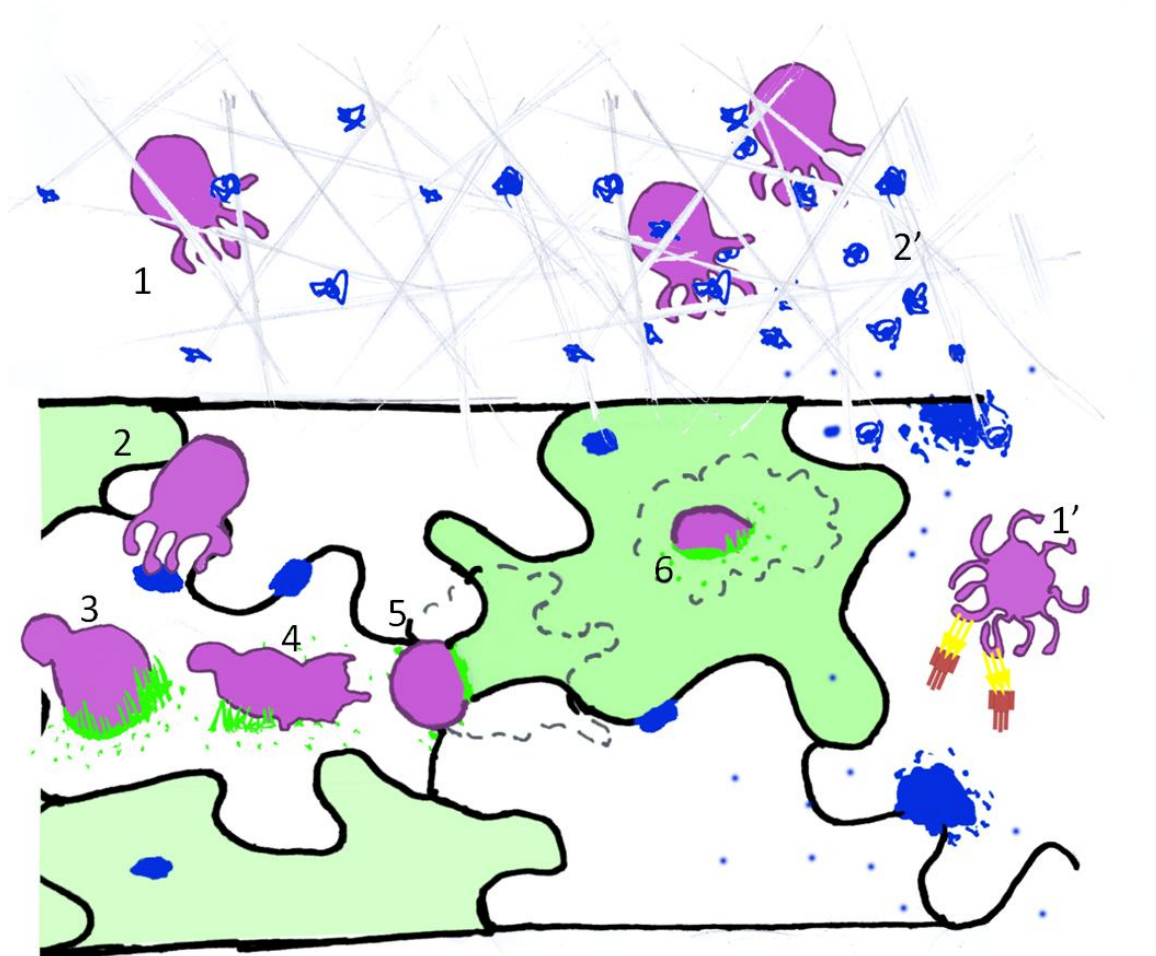
Figure 2. Schematic model of inflammation dependent dendritic cell migration across lymphatic vessel. Sequential processes of DC lymphatic intravasation. 1- DC chemoattracted by CCL21 gradient towards the lymphatic capillaries. 2- DC docking to a CCL21 accumulation on LEC surface. 3- DC adhered and captured by adhesion molecule enriched microvilli projections. 4- DC crawling over the LEC surface accompanied by microvilli projections. 5- DC traversing lymphatic endothelium following a paracellular route. 6- DC performing transcellular intravasation. 1' DC interacting with inflamed LEC and activating CD137 on endothelium. 2' Increased secretion upon CD137 activation
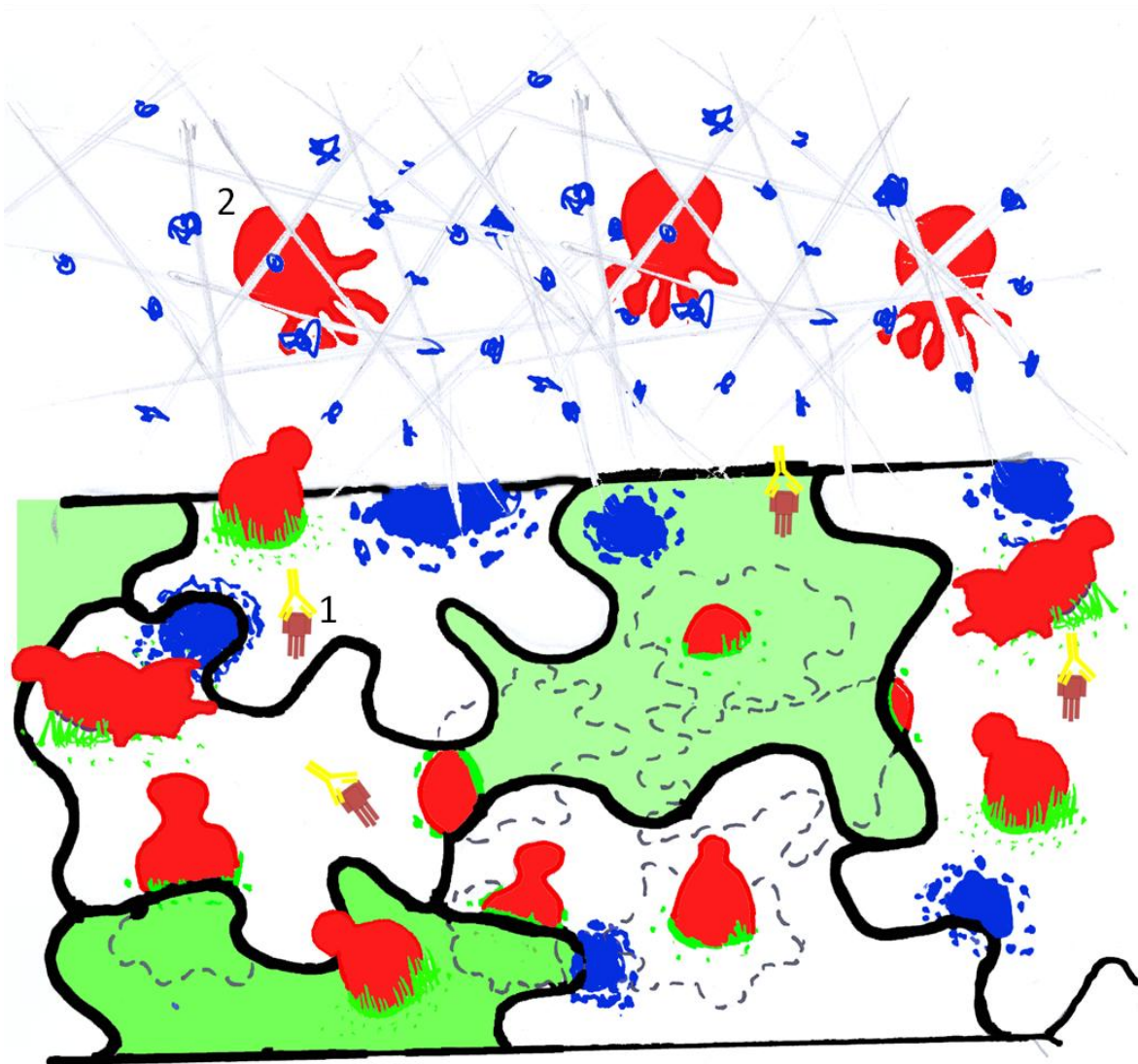
Figure 3. Schematic model of the effects induced by the two treatments proposed
1-Agonistic antibody treatment of CD137 induces increased CCL21 secretion and chemotaxis. 2- IFNα induces upregulation of CCR7 and LFA-1. Activation of LFA-1 is also strongly induced. As a result inflammation dependent adhesion and TEM is enhanced.

CONCLUSIONS

1- Dendritic cells differentiated from monocytes in the presence of IFNα migrate more efficiently across inflamed lymphatic endothelium in *in vitro* and *in vivo* than conventionally differentiated DCs

2- This increased migration observed for IFN-DC is a result of:

    I.   The surface upregulation of CCR7 and CCR5 chemokine receptors.

    II.   The increased expression, affinity and avidity of the integrins LFA-1 and VLA-4.

3- Inflamed LEC are able to protrude microvilli-like 3D structures enriched in integrin ligands upon DC adhesion.

4- For these structures to be formed, integrin receptor binding to high affinity integrin epitopes expressed on DC surface is mandatory.

5- These structures are involved in enhancing DC adhesion, crawling and transmigration across inflamed LEC.

6- We demonstrate for the first time, by confocal microscopy, the existence of these 3D microvilli-like structures in *ex vivo* human and mice inflamed lymphatic endothelial vessels.

7- CD137 is expressed on lymphatic endothelium in *in vitro* primary LEC culture and *ex vivo* inflamed samples. It is also detected in clinical samples obtained from inflamed tissue.

8- In vitro activation of CD137 by agonistic mAbs promotes DC migration across LEC by incrementing the secretion of CCL21 cytokine and by the upregulation of VCAM expression on LEC surface.

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