



Electric signals regulated immunomodulation and wound healing

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APPENDIX 1:

Specimen layout for Thesis Summary and Declaration/Statements page to be included in a Thesis

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SUMMARY

Endogenous electric fields (EFs) are present during a variety of physiologic and pathologic events, including penetrating injury to epithelial barriers. An applied electric field with strength within the physiologic range can induce directional cell migration of epithelial cells, endothelial cells, fibroblasts, and immune cells suggesting a potential role in controlling cell behaviours during wound healing. Dendritic cells (DCs) and dermal fibroblasts were used to explore the molecular mechanisms underlie EF-induced cell activities during two aspects of wound healing: immune response and remodelling.

In this study, we investigated the effects of applied EFs on several types of DCs in response to IL18. DC progenitor cells KG-1 shows dose dependently response to EFs stimulation to increase IFN- γ expression. Moreover, the migration of KG-1-derived DCs and Langerhans cells (LCs) in mouse skin showed increased response to IL18 with directional migration when exposed to EFs *in vitro* and *ex vivo*. Furthermore, the *in vivo* investigation suggested that pharmacologically increased trans-epithelial potential difference (TEPD) induced LCs to emigrate from skin to draining lymph node. The sensitization of DCs to IL18 can be strengthened by EFs through redistribution of IL18 receptors and phosphorylation of p38 MAPK.

We also comparatively studied the responses of human chronic wound fibroblast (CWF) and chronic matched fibroblast (CMF) to applied EFs with addition of platelet derived growth factor (PDGF). The results indicate that 1) EFs induce human dermal fibroblast directional migration in a voltage dependent manner. 2) CWF shows impaired sensitivity in response to EFs compared to CMF and HF. 3) Activation of PDGFR and PI3K are both required for EF-induced directional migration. 4) PDGF attenuates EF-induced migration directedness through PDGFR-ROCK other than PI3K pathway. 5) Optimised concentration of PDGF plus physiological EFs enhance chronic wound healing.

We propose that the EF-induced re-distribution of the receptors on the cell surface results in a shift of membrane receptors between the cathode-facing and the anode-facing membrane of the cell. There would be a higher probability to overcome the threshold of signal transduction at the higher density receptor side. The downstream

signalling cascade therefore can be ignited. Understanding the signalling pathways underlying guidance cues (EFs, cytokines, chemokines) will help to optimise future therapies for immunomodulation, vaccination, wound healing and regeneration.

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CHAPTER 1 INTRODUCTION

1.1 The concept of human skin

1.1.1 The construction of skin

Human skin is the largest organ of the body and is composed of two parts: a stratified, cellular epidermis and an underlying dermis of connective tissue (Figure 1-1).

The epidermis is mainly composed of keratinocytes and can roughly be divided into four layers; from the exterior inwards these are: stratum corneum, granular layer, spinous layer and basal layer. The specific proteins of keratinocytes, keratin and filaggrin, build the structure of the epidermis and contribute about 80-90% of the mass of the epidermis.

The other cells of epidermis are melanocytes, Langerhans cells and Merkel cells. Melanocytes are differentiated from neural crest cells and are responsible primarily for skin colour by secreting the pigment protein melanin. The Langerhans cells are professional antigen-presenting cells and process antigens encountered by the skin to local lymph nodes. They are of mesenchymal origin and originate from bone marrow. Merkel cells are probably derived from keratinocytes and are somatosensory receptor cells which respond to touch.

The epidermis is attached to the dermis via a complex network consisting of protein and glycoprotein. The epidermis-dermis connection is vitally important due to the roles it plays in cellular communication, nutrient exchange, absorption and other functions. In less than 200 μm of connecting area there are over 30 different macromolecules (collagens, laminins, integrins) which interact within the membrane zone.

The dermis is made up mainly of elastic fibres and collagen floating in a polysaccharide and protein network. In contrast to the epidermis, it is vascularised, enabling it to not only provide energy and nutrition to the epidermis but also play a pivotal role in thermoregulation and wound healing. The major types of cells in the dermis are fibroblasts, macrophages and adipocytes.

Fibroblasts mainly produce and organize the extracellular matrix and also express cytokines (e.g. FGF) to communicate with other cells residing in the skin. In addition, fibroblasts are the main force of contraction during wound healing.

Macrophages have their effective function during the wound healing by coordinating the healing process. Their functional phenotype is dependent on the wound microenvironment. During the early and short inflammatory phase macrophages exert pro-inflammatory functions like antigen-presenting, and the production of inflammatory cytokines and growth factors that facilitate the wound healing process; during the proliferative phase, macrophages stimulate proliferation of connective, endothelial and epithelial tissue directly and indirectly.

Adipocytes make up the subcutaneous fat which is an active part of the endocrine system, secreting the hormones leptin and resistin.

The interaction between different kinds of cells, structures, cytokines, growth factors and other large and small molecules in the skin is an extremely complex, and largely unknown part of the physiology and pathology of human skin.

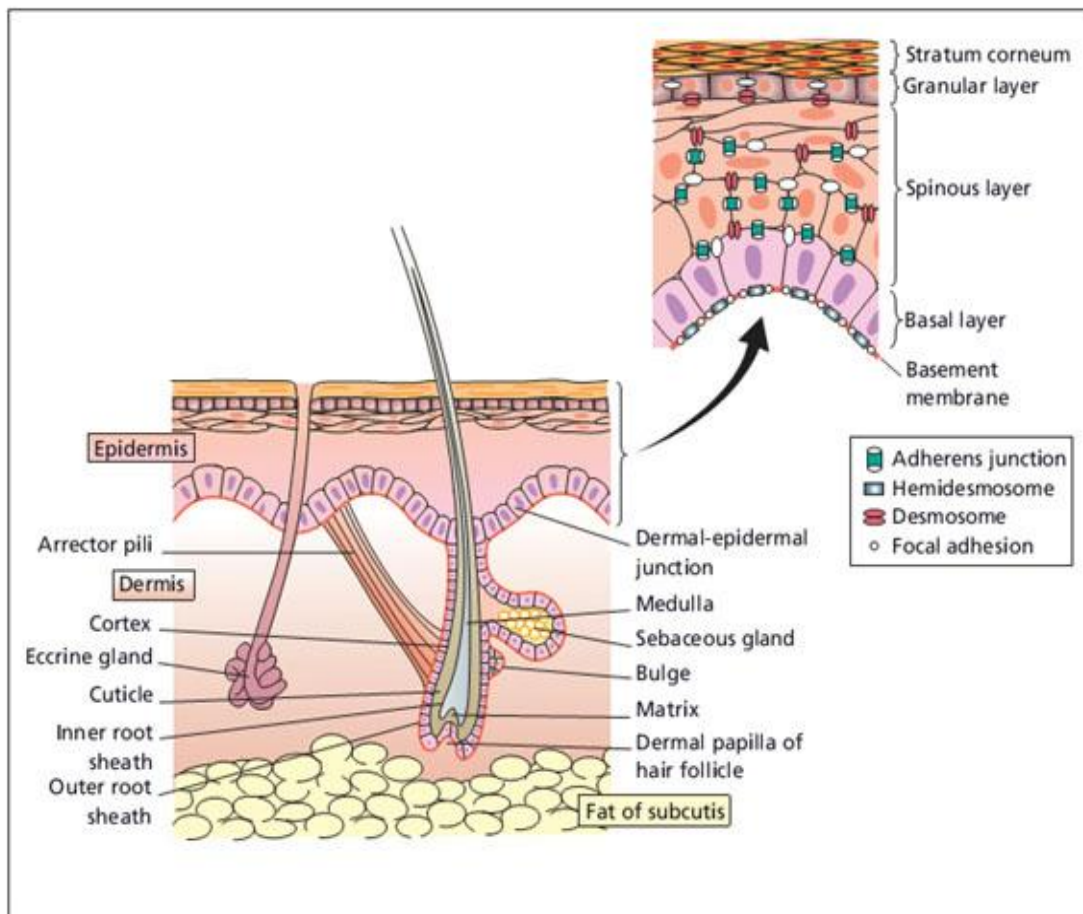


Figure 1-1 The skin and its appendages

The schematic illustrates the structure of the skin (Rook and Burns, 2010). The skin is composed of two layers: epidermis and dermis. The epidermis can be divided into 4 layers: stratum corneum granular layer, spinous layer, basal layer and basement membrane. The dermis consists of connective tissues and is divided into two layers: papillary region and reticular dermis.

1.1.2 The function of skin

The key role of the skin is to provide a barrier to the external environment, keeping certain elements either in or out of the body (e.g. keeping water in and bacteria out), whilst also acting as channel for environmental stimuli and for keeping the body in balance (e.g. thermoregulation).

Water is the most important factor for all cell activity, and the cornified cell envelope and the stratum corneum prevent water loss through the skin. In the epidermis, there also are about 2×10^9 Langerhans cells, which serve as sentinel cells whose primary function is to survey the epidermal environment and to initiate an immune response against microbial threats.

Another important function of skin is thermoregulation. The networks of blood vessels in the deep or superficial plexuses help regulate heat loss through vasodilatation and vasoconstriction, and eccrine sweat glands excrete sweat to lower body temperature *via* evaporation.

Subcutaneous fat has two main functions: trauma cushioning and calorie reserves. In non-obese subjects, about 80% of the body's total fat is found in subcutaneous tissue. Fat also has an endocrine function to regulate hunger and energy metabolism.

1.2 The process of wound healing

Wound healing is a complex and precisely controlled process where various intracellular and extracellular pathways are activated and coordinated to restore tissue integrity and homeostasis. Wound healing involves a complex interaction between epithelial and dermal cells, the extracellular matrix, and controlled angiogenesis and plasma derived proteins, which are coordinated by endogenous and exogenous stimuli. This dynamic process has been divided into three separate phases: inflammation, proliferation and maturation/remodelling (Harding et al., 2002). These different phases are not distinguished temporally as they overlap in time and therefore are distinguished by their separate functions. The entire process is summarised below in Figure 1-2.

When tissue is first wounded, blood comes in contact with collagen, triggering blood platelets to begin secreting inflammatory factors; this is the start of the

inflammatory phase. Platelets also express glycoproteins on their cell membranes that allow them to stick to one another and to aggregate, forming a mass (Midwood et al., 2004). Fibrin and fibronectin cross-link together and form a plug that traps proteins and particles and prevents further blood loss (Sandeman et al., 2000). Additionally, these activated platelets release a number of cytokines and growth factors which activate an inflammatory response (Werner and Grose, 2003). Neutrophils and macrophages respond to these chemicals by migrating to the wound site. Once there, they actively cleanse the wound of debris and bacteria by phagocytosis (Deodhar and Rana, 1997). Macrophages also secrete a number of growth factors and other cytokines, which attract cells involved in the proliferation stage of healing to the area (Werner and Grose, 2003).

The beginning steps of proliferation are the migration of keratinocytes over the damaged dermis and the formation of new blood vessels. Angiogenesis increases the supply of oxygen and nutrients to the wound site, meeting the metabolic requirements of cells that are involved in wound repair. The sprouts of capillaries associated with fibroblasts and macrophages forms a new substrate for keratinocytes migration at later stages of repair process. The keratinocytes are behind the leading edge proliferate and mature and, finally, restore the barrier function of epithelium. Simultaneously with angiogenesis, fibroblasts begin accumulating in the wound site. Later, they become the main cell type that lays down the collagen matrix in the wound site (Stadelmann et al., 1998). Some parts of fibroblasts differentiate into myofibroblast which contract around cells that bring the edges of wound together and form granulation tissue which actually begins to appear in the wound already during the inflammatory phase, and continues growing until the wound bed is covered.

The third stage is remodelling which normally lasts for a bit more time than the first two stages. During this stage all the process activated before gradually slow down and cease. Most of the macrophages, endothelial cells and myofibroblast move away from the wound or undergo apoptosis. The structure change of matrix from type III collagen to Type I is prevail at proliferation stage (Lovvorn et al., 1999). Metalloproteinases secreted by fibroblast, macrophages and endothelial cells is the main force of this process. This process strengths the repair tissue but the tissue never regains the properties of uninjured skin (Gurtner et al., 2008).

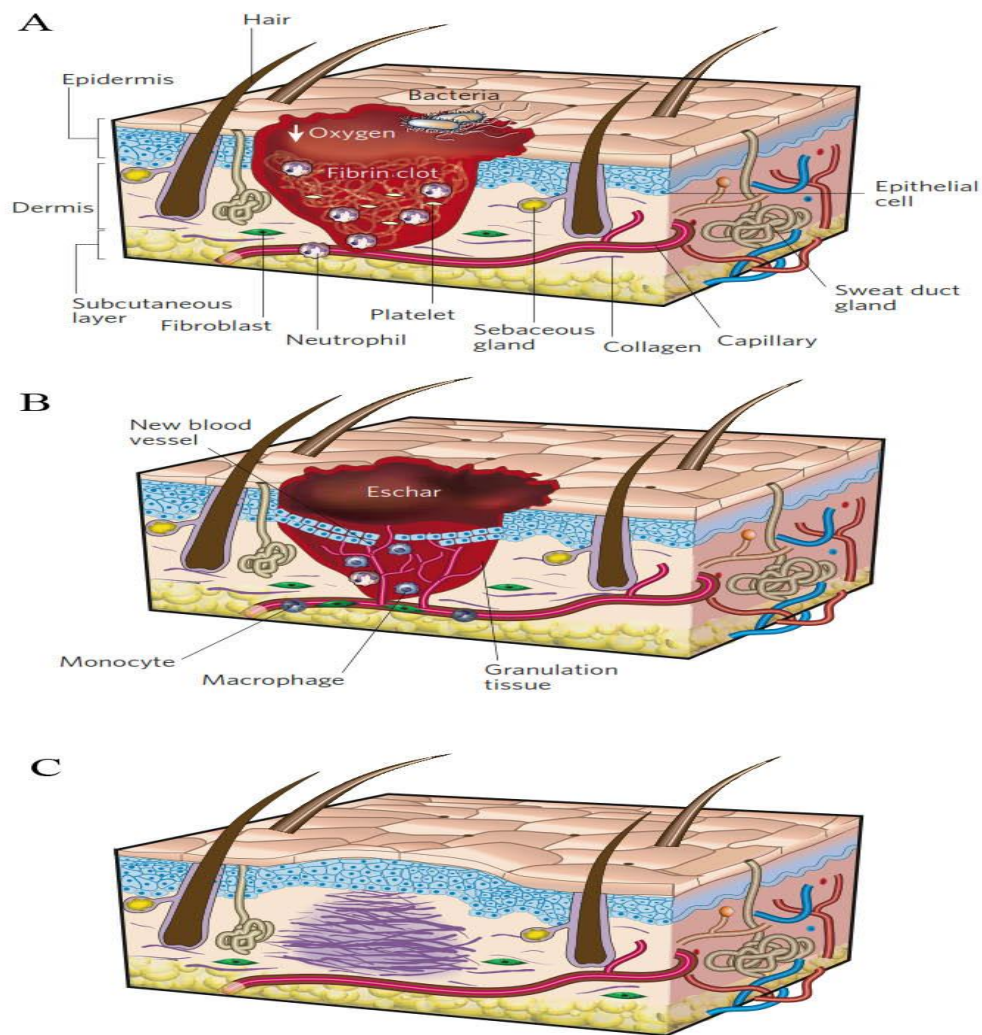


Figure 1-2 Phases of Wound Healing

There are three classic stages of wound healing: inflammation (A), new tissue formation (B) and remodelling (C) (Gurtner et al., 2008). A. Inflammation. This stage starts immediately after injury and lasts about 2 days. The wound is characterised by a hypoxic environment in which a fibrin clot has formed. Bacteria, neutrophil and platelet appear in the wound. B. New tissue formation. This stage starts about 2 days after injury and lasts one to two weeks. An eschar has formed on the surface of the wound; the new blood vessels populate the area. The immune cells start to migrate out from the skin and epithelia cells start to migrate into the wound. C. Remodelling. This stage lasts for a year or longer. Disorganized collagen has been laid down by fibroblasts that have migrated into the wound. The wound has contracted and the re-epithelialized wound is slightly higher than the surrounding surface.

1.3 The inflammatory response

The inflammatory response is the first of a number of events that constitute wound healing and commences almost immediately after wounding. In skin repair, it begins with the passive leakage of circulating leukocytes (largely neutrophils) from damaged

blood vessels. They have multiple functions, not only defending against invading pathogens but also playing roles in tissue degradation and tissue formation. As such, the infiltration of leukocytes migrating into wound site may have profound effects on downstream cell migration, proliferation, differentiation, and ultimately the quality of healing process (Eming et al., 2007). The other types of immune cells which are already resident within the tissue such as mast cells, $\gamma\delta$ T cells and Langerhans cells also become activated and in turn release a rapid pulse of chemokines and cytokines. Those cytokines, together with foreign epitopes such as lipopolysaccharides (LPS), actively recruit neutrophils and macrophages from nearby blood vessels into the wound site. Together, these activities promote local endothelial cell expression of selectins which induce leukocyte rolling and crossing of the endothelial barrier. This is enhanced by vessel dilation, which in turn is triggered by inflammation- associated nitric oxide, mast cell- derived histamine, tissue plasminogen activator and other factors (Shaw and Martin, 2009).

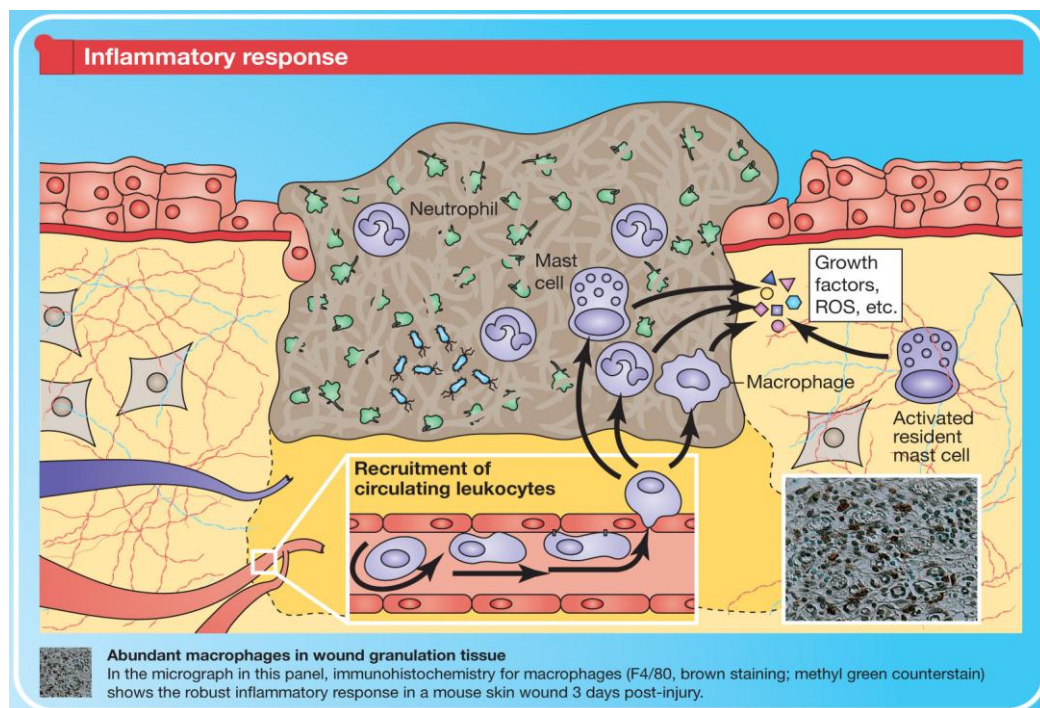


Figure 1-3 Inflammatory response of skin wound

The inflammatory response to wounding starts immediately with the leakage of circulating leukocytes. The resident immune cells also are activated and release a rapid pulse of cytokines and chemokines. Thus the neutrophils and macrophages are attracted by chemotaxis and migrate into wound bed (Shaw and Martin, 2009).

1.4 The role of immune cells

In various experimental animal and human skin wounding models it has been demonstrated that the leukocyte subsets orchestrate the process spatially and temporally. The understanding of the interactions between the cells and well-defined chronology of the process is essential for artificial promotion of optimal wound repair.

1.4.1 Neutrophil

Neutrophils are immediately activated and recruited to a wound site by chemo-attractant released from coagulation of the blood clot. Within a few hours after injury, the wound-released cytokines such as IL-1 β , tumour necrosis factor- α (TNF- α) and interferon- γ (IFN- γ) trigger the activity of neutrophils and induce them to transmigrate across the endothelial cell wall of blood capillaries. In addition, those cytokines increase neutrophil expression of adhesion molecules, including endothelial P- and E-selectins and ICAM-1,-2. The adhesion molecules are crucial for neutrophil diapedesis because they interact with integrins present at the neutrophil cell surface and induce cell morphology transformation (Eming et al., 2007). The main function of neutrophils is to cleanse the wound site and kill invading microorganisms through several strategies, including the release of proteolytic enzymes and bursts of reactive oxygen species (ROS) (Dovi et al., 2004; Shaw and Martin, 2009). Other studies suggest that these cells also influence many other aspects of repair, such as resolution of the fibrin clot and provisional ECM, promotion of angiogenesis, and re-epithelialisation (Kim et al., 2008; Li et al., 2003b; Theilgaard-Monch et al., 2004).

1.4.2 Monocyte

Monocytes appear at the wound site from the circulation soon after neutrophils and their numbers peak at a day or so after injury (Shaw and Martin, 2009). The immigration of monocytes into the wound is regulated by the interaction of late antigen-4 ($\alpha 4\beta 1$ integrin) and endothelial vascular cell adhesion molecule-1 (Eming et al., 2007). These cells mature into macrophages due to stimuli in the wound environment such as growth factors, cytokines and MCP-1. Thus their function can be separated into two phases during the wound healing process according to the concentration gradient of various chemotactic factors. In the earlier phase the

monocytes are recruited and play a major role in phagocytosis and clearance of infection (M1 pro-inflammatory). In the later phase, the monocytes mainly take part in repair and angiogenesis (M2 anti-inflammatory and pro-angiogenesis). These transformations imply major changes in the active phenotype of macrophages caused by significant alterations in gene expression. The membrane receptors of macrophages are thought to sense environmental change and therefore direct the transformation. Those receptors include Toll-like receptor, complement receptor and Fc receptor; however, the details of mutual interplay and the mechanisms of interaction between receptors and outside stimuli still remain unclear. In addition to their immunological function as phagocytes, macrophages possess other functions to facilitate wound repair by the synthesis of numerous potent growth factors, including TGF- β , TGF- α , basic fibroblast growth factor, platelet-derived growth factor and vascular endothelial growth factor, which promote cell proliferation and the synthesis of extracellular matrix molecules by resident skin cells (Eming et al., 2007; Lai et al., 2009).

1.4.3 Mast cell

Mast cells arise from pluripotent hematopoietic progenitor cells present in bone marrow, and these progenitor cells are delivered to various tissues where they reside and terminally differentiate. Over the years, mast cells have been shown to be an important source of a variety of proinflammatory mediators and cytokines (Aller et al., 2007; Bienenstock et al., 1987). Thus it has been widely accepted that they play an important role not only in wound repair but also in autoimmune disorders such as arthritis. In the immune response, mast cells release ROS and reactive nitrogen oxide species (RNOS), both of which are used at high levels to kill phagocytosed organisms. Low levels RNOS may diffuse outside cells to impact the vascular and nervous system. However, some studies of mast cell deficient mice have shown contradictory results concerning their function. Egozi reported that mast cell deficiency had no significant effect on epithelialisation, collagen synthesis or angiogenesis, but resulted in decreased number of neutrophils at a wound site (Egozi et al., 2003), this has been challenged by a report that mast cell deficiency led to a significant reduction in vascular permeability, neutrophil influx and wound closure (Eming et al., 2007).

However, further investigations are needed to better understand the impact of mast cells on the wound healing process.

1.4.4 $\gamma\delta$ T cells

The $\gamma\delta$ T cells are a unique T cell population resident in the epidermis. They arise from foetal thymic precursor cells and express a canonical $\gamma\delta$ T cell receptor (TCR). These cells have a dendritic morphology and are strictly limited in their distribution to the epidermis, where they are thought to play an important role in mediating wound healing. Upon wounding, they recognize an unknown antigen expressed by damaged keratinocytes and then produce epithelial growth factors and inflammatory cytokines. The study on $\gamma\delta$ T cell-deficient mice reveals that the deficiency of $\gamma\delta$ T led to delayed wound healing (Eming et al., 2007). Similar findings in the clinic revealed that $\gamma\delta$ T cells from patients with healing wounds were activated and secreting growth factors, and conversely, $\gamma\delta$ T cells had an impaired response in non-healing patients (Havran and Jameson, 2010). In addition, this population of T cells has recently been shown to participate in EMC deposition and macrophage infiltration. Taken together, these results demonstrate a novel function of $\gamma\delta$ T cells and provide a new perspective of T cells on skin wound healing.

Table 1-1 Inflammatory cells, their functions and mediators (Eming et al., 2007)

Cell type	Function	mediators
PMN	Phagocytosis of infectious agent	ROS, cationic peptides, proteases
	Macrophage activation through phagocytosis Amplify inflammatory response	TNF-alpha, IL-1beta, and IL-6
Macrophages	Stimulate repair response Phagocytosis of PMN and fragments of tissue degradation	
	Amplify inflammatory response	TNF-alpha, IL-1beta, and IL-6
	Anti-inflammatory function	IL-10, TGF-beta1
monocytes	Stimulate repair response: angiogenesis, fibroplasia fibrolysis	t-PA, uPA, u-PAR, and PAI-1/-2
	Control vascular	Histamine

	permeability	
	control influx of PMN	Chymase, tryptase
	regulate tissue remodelling	
T cell; Th1/Th2	regulate tissue remodelling	Cd40l ligand; IL-2, TNF-alpha/IL-4,-5,-10
	Keratinocyte proliferation, differentiation, hyaluronan	
$\gamma\delta$ T cells	synthesis in Keratinocytes	FGF-7, -10 and IGF-1

bFGF, basic fibroblast growth factor; FGF, fibroblast growth factor; PDGF, platelet-derived growth factor; PMN, polymorphonuclear leukocyte; ROS, reactive oxygen species; TGF, transforming growth factor; TNF, tumor necrosis factor, uPA, urokinase-type plasminogen activator; VEGF, vascular endothelial growth factor

1.5 The roles of growth factors

Platelet-derived growth factor (PDGF) is a major mitogen for a number of different cells, including fibroblasts and vascular smooth muscle cells. It has a crucial role in development and is involved in the initiation of wound healing events. The main cellular effects of PDGF are to promote growth and reorganisation of the actin cytoskeleton, initiate chemotaxis, and prevent apoptosis.

The main signal transducers that bind to activated PDGF receptors include phosphatidylinositol 3'-kinase (PI3K), phospholipase C- γ , tyrosine phosphatase SHP-2 and activating proteins for the Rho family of small GTPases. PI3K is able to interact with a number of different downstream effectors to promote actin reorganisation, chemotaxis, growth and protection against apoptosis. Phospholipase C- γ induces mobilisation of intracellular calcium stores and contributes to proliferation and motility of some cell types. Dephosphorylation of tyrosine residues by PDGF-activated SHP-2 serves as a negative feedback mechanism of receptor activation. The mitogen-activated protein kinase (MAPK) cascade can be initiated by a number of PDGFR signal transducers to result in the transcription of genes that are required for cell proliferation, differentiation and migration (Andrae et al., 2008). There is a significant degree of cross-talk between the different transduction mechanisms which can help to amplify signals. For example, PI3K products can serve to increase phospholipase C- γ activity.

Hepatocyte growth factor (HGF), through binding to its receptor (Met), exerts important function in re-epithelialization. Met gene knockdown mice showed strongly delayed re-epithelialization in response to skin wounding (Chmielowiec et al., 2007). Though it was thought that other growth factors are involved in this process, it is clear

that they cannot compensate for the absence of HGF signalling. HGF/c-Met trans-activates epidermal growth factor receptor (EGFR) and then enhances activation of downstream kinases PI3K and ERK. The cross-talk between EFGF and HGF/c-Met may play a key role in regulating retinal pigment epithelial cell migration, proliferation, and wound healing (Xu and Yu, 2007). Mesenchymal stem cells can enhance the quality of wound healing, and may generate *de novo* intact skin. It has been reported that HGF can induce human MSC migration and plays an important role in hMSC recruitment to wound site (Neuss et al., 2004).

Ligands for the IIIb variant of FGF receptor 2 (FGFR2- IIIb), in particular, contribute to wound repair. This was shown in a transgenic mouse experiment in which keratinocytes expressing a dominant-negative mutant of FGFR2- IIIb showed a strong inactivation in re-epithelialization. The mutant receptor binds to FGF but cannot transduce the signal (Werner et al., 1994). FGF7 and FGF 10 are likely to be the most efficient FGFR2- IIIb ligands (Braun et al., 2004). The mice that lack dendritic epidermal T cells, which are a potent source of FGF7 and FGF10, show decreased keratinocyte proliferation and wound closure after injury (Jameson et al., 2002).

Epidermal growth factor (EGF) is believed to play an important role in normal wound healing in tissues such as skin, cornea and gastrointestinal tract. In addition heparin-binding EGF has been shown to have a functional role in keratinocyte migration and accelerates wound healing (Shirakata et al., 2005). Those positive re-epithelialization growth factors such as FGFs, HGFs and EGFs are ligands of receptor tyrosine kinase, the activation of which will stimulate fibroblast and keratinocyte migration and proliferation and lead to wound closure. Some of those processes are mediated by Signal Transducer and Activator of Transcription 3 (STAT3) which responds to a number of growth factors and cytokines. STAT3 is phosphorylated by receptor associated kinases and then forms homo- or heterodimers that translocate into the cell nucleus, where they stimulate gene expression. Another transcription activator, Activator Protein 1 (AP1), is thought to play a similar role to STAT3 in response to cytokines and increases wound closure (Li et al., 2003a).

In contrast to these mitogenic growth factors, TGF- β is a negative regulator of re-epithelialization. It was demonstrated that loss of TGF- β signalling leads to an accelerated re-epithelialization and an increased proliferation of keratinocytes at the

wound edge (Amendt et al., 2002). This finding is supported by studies in mice lacking transcriptional regulator SMAD3, one of the main targets of TGF- β mediated signalling. Smad3 null mice show accelerated cutaneous wound healing, characterized by an increased rate of re-epithelialization (Ashcroft et al., 1999).

1.6 Chronic wounds

Chronic wounds are a major global health problem resulting not only in distress and disability to patients but also enormous economic burden to health care providers (Nwomeh et al., 1998). Chronic wounds encompass a spectrum of disease and can be divided into three main forms: chronic venous leg ulcers, pressure ulcers, and diabetic ulcers. Generally, the normal wound healing responses described above are altered in chronic wounds, with prolonged inflammation, a defective cell activity and a failure of response to cytokines.

1.6.1 Growth factors in chronic wounds

Growth factors are soluble signalling proteins and play essential roles in mediating the process of normal wound healing. Thus the defective function of growth factors always leads to chronic wounds. It has been demonstrated that there is no active PDGF-BB in chronic wound fluid (Castronuovo et al., 1998); and decreased expression of TGF- β in chronic wound fibroblast (Cha et al., 2008a). In addition, EGF and TGF- β were reported to increase fibroblast migration in a chronic wound model *in vitro* (Cooper et al., 1994). Moreover, topical treatment with basic fibroblast growth factor has been showed to effectively heal the chronic cutaneous wounds (Fu et al., 2002).

1.6.2 Skin cells in chronic wounds

The properties of skin cells have been altered by the microenvironment of chronic wound. It has been reported that keratinocytes at the chronic ulcer edge are highly proliferative and have an activated phenotype (K16), but show a reduced expression of LM-3A32 – a key molecule present on migrating epithelium (Usui et al., 2008). Additionally, the fibroblasts from chronic wound showed slow proliferation, impaired response to TGF- β stimulation and collagen disposal capacity (Cha et al., 2008a;

Loots et al., 1999; Stephens et al., 2003). The dysfunction of leukocytes cells was also reported to contribute to chronic wounds (De Ugarte et al., 2002).

1.6.3 The perspective of chronic wound treatment

PDGF-BB shows promise in the treatment of complex wounds with exposed tendon or fascia, but there are still several clinical studies that could not replicate the result (De Ugarte et al., 2002). It has been suggested that the treatment of complex intractable wounds requires consideration of growth factors in combination with adjunctive modalities (Martin, 1997). Adjunctive modalities, including electrical stimulation, hyper baric oxygen, immunomodulatory reagents may enhance the wound healing efficiency (Goldman, 2004).

1.7 Bioelectric signals in biology

In addition to biological factors there are several physical ones; for example electrical signals are also found to regulate wound healing. The detail of how electrical signals control cell behaviour in wound repair will be discussed below in section 1.8.

The first definitive evidence for electric signals being involved in biological activity was discovered by Galvani in the 1790s. His fundamental work proved that it is electricity that causes frog leg muscle to twitch. Evidence has accumulated in nearly every aspect of bioelectrical study with major developments in this field over the last hundred years. Through the 1920s and 1930s Lund found the polarity of cells was somehow controlled by the bioelectric polarity of ion flows *in vivo*. In the 1930s and 1940s, Burr discovered that there was a correlation between the voltage gradient and developmental pattern in a wide range of species and organs (Burr, 1941; Lund, 1928). The main breakthroughs which demonstrated the electrical properties of individual cells, epithelia, neural structures and limb regeneration were based on works by McCaig and his colleagues (McCaig, 1986, 1987, 1989; McCaig and Dover, 1993).

This fascinating research field has shown that endogenous electric signals control limb and spinal cord regeneration, cell and embryonic polarity, growth control, and migration guidance of numerous cell types (Levin, 2009; McCaig et al., 2005). These

endogenous currents and fields were found widely distributed in biological systems (Table 1-2).

Table 1-2 Electrical signals in different biological system (Levin, 2009)

Role	species/Sytem
Cellular polarization (anatomical asymmetry of cell or epithelium)	Alga <i>Fucus</i>
Patterning in gastrulation, neurulation, and organogenesis	Chick, axolotl, frog
directional transport of maternal components into the oocyte	Moth, <i>Drosophila</i>
Growth control and size determination	Segmented worms
Neural differentiation	Xenopus embryo
Polarity during regeneration	Plannaria and annelids

1.7.1 The basis of bioelectrical signals

Electric current is commonly referred as the movement of ions. There is a direct relationship between the voltage, resistance and current. This is known as Ohm's law: $V=I \times R$, where V is the Voltage of the battery, I is the current (in amps), and R is the resistance (in ohms). In physiological environments such as the cell membrane, cytoplasm or the fluids of extracellular spaces charge, is carried not by electrons but by charged ions like Na^+ and Cl^- . Current is generated by the movement of the ions through specific ion channels and pumps within the cell membrane. The bulk resistivity of a physiological solution is about $100 \Omega \cdot \text{cm}$ with some exceptions *e.g.* a single human red cell has a resistivity of only $10 \Omega \cdot \text{cm}^2$ (Johnson and Woodbury, 1964).

Current will flow if a voltage difference exists between any two points in a conductive medium. The electric field (EF) is expressed as the voltage difference per unit distance. The relationship between the current density and electric field is $E = J \times \rho$, where E is the electric field, J is the current density and ρ is the resistivity of the medium. Importantly, all voltage differences, currents and electric fields have directions which are inevitably interdependent. The direction of the EF thus makes it a potential mediator for spatial organization, because it can impose directional

movement on chemicals in the extracellular environment, receptor molecules, cells and tissues (McCaig et al., 2005).

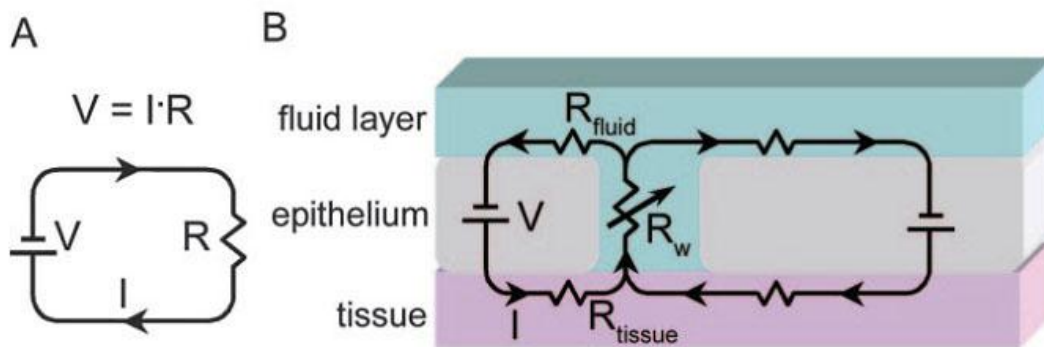


Figure 1-4 Electrical properties of wounded epithelia and surrounding tissue

A: a sample of electric circuit physics. Wires connect a battery (V) to a resistor (R). Electrons, which are negatively charged, carry the current (I , arrows) within the wire move from negative pole (low potential) to positive pole (high potential). The relationship between the voltage difference across the resistor and the current flowing through it is described by Ohm's law: $V = I \times R$. B: In an ion-transporting epithelium, the transepithelial potential difference (TEP) of several tens of millivolts acts as a "skin battery" (V). In biological systems the current (I) is carried by charged ions, such as Na^+ and Cl^- . The ions leak out when there is an injury to the epithelium which produces a low resistance. The total resistances include the wound (R_w) the inner tissue (R_t) and outside fluid (R_f). Because the direction of current flow is taken to be the direction of flow of positive ions (arrows), the wound is more negative than distal regions within the tissue. The wound therefore represents the cathode of the naturally occurring subepidermal electric field. (McCaig, et al. 2005)

An EF in the context of development and regeneration is distinct from those found in the electrophysiology in neurons/cardiomyocytes and electroporation due to the electrical magnitude. To depolarize a neuron and start an action potential require an EF strength of 1-2 V/mm; the common electroporation method to deliver drugs or genes into cells uses an extremely high strength EF, roughly 100-500 V/mm; the EF that plays physiological roles in development and regeneration is between 1-100 mV/mm, three or four orders of magnitude less than electroporation.

With the development of molecular genetics and other state-of-the-art techniques during the last few decades detailed studies of the proteins responsible for bioelectrical signals, the genetic networks that shape them, and the mechanisms that allow cells to convert the information into growth control decisions have been widely

carried out. Several research areas have made tremendous progress in understanding biological events controlled by bioelectrical signals, such as wound healing, neural guidance and cell orientation responses (McCaig et al., 2005; Zhao, 2009).

1.7.2 EFs and wound healing

Though the naturally occurring electric current present in human skin wounds were measured 150 years ago, the confirmation and further understanding of its biological function wasn't gained until the application of modern technologies such as the vibrating probe, and glass and platinum microelectrodes in the last decades.

Epithelial cells are organized in apical-basal pattern which segregate the charges achieved by ion flux, giving rise to a trans-epithelia potential (TEP). The mammalian skin epithelia support a TEP around +70 mV, internally positive. The top layer of cells- the stratum corneum is connected by tight junctions and this forms the major resistive barrier. Intact mammalian epithelial cells transport Na^+ and K^+ inwards from the extracellular fluid and Cl^- in the opposite direction. Upon wounding which disrupts the epithelia barrier the TEP is short-circuited. The voltage potential at the wound thus drops to zero. However, normal ion transport maintains the original potential in unwounded epithelial cells 500-1000 μm away to the wound edge. The resulting gradient of electrical potential, from 0 mV at the wound bed to +70 mV at 500-1000 μm away from the wound, thus establishes a steady, laterally oriented EF with the cathode at the wound (Figure 1-5).

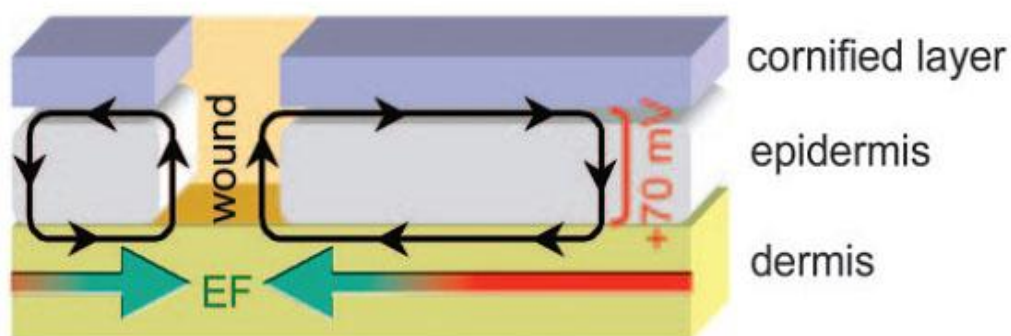


Figure 1-5 Wound generated EFs in epithelia

Mammalian skin keeps a substantial TEP. Wounding collapses the TEP locally thus generates a wound current and an electric field within the sub-epithelium. This wound-induced EF persists until the migrating epithelium reseals the wound and re-establishes a uniformly high electrical resistance (McCaig et al., 2005).

Several studies have investigated these steady wound-induced EFs in a number of tissues. In a cornea wound there is an EF of about 42 mV/mm, whereas in guinea pig skin the wound generates a steep, lateral EF of about 140 mV/mm. The similar strength endogenous EFs have been detected at human skin wounds by using a bio-electric imager (Nuccitelli et al., 2008). In both tissues the voltage gradient dropped off exponentially from the wound edge with a profile that was equivalent to that of a uniform cable that has been disrupted at one point (McCaig et al., 2005). In skin wounds, the distance was about 330 μm , which means that at 330 μm away from the wound bed the voltage gradient would have decayed to 37% of its maximum value at the wound edge. It is similar in human skin wound that the voltage gradient was 140 mV/mm at 250 μm away from the wound bed, 40 mV/mm at 300-500 μm , and about 10 mV/mm at 500-1000 μm .

1.7.3 The endogenous EFs are regulated spatially and temporally

The endogenous current can be tightly regulated in space and in time. Spatially, in different organ or at different distance to wound bed of the same tissue, the current strengths are variable. Amphibian neural tube establishes a potential difference across the tube wall. There is about 90 mV in axolotl at stage 28 and this voltage difference would create a steady voltage gradient across cells in the neural tube wall of remarkable 1800 mV/mm. Levin and his colleagues found H^+ flux might be crucial for creating voltage gradient that drive directed regeneration of spinal-cord axons and tadpole-tail (Adams et al., 2007). On the other hand, in the mammalian skin wound, the strongest currents are found at the wound edge; wound centres have much lower current flow.

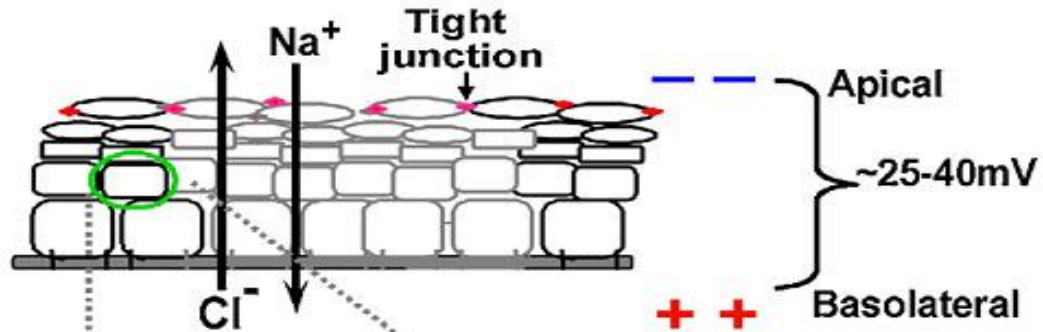
The EF at a wound site varies temporally; the EF at a skin wound is around 140 mV/mm immediately after wounding. This gradually increases to a maximum value of about 250 mV/mm 24 hours after wounding, and then gradually decreasing over about one week until returning to rest the resting TEP of 70 mV/mm (Nuccitelli et al., 2008). Cornea wounds have similar currents that flow out of the wound and rise to a

peak within one hour and persist for several days (Song et al., 2004). During limb development in embryos, current flows out of a precise point on the skin flanking the area from which the limb bud will appear a day or so after the current begins flowing. Injecting a fluorescently tagged charged protein into the extracellular space of non-bud flank skin resulted in radial diffusion. However, the injection into the limb bud resulted in limb development disruption (McCaig et al., 2009). This current also appears in chicken and mouse, which indicates the currents are the guidance of tail regeneration.

1.7.4 Manipulation of epithelial transportation of ions and the effects on wound electric fields

The polarized distribution of ion channels and pumps and segregation of free charges by tight junctions maintain vectorial ion transport (Kucerova et al., 2011; McCaig et al., 2002; Zhao, 2009). The Na^+ channel and Cl^- channel on the cornea epithelium consistently pump Na^+ out of and Cl^- into the tear film, respectively (Figure1-6). The relative magnitudes of net Cl^- secretion and Na^+ absorption largely decide the electrogenic transporting system and potential differences.

(A) Cornea epithelium and the electric potential difference



(B) Transportation of ions in an epithelial cell

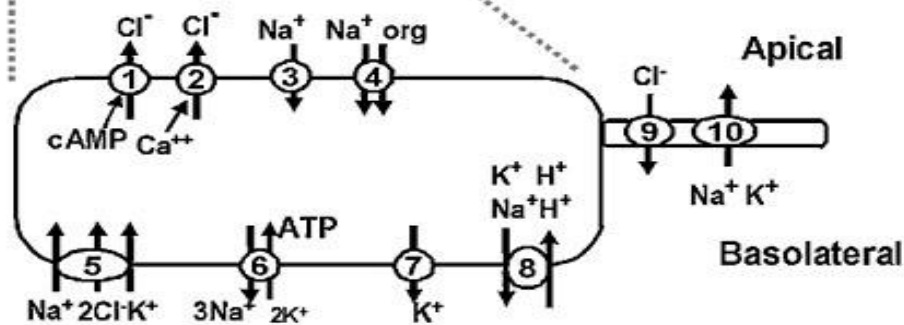


Figure 1-6 Molecular basis of ion channels generate electric potential difference

A: skin is like a battery and maintains an electric difference across the whole layer due to the ions exchange B; An electric potential difference, positive at the basal side relative to the apical side is generated and maintained by net directional ions movement (Zhao, 2009).

Several experiments have proved that a reduction in Cl^- in the bathing solution at the topical side of cornea increases both the electric and chemical forces that drive Cl^- to move to the tear side, therefore the movement of Cl^- is significantly increased and the voltage difference has been doubled. Oppositely, a decrease of Na^+ in the bathing solution decreases both electric and chemical forces of Na^+ movement and reduces the voltage difference.

Pharmacological manipulation of ion channels or pumps- especially Cl^- and Na^+ channels- has a large effect on wound electric fields. The application of Cl^- channel activators PGE2 (prostaglandin E2), Aminophylline or Na^+ channel activators ascorbic acid and AgNO_3 have been proven to achieve an increase of wound TEP (Nuccitelli et al., 2008; Song et al., 2004; Zhao et al., 2006). Conversely, inhibition of

Cl⁻ or/and Na⁺ channel decreases wound TEP. Na⁺-K⁺-ATPase, Na⁺ channel, and Na⁺-K⁺-Cl⁻ transporter can be inhibited by ouabain, benzamic and furosemide respectively, and those inhibitors have been experimentally confirmed to reduce wound TEP (Nuccitelli et al., 2008; Song et al., 2004; Zhao, 2007). Though these ion channel activators or inhibitors have different pharmacological or physiological effects, their applications all converge on one common denominator- the increase or decrease of wound-induced EFs, respectively (McCaig et al., 2005; Song et al., 2004; Zhao et al., 2004; Zhao et al., 2006). The latest investigations imply that wound-induced EFs have major effects on directional cell migration and open another window for wound management (Zhao, 2009).

1.8 Electric fields are an overriding guidance cue that directs cell migration in wound healing

During the wound healing process, endogenous or exogenously applied EFs have been shown to be an overriding mediator for directional cell migration. Many types of cells respond to applied EFs by directional migration. Although EFs are applied experimentally, the same EFs occur naturally *in vivo*.

Cells involved in wound healing must acquire a spatial asymmetry polarization, protrusion towards the wound bed which enables translocation of cells and wound closure. The Golgi apparatus is one of the important elements in mediating cell polarization and protrusion. In detail, Golgi apparatus reorganize the cytoskeleton to direct secretory traffic and recycle the endosomes to the leading edge of the migrating cell (Zhao, 2009). This redistribution of traffic and re-organization of cytoskeleton leads to the enrichment of cell-surface receptors and adhesive molecules at the “front” of the cell that are required for cell protrusion. The movement of the Golgi apparatus to the anterior side of the nucleus decides the direction of migration, thus the cell organizes into a “head-tail” polarization. Pu and colleagues found that Golgi apparatus could be polarized toward the cathode by EFs and disruption which inhibited the directional cell migration. Remarkably, when an EF was applied against the default polarization of cells in a scratched wound (toward wound bed), the Golgi polarization and cell migration followed the direction of EF and ignored the other co-existing directional cues (Pu and Zhao, 2005).

1.8.1 Electric signals in inflammation

As with epithelial cells in wounds an applied EF of physiologic strength can induce directional migration of immune cells such as neutrophils, dendritic cells and lymphocytes. Both freshly isolated neutrophils and HL-60 cell line-differentiated neutrophils showed a strong response to applied EFs. The migration speed and directedness appeared to follow a dose-dependent pattern based on the strength of the EFs. The threshold voltage to induce directional migration was around 200 mV/mm (Zhao et al., 2006). I found applied EFs increased the expression of interferon γ (IFN- γ) in KG-1 cells and induced directional migration in KG-1-derived dendritic cells in 3D culture. I also found EFs could increase the response of dendritic cells to stimulation by IL18 by increasing co-localization of IL18 receptors. Human peripheral blood had previously been investigated to determine whether any subset of cells response to applied EF. Compared with spontaneous migration in the absence of EFs, there was a significant increase in migration of all lymphocyte subsets except NK cells when the EF was applied. In addition, the magnitude of the effects was similar for CD4 T cells, CD8 T cells, naïve T cells, memory T cells, B cells and for monocytes. This observation is in contrast to the chemotactic receptor-driven responses, which are characteristically subset-selective; the electrotaxis response is remarkably uniform among the major lymphocyte classes (Lin et al., 2008).

1.8.2 Electric signals control cell behaviours in proliferation phase of wound healing

During the proliferation phase of wound healing there are mainly two cell types playing the central role. Keratinocytes are the main force for re-epithelialization and are a good model for chemotaxis studies. To study how keratinocytes would respond when both EFs and cytokines are present, the cross flow experiment was used as the method excludes indirect effects of chemoattractant gradients in an electric field. The culture medium containing chemoattractant was circulated through the electrotactic chamber perpendicularly to the field vector at a rate of 20 ml/h, thus eliminating build-up of chemoattractant gradients. The directedness of keratinocyte migration was exactly the same at that of the cells in the same strength EF but absence of medium flow (Zhao et al., 2006).

Fibroblasts in close proximity to a wound respond by forming stress fibres, which enable connective tissue contraction, and also migrate and proliferate to form granulation tissue. Freshly isolated human dermal fibroblast showed a response to applied EFs as low as 100 mV/mm, and the stronger EFs (200-400 mV/mm) had more prominent effects to induce directional cell migration. From the scratch wound experiment, EF stimulation for 6 hours significantly increased wound closure (Guo et al., 2010; Zhao et al., 2006). This evidence clearly verifies that EFs are the overriding signals during wound closure.

1.9 The hypothesis of how cells sense the EFs

The molecular mechanism by which cells detect and migrate directionally in response to an EF remains vague. The early theory that the EF redistributed molecules in the extracellular medium, thus creating a chemical gradient which guides cell response, is not entirely true. This is because cells retain their characteristic electrotropic response to EFs with perfusion of fresh medium orthogonal to the vector of the EFs (Patel and Poo, 1982; Patel and Poo, 1984). Another proposal was that a direct voltage difference across a cell might produce a cytoplasmic voltage gradient which means the cell has positively and negatively charged ends. However, because of the very high resistance of the plasma membrane, the vast majority of the voltage drop remains outside the cells, with almost no internal gradient arising (Betz et al., 1980). The focus then changed to examining which molecular elements of the cells might be asymmetrically affected on the exposure to an EF.

1.9.1 Nerve Cells

It was very controversial finding that showed that growing nerve cell processes (neurites) respond to an electric field; Jaffe and Poo found that neurites grew faster toward the cathode than toward the anode by using embryonic chicken dorsal root ganglia (Jaffe and Poo, 1979). But continued research revealed that it was indeed an electrotactic response, and neurites even turned 180 degree in order to move to the cathode. Further study discovered that EFs also increased total neurite production, and what's more, there were more neurites initiating on the cathode-facing side of the cell body than the anodal side.

Rajnicek and Mccaig first tried to explain the molecular mechanism behind the EF-induced neurite migration (Rajnicek et al., 2006a, b). They found that dynamic microfilaments and microtubules are very important for EF-induced growth cone migration. Further, growth cone turning can be divided into two stages: filopodia reorientation is in advance of turning and lamellipodia reorientation is coincident with the turning. Finally they suggested that Rac/Cdc42-mediated dynamics of microfilaments and microtubules act cooperatively to generate cathodal steering.

1.9.2 Muscle Cells

Myoblast cells from frog embryos were aligned perpendicularly to the vector of an applied EF. The threshold to induce the alignment of myoblasts was as low as 0.3 mV/mm. Mccaig and Dover (McCaig and Dover, 1991) found that calcium potentially contributed to the EF-induced orientation change, because a calcium inhibitor blocked the perpendicular orientation of myoblasts. Calcium entry is essential for EF-induced perpendicular orientation of mouse embryonic fibroblasts as well (McCaig and Dover, 1991). Further experiments indicated that L-type calcium channels might not be as important as T and N type calcium channels in this effect. Another factor which affected the EF-induced orientation change was recognized to be cytoskeleton proteins, and pharmacological inhibitor studies implied that the limited amount of F-actin present played a more important role than microtubules in the morphology transformation.

1.9.3 Neural Crest Cells

Neural crest cells are a transient, multipotent migratory cell population that first accumulates on the dorsal side of the vertebrate neural tube, and then disperses as these cells follow a characteristic pathway to form a remarkable number of derivatives, including melanocytes, craniofacial cartilage and bone, smooth muscle, peripheral and enteric neurons and glia. A number of studies were carried out mainly on amphibian and quail material, and the results were consistent, which were that the cells responded directionally to a transcellular voltage difference of 0.7 mV. In agreement with muscle cells, the neural crest cells have similar morphology transformations in applied EFs, which is difficult to explain because any asymmetries induced by the field will be parallel to the field. Cooper and Keller (Cooper and Keller, 1984) have

explained that the perpendicular alignment minimizes the perturbing effect of the field on the membrane potential and suggest that cells respond in such a way as to achieve this minimization.

1.9.4 Epithelial Cells and Fibroblasts

There are a quite a number studies discussing how epithelial cells and fibroblasts respond to an applied EF. The threshold of the field able to induce directional migration in these two cell types is around 100 mV/mm. Cornea epithelia cells in an EF of this magnitude have a migration speed of 10 $\mu\text{m}/\text{hour}$ with directedness of 0.6, and fibroblasts appear to have a similar response. However, most of the fibroblasts studied have shown migration towards the anode, which is contrary to the response of the majority of other types of cells (Guo et al., 2010; McCaig et al., 2005). The mechanism of the difference is largely unknown so far.

1.9.5 Mechanism of interaction with electric fields

Although it is still unclear how cells sense and respond to an electric field as small as 0.1 mV, their responses to larger voltage gradients are partially due to the influence of the difference between calcium entry into the cathode-facing sides and anode-facing sides of the cells. The symmetric zygotes of the brown algae *focus* and *pelvetia* will polarize and develop their rhizoids on the anodal side in an electric field; the argument is that the imposed electric fields polarize the zygotes by driving in calcium on the anodal side, which mimics the normal physiological calcium drive process (Figure 1-7).

However, this straight-forward explanation with perturbation of calcium flux cannot explain all observed scenarios. An alternative mechanism whereby an applied electric field might induce asymmetric distribution of membrane proteins is by electrophoresis or electroosmosis.

In the case of ion channels on the cell membrane, it has been proposed that an electric field might redistribute and polarize charged and mobile components in the cell membrane. The degree of redistribution a molecule achieves in a given electric field has been shown to be dependent on the diffusion coefficient of the electrophoretic mobility and the voltage drop per cell required to produce asymmetry of 0.1 (0 representing uniform distribution and -1 or +1 representing completely

redistribution of the component to the one pole or the other). A variety of membrane receptors has been shown to be redistributed to the cathode side of the cell in an applied EF (McCaig et al., 2005; Robinson, 1985), including the Con A receptor, acetylcholine receptor, and the Fcε receptor.

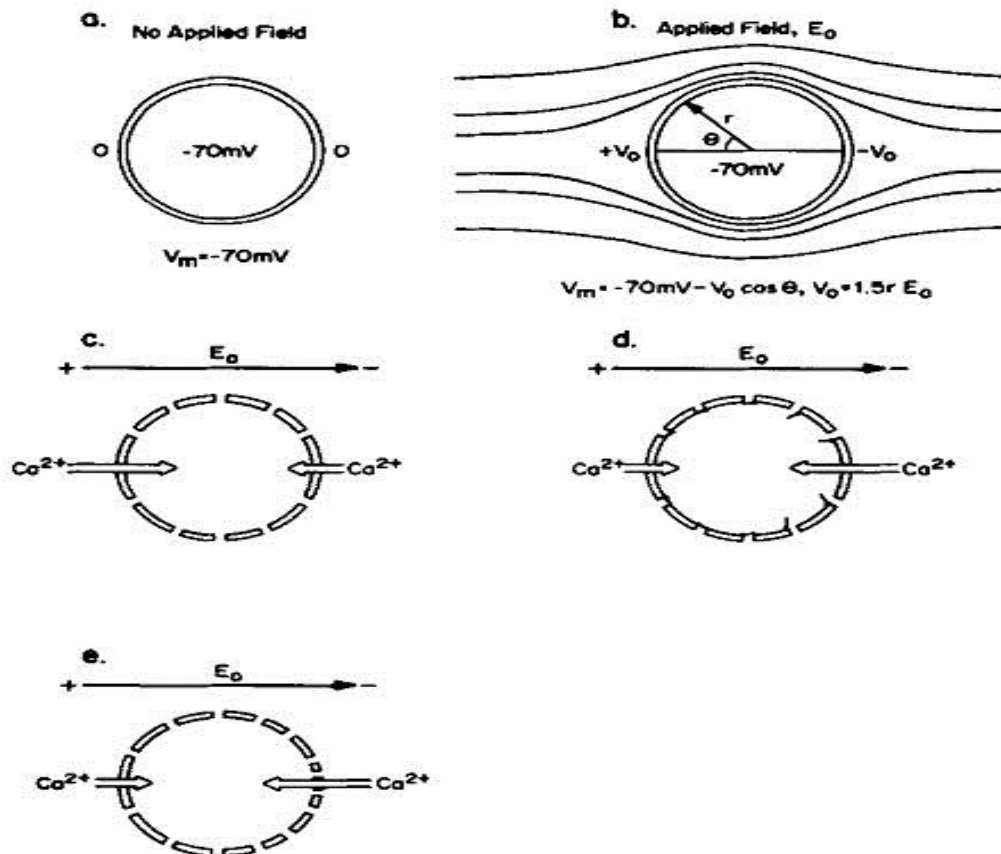


Figure 1-7 Electric Fields interact with a cell by changing calcium uptake

(a) A spherical cell with a membrane potential of -70 mV. (b) The effect of a uniform applied electric field. The electric field is distorted by the highly resistive cell as shown. The potential outside the cell will vary sinusoidally and thus the transmembrane potential, will vary as well so that the anode-facing side is hyper polarized and cathode facing side depolarized. (c) The effects of a field on one voltage gated calcium channel. Because the anodal side is hyper-polarized meaning cytoplasm is more negative, the force driving calcium inwards is increased, while the calcium driving force on the other side is decreased. (d) The effects of the field on calcium flux through voltage-gated channels. The calcium channels on the cathodal side are open due to depolarization, while anodal side channels remain closed. (e) Electrophoretic redistribution of calcium channels. The other proteins studied have been shown to be redistributed by the field. Calcium channels here are only for illustrative purposes, similar consideration applies to other ion channels or protein (Robinson, 1985).

Surprisingly most of the studied receptors with a high degree of redistribution were

glycoproteins which have a net negative charge at physiological pH. This observation has led to the suggestion that the mechanism of electric field-induced receptor redistribution is due to electroosmotic water flow near the surface of the cell, produced by the immobile negative charge on the surface (Robinson, 1985). The evidence has come from Poo's experiment that showed the direction of migration of the Con A receptor was reversed following the treatment of the cells with neuraminidase, which would be expected to remove much of fixed negative charge, allowing a direct electrophoretic response by the receptors (McLaughlin and Poo, 1981).

Another model is cornea epithelia cells as wounds in the cornea epithelium generate endogenous currents that control the process of reepithelialization. This small wound-induced electric field can affect both cell migration and division. During electrotaxis, several growth factors have also been found to act together, perhaps using parallel signalling pathways to transduce the effect of EF. Most electrotaxis work has concentrated on EGF, because during cornea wound healing EGF is upregulated and EGF receptor is activated at the leading edge, which experiences the strongest electric field. Flow cytometry results have confirmed that applying EF increases the expression of EGF receptors and resulted in redistribution of EGF receptors and F-actin to the cathodal side of the cells.

EF-induced asymmetry of EGFRs also induced asymmetric intracellular signalling through the mitogen-activated protein (MAP) kinase signalling cascade. Western blot results have shown increased activation of dual phosphorylated ERK1/2 distributed at the cathodal side. Moreover, activated ERK1/2 and F-actin have become colocalized at the leading lamellae in CECs migration.

In short, the mechanisms driving EF-induced cell directional migration in different kinds of cells share several elements in common. They all can be transduced by an induced asymmetry of membrane receptors which interact with the chemical gradient. They all involve signal transmission at the leading edge by second messenger pathways and eventually connect to cytoskeleton proteins. Figure Figure1-8 compares and contrasts the mechanisms that control electrically and chemically directed cell movement (McCaig et al., 2005).

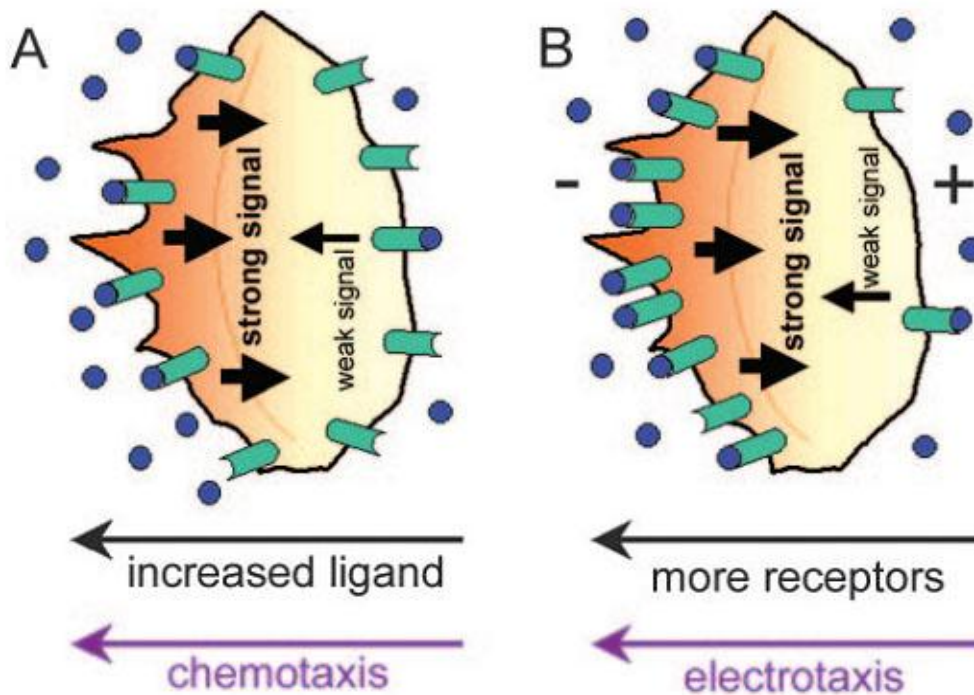


Figure1-8 Model proposed for chemotaxis (A) and electrotaxis (B)

(A) In chemotaxis, a gradient of ligand concentration stimulates activation of more receptors on one side the cell than the other. This results in increasing intracellular signalling pathway activation on one side of the cell even if the receptor density is evenly distributed on both sides of the cells. Therefore, cells migrate toward the high concentration of ligand. (B) In electrotaxis, asymmetric signalling comes from EF-induced polarized receptor distribution, mainly on the cathodal side. Thus, cells migrate towards the side with more polarized receptors (McCaig et al., 2005).

1.10 Cell migration

Cell migration is an important biological process and plays a key role in physiological and pathological processes. During embryonic development, cell migration is required for morphogenesis ranging from gastrulation to development of the nerve system. Migration remains prominent in the adult organism, in physiology as well as in pathology. In the inflammatory response, for instance, leukocytes migrate into areas of insult, where they mediate phagocytic and immune function (Lauffenburger and Horwitz, 1996). In wound healing, migration of epithelial cells, fibroblasts and vascular endothelial cells is essential. In cancers, abnormal cells can migrate from initial tumour mass into the circulatory system. Finally, cell migration is

also crucial to technological application or engineering such as tissue regeneration, tissue engineering and biomaterial scaffolding.

Over the past few years, immense work has been done in order to understand the various aspects of cell migration, including the establishment of polar structures, the regulation of dynamic process of actin, microtubule polarization, and regulation of spatial and temporal signal transduction. Cell migration is a precisely controlled and highly orchestrated multistep process. Migration varies from one cell type to another. For example, the fibroblast is a very typical cell type used for cell migration study. It exhibits relatively slow and uncoordinated movement with protruding and retracting lamellipodia, and they contain many stress fibres connected to large focal adhesion. In contrast, fish keratinocytes move faster and have characteristic unique persistent lamellipodia, small areas of adhesion and absence of stress fibres and filopodia (Le Clainche, 2008). Nevertheless, different types of cells share some common features of cell migration: cells need to establish morphological polarization in response to an extracellular response. At the “front”, cells form lamellipodia which connect to the adhesions; at the “rear” cells retract by combining actomyosin and disassembly of adhesions (Figure 1-9).

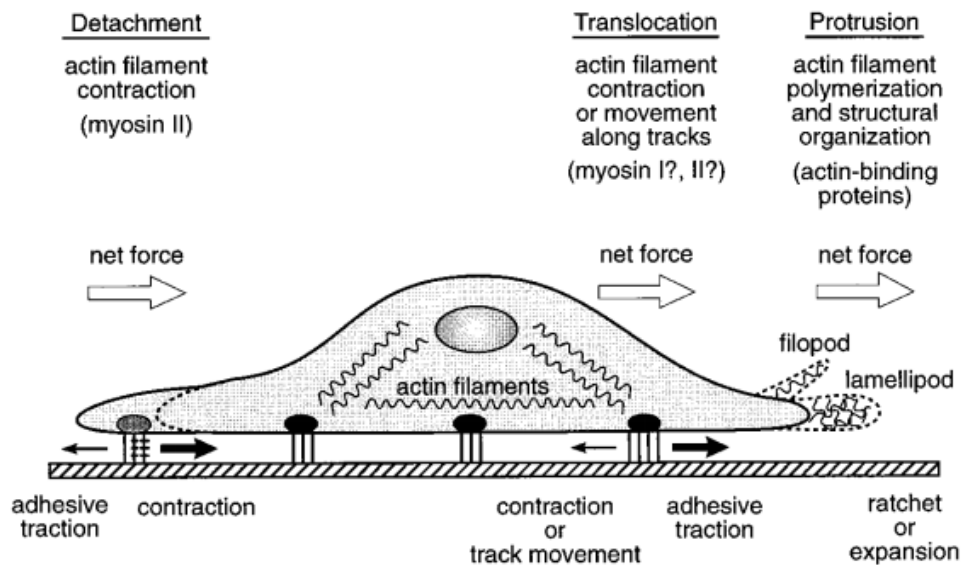


Figure 1-9 Illustration of Cell Migration

Protrusion of membrane lamellipodia is achieved by actin polymerization. Cell starts to move forward once the membrane protrusion has become adherent to the substratum; this may occur by myosin interactions with actin filaments. The rear of the cell follows, detaching and contracting (Lauffenburger and Horwitz, 1996).

1.10.1 Actin-based directional cell migration

The actin network is the most important component of lamellipodia formation and protrusion. The constant and rapid actin assembly maintains the cell lamellipodium and migration direction. Moreover, actin assembly also initiates, regulates and mechanically couples to adhesion to enable protrusion and traction of the cell body.

How actin assembly is converted into a protrusive force to enable cell migration is the basic question in understanding cell migration. Based on seminal experiments, the actin network in cell protrusion is summarised in Figure 1-10, showing that actin is organized in a bidimensional dendritic array of branched filaments.

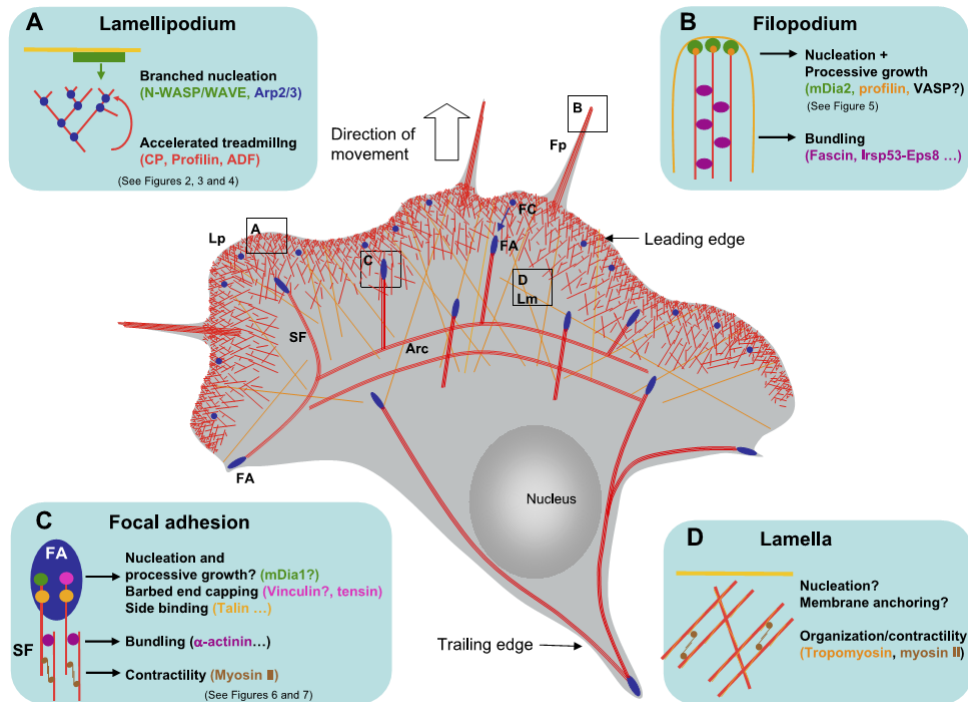


Figure 1-10 Schematic illustration of actin skeleton in a migrating cells

This schematic cell shows the major structures containing actin in migrating cells but does not correspond to a precise cell type. A. In lamellipodia, branched actin filaments are generated at the plasma membrane in response to WASP-Arp2/3 signalling and are maintained in fast treadmilling by a set of regulatory proteins (profilin, ADF). B. During cell migration, cells extend finger-like protrusions called filopodia beyond the leading edge of the protruding lamellipodia to sense the environment. C. Slow moving cells forming focal adhesion in response to RhoA signalling. Focal adhesion connects the extracellular matrix to contractile bundles made by actin filaments, myosin II and bundling proteins like α -actinin. D. The lamella is characterized by a slow actin turnover and contains the signature proteins tropomyosin and myosin II (Le Clainche and Carlier, 2008).

Lamellipodia are broad, flat, sheet-like structures whereas filopodia are thin, fingerlike projections. Both can extend into 3D dimensions around the cell in steady or motile states. FRAP (fluorescence recovery after photo bleaching) experiments in lamellipodia using microinjected fluorescent actin has demonstrated that actin filaments polymerise at the leading edge and depolymerise at the rear. The movement speed of the cell correlates with the rate of actin assembly; the movement of fluorescent actin is at the same relative speed as cell migration. The constant actin nucleation and depolymerisation at front edge of lamellipodia is described as “treadmilling”. Recent discoveries indicate that treadmilling of actin filaments was

sufficient to support the protrusion of the leading edge. Moreover, there are two different types of actin network involved in the process: fast-moving speckles in the lamellipodia and slow-lived speckles in lamella (Ponti et al., 2004). The lamella is behind the lamellipodia and is characterized by a loose array of unbranched actin filaments that is enriched with tropomyosin and myosin II (Svitkina and Borisy, 1999; Svitkina et al., 1997). Microinjection of tropomyosin to replace the lamellipodium-forming components Arp2/3 and actin depolymerising factor (ADF) results in the loss of lamellipodia but also an increase in the level of protrusion and in migration speed (Gupton et al., 2005). This indicates that lamella play a major role in protrusion and the lamellipodia is dispensable. Lamellipodia may serve other functions such as sensing the environment; another possibility is that lamellipodia and lamella may have redundant function in cell migration (Le Clainche and Carlier, 2008).

1.10.2 Molecular basis of actin treadmilling

Actin assembly is a dissipative biochemical process where actin polymerization costs ATP, thereby forming two types of actin formats: ATP-bounded barbed end and ADP-bounded pointed end. Therefore the balance between depolymerisation and polymerisation which reflected by the ratio of pointed-end and barbed-end actin determines the protrusion force. It has been discovered that a set of proteins- ADF, profilin and capping proteins- cooperate to accelerate treadmilling of actin.

ADF binds to pointed end actin and changes its structure to enhance depolymerisation *in vitro* (Carlier et al., 1997). As a result, the concentration of G-actin (monomeric actin) increases up to a level that allows G-actin to bind to barbed end actin, thus keeping the concentration of G-actin in balance. ADF therefore promotes barbed end growth and potentially fosters actin-based motility. In good agreement with its biochemical function, ADF increased the velocity of *listeria* and N-WASP-coated beads in reconstituted motility assays, but decreased the length of the tails (Wiesner et al., 2003). Identified effects have been discovered in *Drosophila* where the RNAi knockdown of ADF leads to an increase in the width of the lamellipodia of cells.

Profilin is another regulative protein of the treadmilling process and it is abundant at lamellipodia. Profilin binds to monomeric actin and forms a profilin-actin complex which exclusively binds to barbed end actin; thus it enhances directional treadmilling

(Didry et al., 1998). Recent studies have revealed that the WH2 domain (WASP homology 2) protein shares similar functions with profilin in promoting barbed end assembly (Co et al., 2007; Hertzog et al., 2002).

There are also a group of proteins named capping proteins which have high affinity to barbed end actin. However, this group of proteins is also involved in other cellular functions and controlled by distinct signalling pathways. The dynamics of capping protein interaction with barbed end actin have been addressed *in vitro* and *in vivo*. Surprisingly, the kinetics in the two situations show huge differences, with the dissociation constant *in vivo* being 3 orders magnitude longer than *in vitro* (Iwasa and Mullins, 2007; Miyoshi et al., 2006).

1.10.3 Nucleation of actin filaments by the Arp2/3 complex in response to signaling

During migration cells need to keep a balance between the nucleation and depolymerisation of actin filaments. In the lamellipodia of migrating cell, the nucleating factor is the Arp2/3 complex activated by the signalling WASP proteins (Gournier et al., 2001). The Arp2/3 complex acts as an accelerator for actin treadmilling and therefore the localized activation of Arp2/3 determines the direction of protrusion. Arp2/3 localizes at the leading edge of lamellipodia, where it nucleates branched actin filaments. Arp2/3 contains seven conserved subunits including the two actin-related proteins Arp2 and Arp3 and ARPC1-5. Arp2/3 complex can be activated by WASP proteins and then nucleates actin filaments by branching the side of preexisting filaments (Amann and Pollard, 2001; Blanchoin et al., 2000).

Actin treadmilling is an energy consuming process, in which ATP exchange and hydrolysis play a critical role. It has been reported that inactive Arp2/3 complex has a high affinity with ATP (Le Clainche et al., 2001). Whilst both the Arp2 and Arp3 subunits have an ATP binding site, those sites are quite different. Arp3 has a high affinity binding site whereas Arp2 has a low affinity site. ATP is hydrolysed at Arp2 without affecting the branched nucleation but strongly inhibits debranching.

The Arp2/3 complex must be activated before initiating lamellipodia protrusion. There are several Arp2/3 activators: WASP/N-WASP, SCAR/WAVE and cortactin (Le Clainche and Carrier, 2008). The WASP (Wiskott Aldrich syndrome proteins) and N-WASP (Neuronal WASP) share the several domains: The NH₂-terminal WASP

homology 1 domain, a GTP binding domain, and a COOH-terminal catalytic domain. Both WASP and N-WASP can be activated by Cdc42 and PIP2, whereby their C-terminal conformations are changed and bind to Arp2/3 complex (Stradal and Scita, 2006; Takenawa and Suetsugu, 2007). SCAR (Suppressor of cAMP Receptor) and WAVE (WASP-family Verprolin homology protein) were identified as WASP related proteins. WAVE/SCAR activates Arp2/3 in quite a different way in comparison with WASP/N-WASP. Neither can be activated by Rho-GTPase due to the lack of GBD domain. However, WAVE/SCAR activates Arp2/3 followed by binding to Rac1, which induces membrane ruffling (Eden et al., 2002). Another candidate as an activator of Arp2/3 is cortactin, because it is enriched at the leading edge of the lamellipodia where it colocalized with actin and Arp2/3 (Cantarelli et al., 2006; Weed et al., 2000). However its role in the dynamics of lamellipodia is still unclear.

A detailed description of the interaction of these molecules and their signalling pathways will require an in-depth investigation and biological dissection of the spatial and temporal regulators. The new high throughput model in yeast and newly designed fluorescence probe probably will help to establish the specificity of these different pathways (Lorenz et al., 2004; Sun et al., 2006).

1.10.4 Integrin signaling during cell migration

For the migration to occur, a protrusion must be stabilized and attached to its surroundings. Although many different receptors are involved in this process in different cell types, the integrins are a major family of migration-promoting receptors (Ridley et al., 2003). These receptors act as the “feet” of migrating cells by supporting the adhesion to the ECM or by linking via adapters of actin filaments inside of the cells.

Integrin and adhesion types

The adhesion is like a “hub” to cell migration and at the centre of the migration cycle. It is involved in leading edge protrusion, adhesion to the ECM, contraction of the cell body and release of the adhesions at the rear. In mammalian cells, there are 18 α subunits and 8 β subunits that can bind as about 24 different heterodimers to determine ligand specificity. Structurally, integrins contain three domains: a ligand binding extracellular globular head, a hydrophobic transmembrane domain and

cytoplasmic tail. There are two conformations of integrins: extended and intermediate in accord with if interacting to extracellular ligand (Askari et al., 2009). Those two statuses are reversible and are vital for signalling transduction. Integrin can also be activated by intracellular molecules which bind to the cytoplasmic tail of the β subunit (Zaidel-Bar et al., 2007).

Integrin-based adhesions have been broadly classified into four groups based on their location, lifetime and protein composition (See Figure 1-11). They are: nascent adhesions (NAs), focal complexes (FCs), focal adhesions (FAs), and fibrillar adhesions (FBs) (summarized in Figure). NAs are very small and punctuate structures at the leading edge of lamella with a very short turn over time (less than 1 minute). If NAs remain stable (for more than 5 minutes), they mature into FCs. If FCs become bigger and stable for a further 10+ minutes, they change into FAs and can interact with zyxin and focal adhesion kinase (FAK). FBs are stable contacts found parallel to bundles of fibronectin (Mercurio et al., 2001). While the turn-over process of transformation and identity of recruited proteins are still not very clear, super-resolution imaging technology and high through-put proteomics may help to reveal more details.

Integrin signalling in adhesion dynamics

Talin is a ubiquitous cytosolic protein with a high concentration in the focal adhesion area, thus it is suggested as a linker between integrin and actin. Recent studies discovered that talin activated integrin by binding the cytoplasmic tail of integrin β -subunits (Anthis et al., 2009). The interaction between talin and integrin has two stages; reversible loose interaction and high affinity binding. It has also been suggested that integrin might dictate talin mobility, because $\beta 1$ and $\beta 3$ integrins could lead to changes in talin (Himmel et al., 2009). The other β integrin activators are kindlin family proteins, which bind at a site distal to that of talin. It is hypothesized that kindlin synergistically active β integrin with talin due to an effect of stabilizing talin or preventing binding of talin competitors (Ma et al., 2008).

In addition to integrin activators, the molecules that maintain integrin in an inactive state also play a very important role in coordinating adhesion dynamics (Scales and Parsons, 2011). The typical example is filamin which binds directly to the cytoplasmic tail of integrin and competes with talin to limit integrin activation (Xu et

al., 2010). Other proteins like Dok1 and ICAP1 may also play similar roles (Calderwood et al., 2003).

The adhesion complex is formed by a variety of proteins, in which α -actinin acts as an organizer by linking integrins, actin and vinculin systematically (von Wichert et al., 2003). Knockdown of α -actinin prevents nascent adhesions (NAs) becoming mature (Choi et al., 2008). Paxillin and vinculin are the key proteins in stabilizing adhesion. Paxillin is rich in of protein-protein interaction sites at its N-terminal. The proteins binding to paxillin are diverse and include protein tyrosine kinases, such as Src and FAK, and structural proteins such as vinculin. Vinculin links integrin, paxillin, F-actin, α -actinin and Arp2/3 complex, thus strengthening adhesions. Vinculin knockout mouse fibroblasts showed reduced adhesion and increased random migration rate, potentially because of a reduction of adhesion stability (Xu et al., 1998).

There are other molecules which also interact with adhesions, such as FAK and small G proteins. By coordinating temporally and spatially, all these molecules together regulate cell adhesion and migration. However, the details of physiology and pathology should be investigated further.

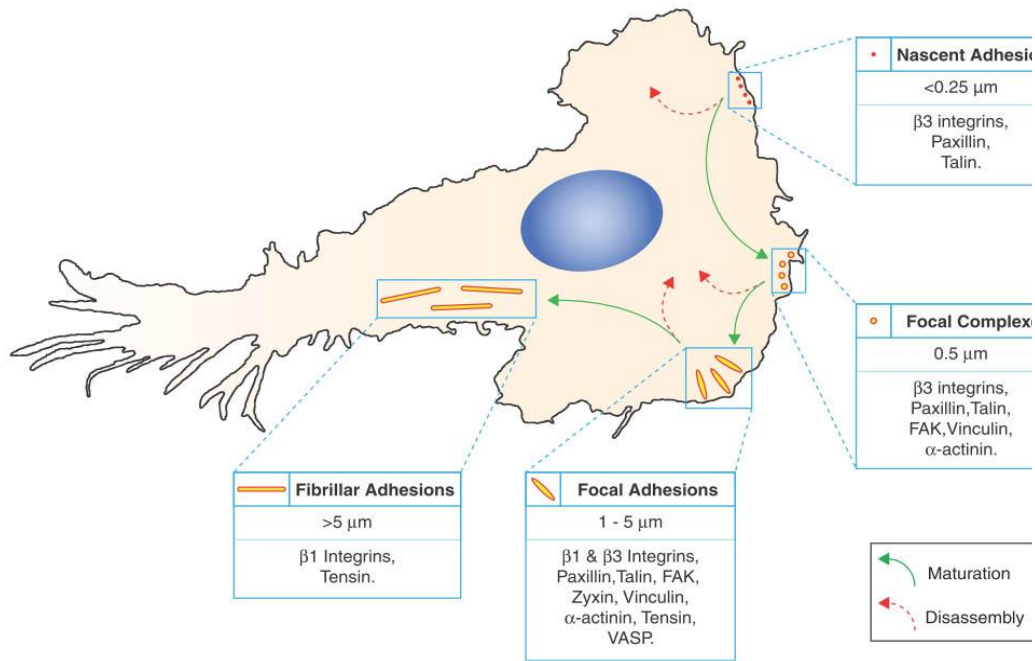


Figure 1-11 Schematic illustration of classic integrin-based adhesions

Shown are the typical localizations of nascent adhesions (NAs), focal complexes (FCs), focal adhesion (FAs), and fibrillar adhesions (FBs) and the hierarchy of their maturation (green arrows) and turn over (red arrows) (Scales and Parsons, 2011).

1.10.5 The roles of Rho-family GTPases in cell migration

Rho-family GTPases, including Cdc42, Rac1 and RhoA, mediate the interaction of cytoskeleton proteins during cell adhesion and migration. These GTPases have been implicated in several different cell types: fibroblasts, T cells, macrophages, astrocytes, epithelial cells and neuronal cells (Fukata et al., 2003). Recent intensive analyses have started to clarify how Rho-family GTPases regulate cell migration: basically they re-organise the cytoskeleton by producing a gradient of signalling molecules (Fukata et al., 2003; Raftopoulou and Hall, 2004; Scales and Parsons, 2011).

The Rho GTPase switch

Rho-family GTPases are ubiquitously expressed and 20 members have been identified in mammals. The Rho-family GTPases cycle between a GDP-bound inactive form and a GTP-bound active form (Fukata et al., 2003). This process is regulated by three factors: guanine nucleotide exchange factors (GEF), Rho GDP

dissociation inhibitor (GDIs), and GTPase-activating proteins (GAPs). Their best-characterised function is to regulate actin dynamics in the lamellipodium. The *in vitro* analyses focused mainly on fibroblasts have clarified that Rho regulates the assembly of actin and myosin filaments, while Rac1 and Cdc42 organize polymerization of actin to form peripheral lamellipodia and filopodia protrusions, respectively (Raftopoulou and Hall, 2004). Moreover, these three GTPases are all involved in the assembly of integrin-based adhesions (Nobes and Hall, 1995).

Regulation of Rho GTPase during cell migration

High resolution fluorescence images have shown that Rac is found at the front of lamellipodia and regulates actin polymerization during cell migration (Chen et al., 2008). Rho, on the other hand, is thought to regulate the contraction and retraction at the rear of cell body, and would be expected to follow an inverse distribution to Rac. For example, by using FLAIR technology, Rac can be visualized in migrating fibroblasts with a high concentration at leading edge and Rho at the rear (Kraynov et al., 2000; Stuart et al., 2008). Cdc42, unlike Rac and Rho, is thought to establish cell polarity in order to initiate migration as live fluorescence imaging showed that Cdc42 was located in the Golgi apparatus where it mediated secretion in endocytic transport (Kroschewski et al., 1999). In the 3T3 fibroblast scratch wound model, a gradient of Rac1 activity exists across the flank of the wound with highest concentration at the leading edge of the wound. A comparison of localization between Rac1 and Cdc42 showed that the highest concentration of Rac is closer to the leading edge than that of Cdc42 (Itoh et al., 2002).

PI3-Kinase and PIP3 in regulation of Rho GTPases activity

PI3K and its product PIP3 have been widely implicated in a pivotal role in regulating cell polarity and migration in physiological and pathological conditions (Stephens et al., 2002). During chemotaxis, PI3K is required for lamellipodium extension and migration *via* signalling from G-coupled receptors (Li et al., 2000). Fluorescence imaging of GFP-tagged PIP3 has shown that extracellular stimuli induce their redistribution to the leading edge of migrating cells and co-localize with centres of actin polymerisation (Haugh et al., 2000; Servant et al., 2000).

But how does the PI3K signalling pathway interact with Rho-family GTPase activation? It has been found that both regulatory and catalytic subunits (p85 and p110, respectively) of PI3k are present in the eluates of GTP γ S-bound affinity column chromatographs (Kobayashi et al., 1998; Raftopoulou and Hall, 2004). In addition GTP-bound Rac1 and Cdc42 can enhance PI3K activity and work as the upstream signals of PI3K (van Leeuwen et al., 1999). Due to the evidence that Rac1 GEFs are activated by PIP3, a positive feedback loop for cell migration is proposed for the PI3K signalling pathway: PIP3—GEFs—Rho GTPases—PI3K—PIP3 (Raftopoulou and Hall, 2004). Recently, PTEN, a lipid phosphatase and a negative regulator of cell survival mediated by the PI3k-Akt pathway, exhibits reciprocal localization to PI3K in *Dictyostelium* (Funamoto et al., 2002).

Downstream effects of Rho family GTPases during cell migration

Rac and Cdc42 are both required for cell migration, with Rac needed for the generation of protrusion force and Cdc42 for polymerisation of actin in filopodia. Recent studies on *Drosophila* have shown that Cdc42 loss of function mutations do not affect the migration speed of peripheral glial cells but instead reduce the directedness of migration (Sepp and Auld, 2003). The interactions between Rac/Cdc42 and actin dynamics have been intensely investigated. The Ser/Thr kinase PAK is commonly activated upon Rac or Cdc42 activation and is believed to play a role in regulating actin dynamics and adhesions. In addition, PAK phosphorylates and activates LIM kinase, which in turn inactivates cofilin. Cofilin is crucial for actin treadmilling at the front of migrating cells by dissociating actin subunits from pointed-end actin filaments (Arber et al., 1998) (Figure 1-12).

The WASP/SCAR/WAVE family of scaffold proteins are the essential regulators of actin dynamics. They are able to stimulate the Arp2/3 complex, which can initiate actin polymerisation either *de novo* or at the barbed end or sides of pre-existing filaments (Amann and Pollard, 2001). Cdc42 activates WASP/N-WASP directly and PIP3 is an important cofactor (Rohatgi et al., 2001), whereas Rac1 activates SCAR/WAVE family members indirectly (Eden et al., 2002). Rho activity is responsible for focal adhesion assembly and cell contractility. In addition, Rho activity is associated with actin and myosin interaction and therefore induces the contraction of the rear of the cell body *via* ROCK (Alblas et al., 2001) (Figure 1-12).

Clearly, if Rho activity is at the front of a migrating cell this will block membrane protrusion, hence mechanisms must be in place to inhibit its activity at the leading edge. Rac may have this function because expression activated Rac has been shown to inhibit Rho activity in many cell types, ranging from fibroblast to neurons (Raftopoulou and Hall, 2004).

Rho-family GTPase and polarity

Although Rho-family GTPases have received attention mainly for their actin modification effects, it is now clear they also possess functions for the regulation of microtubules and cell polarity (Wittmann and Waterman-Storer, 2001). While it seems that microtubules are not involved in lamellipodium dynamics and chemotaxis over a short distance, long distance migration and the persistence of cell polarity are achieved by the microtubule cytoskeleton. Rho was shown to promote the stabilization of microtubules by mDia, which is involved with microtubule capping (Ishizaki et al., 2001). Rac, on the other hand, may elongate microtubules through PAK and the inactivation of the microtubule destabilizing protein, stathmin (Daub et al., 2001).

Cdc42 plays an essential role in defining cell polarity in response to external environmental stimuli. Inhibition of Cdc42 blocked macrophage chemotaxis to CSF without impairing their motility (Ridley, 2001). The polarized migration in many cells is reflected in the reorganization of the microtubule skeleton and centrosome, which usually face the direction of migration (Raftopoulou and Hall, 2004). Cdc42 has been shown to regulate reorientation of the microtubule skeleton and centrosome in migrating astrocytes and fibroblasts. The mechanism involves GTP-bound Cdc42 activating Par6 and PKC ζ at the leading edge of cells, which is essential for determining the orientation of cell migration (Etienne-Manneville and Hall, 2001).

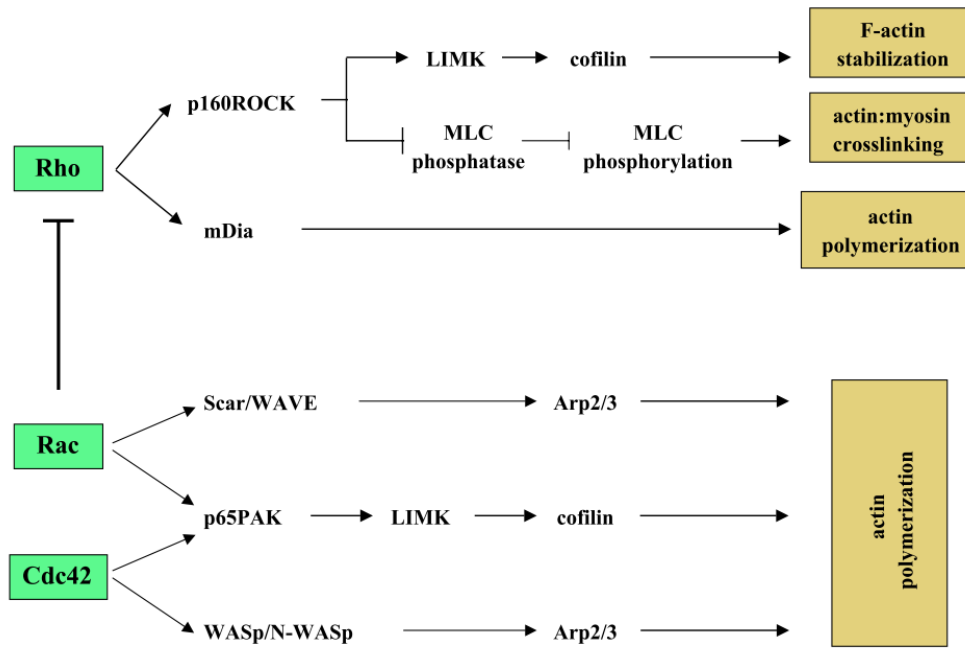


Figure 1-12 Rho-family GTPases regulated pathways affect actin filaments organization

Rho promotes contractile actin:myosin filament assembly through two effectors, mDia and ROCK. Rac and Cdc42 both regulate actin polymerization through the WASP/SCAR/WAVE family of proteins acting on Arp2/3 complex (Alblas et al., 2001).

1.11 Mechanisms of dendritic cell migration

Dendritic cells (DCs) are professional antigen-presenting cells that initiate adaptive immune responses against foreign antigens and maintaining T cell tolerance to self (Steinman and Banchereau, 2007). DCs consist of several subsets which can be distinguished by surface and intracellular phenotypic markers, immunological function and anatomic distribution. In mice all DCs express CD11c integrin and MHC class-II (MHC-II) molecules; by combining tests for these with those looking at the expression of CD8 α , CD4, CD11b, Langerin, PDCA-1 and other markers they can be further distinguished (Shortman and Naik, 2007). When DCs become activated, they normally exert their function in discrete locations remote from their place of origin, which implies that DCs possess migratory capacity to navigate through the body (Alvarez et al., 2008).

All DCs have been found to be derived from hematopoietic stem cells in the bone marrow. However, the facultative DC progenitors are also found in multiple locations other than bone marrow, such as the thymus, blood, lymph and in most visceral organs (Liu et al., 2007). These progenitor cells differentiate into DCs upon challenge of peripheral tissues. The immature DCs in healthy organs are characterised by having highly active endocytic machinery of sampling of foreign antigens but without the full capacity for priming naïve T cells (Banchereau et al., 2000). The most enriched sites of DCs are skin and lymphoid organs where the first entrance of microbial pathogens and adaptive immune response initiates. Indeed their migratory ability allows the DCs in non-lymphoid tissues to transport and present antigenic cargo into and within lymphoid organs. The antigen-bearing DCs become mature with increased expression of MHC complexes and up-regulation of co-stimulatory molecules and cytokines.

Clearly, the ability of DCs to migrate through the body is a critical prerequisite of its immunological function. The “migration” continues throughout the whole lifespan of the DC under different biophysical conditions. Specifically, DC migration encompasses the following events: 1) newly formed DCs or their progenitors exit their place of birth and enter the blood, 2) recruitment of the circulating cells into target tissues; 3) extravascular lodging and interstitial motility to sample antigens; 4) the capacity to access lymph vessels to travel either to lymph nodes (LN) or back to

blood; and 5) the ability to interact with migrating lymphocytes and other immune cells (Alvarez et al., 2008) (Figure 1-13).

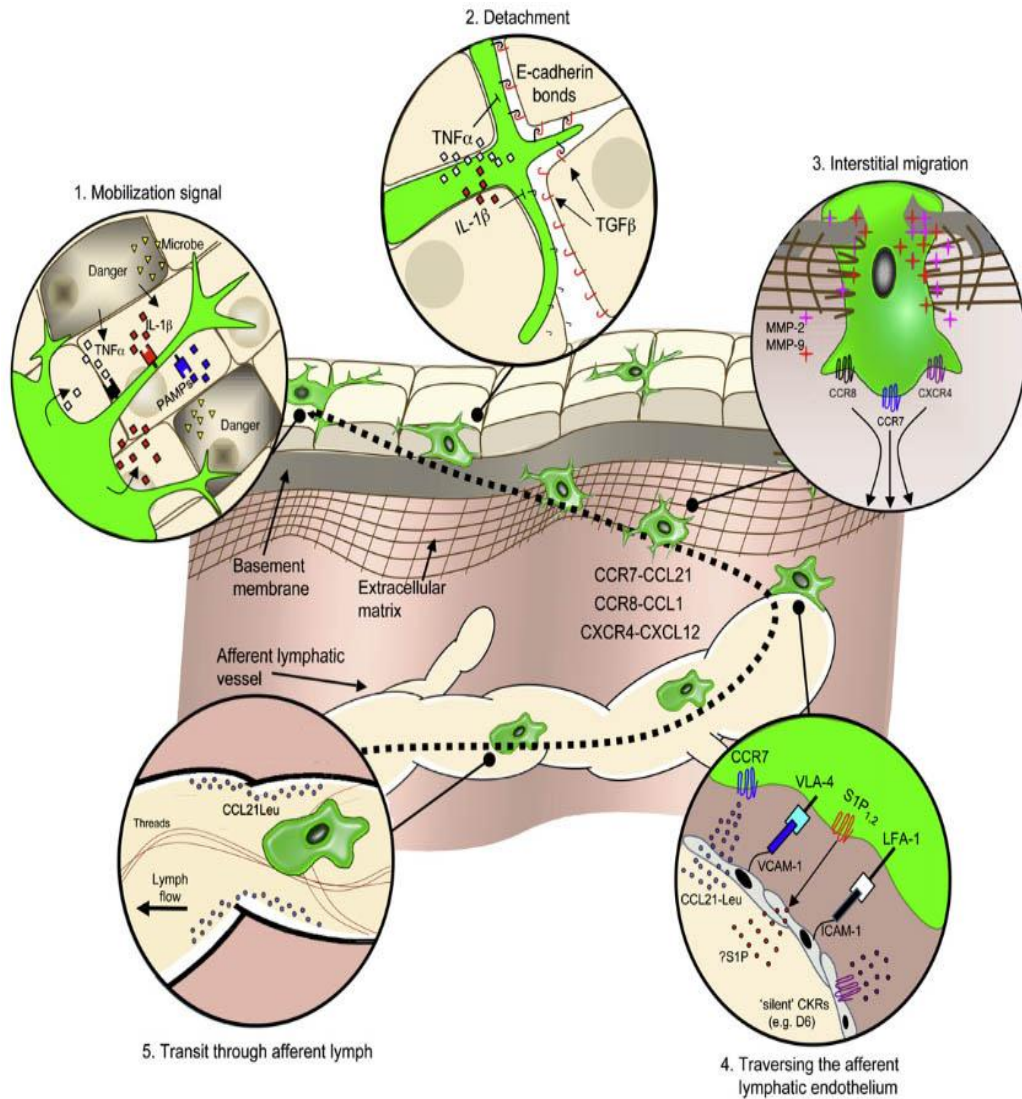


Figure 1-13 DC trafficking in peripheral tissues

This schematic illustrates a proposed model for the interstitial migration of skin DCs from the cutaneous microenvironment to the afferent lymphatics *en route* to the LN. The migratory cascade is divided into five discrete steps (clockwise from top left), starting with recognition of a mobilizing signal (inset 1), detachment from structural tissue elements (inset 2), trafficking through interstitial space (inset 3), transit through the afferent lymphatic endothelium (inset 4), and transit through the afferent lymph vessels (inset 5). Major chemokine-chemokine receptor (CKRs) pathways and other trafficking molecules controlling DC migration are highlighted (Alvarez et al., 2008).

1.11.1 Dendritic cells in the skin

The skin can be divided into two areas: epidermis and dermis. DCs can be found in both as Langerhans cells (LCs) in the epidermis and dermal DCs (DDCs) in the dermis.

Langerhans cells, the first type of DCs to be identified and characterised, are a homogenous population of cells typically found in tight association with the surrounding keratinocytes. They can also be found in the papillary dermis, particularly around blood vessels, as well as in the mucosa of the mouth, foreskin and vagina (Roediger et al., 2008). LCs exhibit stellate morphology with long branches. Dermal DCs are a heterogeneous cell population, both in human and mouse. In contrast to LCs, DDCs display an amoeboid shape and show a stronger ability to migrate (Ng et al., 2008). In terms of function, both LCs and DDCs are capable of capturing and presenting both foreign and self antigens to naïve T cells *in vivo*. However, it is still not clear whether antigen presentation to T cells by the different dendritic cell subsets leads to distinct functional outcomes during the immune response (Ng et al., 2008).

1.11.2 Traffic molecules in DC migration

Circulating DCs and their progenitors can be recruited by attractive signals displayed on vascular walls and exit into specific tissue. The majority of these signals are inflammatory chemokines with rare exceptions, such as the recruitment of progenitors for DC renewal (Elbe et al., 1989). In response to these chemokines, DCs express specific adhesion molecules and chemoattractant receptors that allow them to traffic into target tissues (Sozzani et al., 1997). For instance, relying on chemokine-ligand pathways, such as CCR2-CCL2, CCR5-CCL5 and CCR6-CCL20, immature DCs can access non-lymphoid peripheral tissues and navigate within them (Banchereau et al., 2000). When DCs become mature, they down-regulate their previous level responsiveness to these inflammatory chemokine pathways and up-regulate CCR7, which induces them to traffic to draining lymph nodes (Dieu et al., 1998). During DC rolling and tethering on their journey three members of the selectin family and occasionally $\alpha 4$ integrin mediate the migration (Vestweber and Blanks, 1999).

The combinational use of selectins, chemoattractant receptors, integrins, and their respective ligands results in a great deal of diversity and selectivity in regulating DC migration to different tissue (Springer, 1994).

1.11.3 Lymphatic Vessels

Lymphatic vessels are thin walled, valved structures through which lymph material including soluble protein, antigens, migratory DCs and recruiting T cells travel to lymph nodes (LNs). The Lymphatic system is very important for the maintenance of normal tissue homeostasis, as well as in inflammatory situations (Ryan, 1989). In the skin, afferent lymphatic vessels begin as a plexus of lymphatic capillaries that drain interstitial fluid. These initial absorbing lymphatic vessels ultimately converge in large collecting ducts, such that only a few vessels enter and terminate in the subcapsular sinus of the draining LNs (Roediger et al., 2008).

DCs migration into lymphatic vessels is dependent upon CCR7 mediated chemotaxis. In CCR7-deficient mice it has been shown that CCR7 is essential for DC mobilization to LNs from peripheral tissues (Forster et al., 1999). CCR7 recognizes the ligands CCL19 and CCL21, which together coordinate the traffic of both DCs and T cells to secondary draining LNs under both steady-state and inflammatory conditions (Roediger et al., 2008). However, the function of CCR7 is dependent upon the intracellular calcium concentration, and therefore relies on additional molecules controlling calcium levels. Prostaglandins, particularly PGE₂, have been shown to influence CCR responsiveness by controlling intracellular cAMP, an important intracellular calcium concentration regulator (Scandella et al., 2002). In agreement with this, the ADP-ribosyl cyclase CD38 and the Ca²⁺-activated non-selective channel TRPM4 have been shown to promote DC migration through modulation of intracellular Ca²⁺ influx (Barbet et al., 2008). In addition to CCR7, other cytokines such as IL18 or cytokine receptors like CXCR4 may further facilitate DC migration, particularly in inflammatory conditions (Kabashima et al., 2007).

1.11.4 Mobilization signals

It is firmly established that DCs are mobilised in response to a large variety of pro-inflammatory stimuli, including chemicals (contact sensitizers and irritants), physical ones (UV radiation or trauma), and biological ones (microbial, or tissue necrosis). The

initial exposure to such inflammatory stimuli induces the mobility and antigen uptake of DCs and thus enhances DC maturation. During maturation, DCs start to upregulate their motility, re-arrange their repertoire of chemokine receptors, upregulate their antigen presentation machinery, and eventually migrate to LNs (Granucci et al., 1999). Although DCs respond to a diverse number of signals TNF- α and IL-1 β are the most common intermediate messengers to their mobilization. The importance of IL-1 β and TNF- α in contact hypersensitivity and DC migration has been substantially validated by experiments involving cytokine neutralization, and in mice deficient in the TNF α receptor, the IL-1 receptor, and caspase-1, a protease is required for the release of active IL-1 β (Alvarez et al., 2008). In addition to these findings, Cumberbatch (Cumberbatch et al., 1997a, b) found that subcutaneous administration of either IL-1 β or TNF-a was sufficient to promote DC migration. The mechanisms by which these cytokines promote DC migration probably include altered expression of adhesion molecules and chemokine receptors or the changes of local environment.

1.12 IL-18 and IL-18 receptors

Cytokines are the key mediators of the immune system; the IL-1 family may have been the most thoroughly studied of the cytokines. IL-1 β and IL-18 contribute to host defence against infection by augmenting phagocytosis, promoting DC migration and initiating Th1 and Th17 adaptive immune responses (Li et al., 2004a; Smith, 2011; van de Veerdonk et al., 2011).

IL-18 was found in 1995 to be a strong stimulator of the production of gamma interferon (INF- γ) in Th1 cells (Dinarello, 1999). IL-18 is constitutively expressed as an inactive precursor and can be activated by caspase 1 in response to inflammatory and infection stimuli. In addition, IL-18 is a strong stimulator of the activity of natural killer cells alone or with IL-15. Together with IL-2, IL-18 also stimulates the production of other Th2 cytokines such as IL-13. Now, it is clear that IL-18 is associated with or demonstrated to contribute to numerous inflammatory disorders including infections, autoimmune diseases, rheumatoid arthritis, and metabolic syndrome (Alboni et al., 2010; Bachmann et al., 2007a; St Louis et al., 1999).

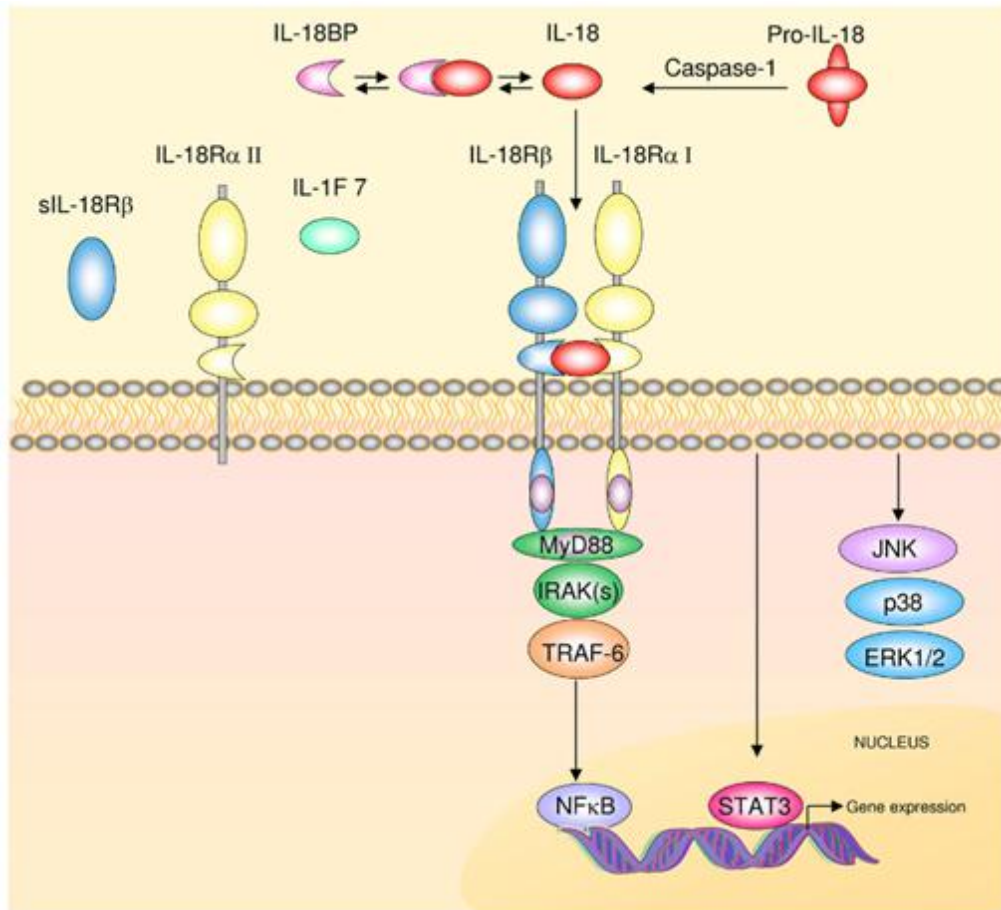


Figure 1-14 IL-18 system

Schematic illustrates the inflammasome components on the activation of IL-18 signaling pathways. Recent evidence suggests that Caspase I is responsible for IL-18 maturation. It also has been demonstrated that IL-18 binds to its receptors thus forming a trimer to initiate downstream cascade, which leads to the activation of a panel adaptor proteins such as MyD88. MyD88, IRAK and TRAF-6 have also been shown to influence the activation of NF-κB and subsequent activation of cytokine transcription (Novick et al., 1999).

1.12.1 Components of IL-18 system

Pro-IL-18 commonly exists as a 24-kDa precursor protein and becomes an 18-kDa secretable mature form after caspase-1 cleavage. Pro-IL-18 can also be processed into its active form by various extracellular enzymes such as protease 3, serine protease, elastase and cathepsin G (Arend et al., 2008). It has been found in an alternatively spliced form during IL-18 gene expression in rat adrenal glands as a shorter IL18 form lacking 57bp/19 aa (Conti et al., 1997).

The IL-18 receptor belongs to the interleukin 1 receptor/Toll like receptor superfamily. There are two subsets: IL-18R α and IL-18R β , both with three extracellular immunoglobulin-like domains and one intracellular Toll/IL-1 receptor (TIR) domain (Smith, 2011). IL-18 is supposed to directly bind IL-18R α and then recruit IL18R β to form a high-affinity heterotrimeric complex, thus inducing downstream signals (Thomassen et al., 1998). IL-18R shares the same adaptor molecules as MyD88, IRAK and TRAF6 and results in similar responses: activation of NF κ B, JNK and P38 MAP kinase as IL1R. Although the IL-18R complex is remarkably similar to IL-1R complex, regulation of IL-18 action is different from IL-1 regulation. It has been claimed that IL18-binding protein (IL-18BP) can bind IL-18 and prevent IL-18 and IL-18R interaction (Novick et al., 1999).

Another negative regulator of IL-18 activity is IL-1F7 a different member of the IL-1 family. IL-1F7 can bind IL-18BP and the IL-1F7/IL-18BP complex is able to interact with IL-18R β , preventing IL-18 signal transduction (Bufler et al., 2002).

1.12.2 IL-18 signaling

Canonical IL-18 acts similarly to IL-1 β *via* the recruitment of adaptor myeloid differentiation factor (MyD88). The sequence of events starts with the activation of the IL-1R-associated kinase (IRAK)/tumour necrosis factor receptor-associated factor 6 (TRAF6) pathway, leading to NF κ B-modulated gene expression (Gracie et al., 2003) (See Figure 1-14). In addition, IL-18 has also been reported to activate the transcription factors tyk-2, STAT3 and NFATc4, and promotes mitogen-activated protein kinases pathways including ERK1/2 and P38 MAP kinase (Alboni et al., 2010). Recently, use of PI3k and NF κ B inhibitors implicated that both were required for IL-18 induction of vascular cell adhesion molecule-1 (VCAM-1) in fibroblasts (Perejaslov et al., 2008).

1.12.3 Functional effects of IL-18

Although the first discovery pertaining to IL-18 was its strong capability of inducing INF- γ production in mice, the effector role of IL-18 is rapidly expanding. IL-18 enhances T and NK cell maturation, cytokine production and cytotoxicity *via* the FasL pathway (Dao et al., 1996). IL-18-deficient mice have shown a reduced cytolytic ability in NK cells which can be rescued by exogenous IL-18 (Takeda et al., 1998). IL-18 has also been found to induce murine T cell Th2 differentiation alone or in combination with IL-4. Thus, IL-18 can promote Th1 and Th2 lineage maturation based on underlying genetic influence and ambient cytokine stimulation (Takeda et al., 1998).

On non-T cell populations, IL-18 has shown direct effects on macrophages and DCs (Li et al., 2004a). IL-18 and IL-12 have been shown to synergistically induce INF- γ production by bone-marrow derived macrophage and splenic DCs (Munder et al., 1998). Studies of knockout mice also revealed that IL-18-induced IL-6 production of macrophages is independent of TNF- α and IL-1 β (Munder et al., 1998). Similarly, IL-18 promotes neutrophil activation, reactive oxygen intermediate synthesis, cytokine release, and degranulation (Leung et al., 2001).

In addition, other non-haemopoietic cell responses to IL-18 are likely with direct effects on chondrocytes and cartilage matrix degradation (Smith, 2011). Keratinocytes have been shown to not only produce but process IL-18 when treated with pro-inflammatory mediators such as LPS (Mee et al., 2000). In addition to keratinocytes, LCs also produce IL-18, which in turn contributes to the regulation of LC migration (Antonopoulos et al., 2008; Cumberbatch et al., 2001; Wang et al., 2002).

Based on the previous investigations, I postulate that electric signals may modulate the response of DC to cytokines and then mediate its maturation/migration. First, EFs generation and DC maturation/migration take place in peripheral organs simultaneously, which provides the spatial and temporal precondition for the interaction. Secondly, it has been proved that cytokines and their receptors decisively mediate the fate, development and function of DCs (Merad et al., 2008; Takashima and Bergstresser, 1996). Thirdly, one mechanism that EFs regulate cells behaviour is through modification of cytokine/ growth factors receptors (McCaig et al., 2005; Zhao et al., 2006).

Above all, I hypothesize that electric signals may regulate DC functions (migration/maturation) in response to cytokines during inflammation or wound healing.

CHAPTER 2 MATERIAL AND METHODS

2.1 Chemicals and reagents

Table 2-1 Chemicals and reagents used in this study

Name	Catalogue No.	Manufacturer
L-glutathione, reduced (GSH)	G6013	Sigma-Aldrich
Tyrphostin AG1296		Sigma-Aldrich
Protease inhibitor cocktail	P2714	Sigma-Aldrich
Phosphatase inhibitor cocktail	P5726	Sigma-Aldrich
Y27632 (Rho-associated protein kinase inhibitor)	688000	Calbiochem
LY294002	440202	Calbiochem
phalloidin, rhodamine-labelled	R415	Invitrogen
TRIZol reagent	15596-026	Invitrogen
Promega Reverse Transcription	A3500	Promega, UK
DMEM:F12+GlutaMAX-1	31331	Invitrogen
dispase II	D4693	Sigma-Aldrich
1M HEPES buffer	15630	Invitrogen
	H0887	Sigma-Aldrich
Human Platelet derived growth factor (PDGF)	PHG0043	Invitrogen
Competent subcloning <i>E.Coli</i>	18265017	Invitrogen
LB ager powder	684295	Sigma-Aldrich
Ampicillin	11593027	Invitrogen
SOB medium,		Invitrogen
SOC medium	15544034	Invitrogen
T4 ligase	15224041	Promega
GoTaq PCR kit	M7650	Promega
DEPC-treated water	D5758	Sigma-Aldrich
Ethanol	E7023	Sigma-Aldrich

Isopropyl alcohol	• I9030	Sigma-Aldrich
25X TAE buffer	AM9870	Invitrogen
Taqman qPCR kit	4391848	Invitrogen

2.2 Cell and tissue culture and gene expression

2.2.1 KG-1 cell culture

KG-1 cell is human myeloid cell line and considered to be progenitor of dendritic cell. In this research, KG-1 cells were taken from liquid nitrogen storage and quickly thawed at 37°C in a water bath. Cells were transferred to 37°C RPMI-1640 medium (Nonza, Germany) containing 10% FBS (Invitrogen) and 100 U/ml penicillin, 100 µg/ml streptomycin and spun down at 1500 rpm for 5 min. Supernatant was aspirated and pellet was re-suspended with ~1 ml RPMI-1640 medium. Cell number was counted using a haemocytometer and $\sim 5 \times 10^5$ cells were inoculated into a T75 culture flask (Greiner, Germany). Medium was changed every other day and cells were split from 1 flask into 2 flasks every 4 days.

In vitro generation of KG-1 cell-derived DCs

KG-1 cell-derived DCs were generated under the conditions essentially as described (Teobald et al., 2008). Briefly, KG-1 cells were stimulated under two different conditions for comparison study. The first condition was Phorbol 12-myristate 13-acetate (PMA) (10 ng/ml) plus ionomycin (500 ng/ml) (sigma-aldrich); the second condition was GM-CSF (50 µg/ml) plus IL-4 (50 µg/ml). KG-1 cells were kept in these two conditional media for 10 days.

2.2.2 Mouse epidermal Langerhans cells isolation and culture

Mouse ear skin digestion

Mouse epidermal Langerhans cells were isolated by using the positive-selection microbead method according to the kit manual (Miltenyi Biotec, UK). Mouse (C57BL/6)

ear skin was cut into ~5 mm² pieces, washed with PBS, and incubated at 4 °C overnight in dispase I (5U/ml) (Roche, Switzerland) in PBS. The next morning the epidermis was removed from underlying dermis with fine forceps and incubated at 37 °C for 15 minutes in trypsin (0.05%)/EDTA (Invitrogen). The single cell suspension was obtained by agitation and aspiration using 1ml pipette. The cells were then washed with RPMI 1640 full medium.

Separation of Langerhans cells using microbeads

The cell suspension was centrifuged at 300×g for 10 minutes and re-suspended in 80 µL of buffer per 10⁷ cells. The cell suspension was then blocked with 10 µL FcR (Fragment crystallisable region receptor) blocking reagent at 4 °C for 10 minutes. 10 µL Langerhans cell microbeads were then added. The mixture was incubated at 4 °C for another 15 minutes, followed by 2 buffer washes. The cells were then re-suspended up to 5 × 10⁷ cells in 500 µL buffer prior to magnetic separation.

A suitable MACS column was washed 2-3 times in buffer to eliminate unspecific binding and then the final cell suspension was passed through. The column was removed from the MACS separator and the Langerhans cells washed out. The detached beads were removed after 2 or 3 times medium change.

Langerhans cell culture

The freshly isolated Langerhans cells were cultured with RPMI 1640 medium with 10% FBS, 50 U/ml penicillin, 50 µg/ml streptomycin and 2 mM L-glutamine. Culture medium was changed every two days and cells were incubated at 37 °C with 5% CO₂ up to a maximum of 7 days.

2.2.3 Coating

A variety of extracellular matrix components have been used for coating in order to induce cell adhesion or migration.

Fibronectin and Laminin

Both fibronectin and laminin were slowly thawed, gently shook, and dissolved in PBS at a concentration of 10 $\mu\text{g/ml}$ and 100 $\mu\text{g/ml}$, respectively. Cell culture plasticware or cover slips were incubated with various amounts of solution (indicated below) at 37 $^{\circ}\text{C}$ for 2 hours, and then washed with PBS 3 times. The coated cell culture plasticware and cover slips were used directly or stored for \sim 1 month at 2-8 $^{\circ}\text{C}$.

Collagen

Collagen I was dissolved in 0.01 M HCl at a concentration of 5 mg/ml and stored at 4 $^{\circ}\text{C}$ until needed. Stock collagen was diluted 1:5 with culture-grade water and the diluted solution was dispensed in the appropriate amount into desired tissue culture plasticware or cover slips. These were left at room temperature overnight in a tissue culture hood. The following day, the collagen solution was aspirated and the dishes washed with PBS 3 times. The dishes were then air dried and stored at 4 $^{\circ}\text{C}$ until needed.

Table 2-2 Collagen coating parameters

24-well	500 µl per well
6-well	2.4 ml per well
10mm petri dish	5 ml
Electric field chamber	500 µl

2.2.4 Skin explant culture

C57BL/6 mice were killed by CO₂ inhalation and the ears cut at the base with scissors. The ears were washed twice with PBS and then with 70% ethanol to sterilise. The ears were then spread out on a petri dish and allowed to dry. 2ml of RPMI 1640 full medium was aliquot into the required number of wells of a 24-well plate. Ears were split into dorsal and ventral halves with forceps under the dissection microscope, and the dorsal halves were floated individually on the medium. The explants were incubated at 37 °C in a 5% CO₂ incubator. In EF-stimulation experiments the explants were transferred to fresh medium after 12 hours.

Experiments were designed to test the effect of either IL-18, EF or both on LC migration from the skin explants. The detailed protocol of IL-18 and EF treatment at various time points were as described below.

2.2.5 Cos7 cell culture

Cos7 cells were maintained with DMEM full medium and incubated at 37 °C in a 5% CO₂ incubator. Cells were split every two days.

2.2.6 Human skin fibroblast culture

Cultures of Chronic Wound Fibroblasts (CWF), patient-matched uninvolved dermal fibroblasts (CMF) and age-matched healthy person dermal fibroblast (HF) were obtained with approval from the Medical Ethical Committee of Cardiff University and Local Research Ethical Committee and after written informed consent from patients with established venous leg ulcers attending the Wound Healing Clinic at the University

Hospital of Wales, Cardiff. Fibroblasts were maintained with Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine (2mM), nonessential amino acids (1 ×), antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin and 0.25 mg/ml amphotericin B) and 10% FBS. The cultures were maintained at 37 °C in a 5% CO₂ atmosphere. At confluence, fibroblasts were split at a ratio of 1:3 and cells were utilized for all experiments between passage 3 to 10 (Cook et al., 2000).

2.2.7 Gene expression and Molecular cloning

RNA isolation by TRIzol method

Total RNA was isolated from CWF, CMF and HF using the TRIzol method according to manufacturer's protocol. In brief the cell monolayer was first rinsed by pre-cooled PBS and then lysed by adding 1 ml TRIzol reagent per 3.5 cm diameter dish. Sequentially, cells were gently scraped out of the dishes and the cell lysates aspirated into an EP tube. The cell homogenates were kept for 5 minutes at room temperature to allow complete dissociation of the nucleoprotein complex.

Following 1.2×10^4 rpm, 15 minute centrifugation at 4 °C the supernatants were transferred into new PE tubes. 0.2 ml chloroform was then added. After around 15 seconds of vigorous vortex mixing, the solutions and then were centrifuged at 1.2×10^4 rpm for 15 minutes at 4 °C. The supernatants were then separated into lower phenol-chloroform phase, interphase, and a colourless upper aqueous phase. The aqueous phase containing nucleic acid was carefully transferred into a new EP tube without disturbing the interphase.

0.5 ml isopropyl alcohol was added to the tube to precipitate the nucleic acid. The mixture was incubated for 10 minutes at room temperature followed by a 1.2×10^4 rpm, 15 minute centrifugation at 4 °C. The RNA precipitate is often visible at this stage.

Following supernatant removal the RNA pellet was washed with 0.5 ml 75% ethanol, which was added into the tube and then the mixture was incubated for another 10 minutes at room temperature. The sample was then spun at 7.5×10^3 rpm for 5 minutes at 4 °C. The above wash process was repeated once more and the remaining ethanol removed.

Finally the RNA was air-dried and re-dissolved in DEPC-treated water. The concentration and purity of the RNA samples were measured by Nano-drop (GE healthcare).

Reverse Transcription

Reverse transcription from RNA to cDNA was performed in a 20 μ l volume using oligo-dT primer, 10 mM dNTP Mix and AMV Reverse Transcriptase (Promega, UK) according to manufacturer's instruction. In brief, the reaction mixture contained 1 μ l oligo-dT primers, 1 μ l dNTP mix, 0.5 μ g RNA, 1 μ l transcriptase, 2 μ l 2X buffer and 14 μ l ddH₂O. The reactions were performed in a thermocycler with the program: 1) 42 $^{\circ}$ C for 60 minutes; 2) 95 $^{\circ}$ C for 5 minutes; 3) 4 $^{\circ}$ C for storage. Following reverse transcription, the sample concentrations were measured, diluted and stored at -80 $^{\circ}$ C for future use.

Classical PCR

The GoTaq PCR system (Promega) was used to perform classical PCR to amplify DNA of interest. The protocol was followed according to the manufacturer's instruction. In brief the reaction consisted of 25 μ l master mix, 5 μ l cDNA or DNA of interest, 1 μ l Taq enzyme, 100 mM primers and ddH₂O to a final volume of 50 μ l. The PCR programs used differed in annealing temperatures due to different products and primers. The outline of the programs is as follows: 95 $^{\circ}$ C for 1 minute for denaturation; 55-65 $^{\circ}$ C 1-2 minutes for annealing; 72 $^{\circ}$ C for 2 minutes for elongation; 30-40 cycles the one PCR reaction. The PCR products were analysed by agarose gel electrophoresis.

Taqman qPCR

For quantification of PDGFs and their receptor expression in fibroblasts, TaqMan qPCR was used according to manufacturer's instructions. The reaction was carried out in a 20 μ l volume in 96 well plates. Each reaction contained 10 μ l of Master Mix, 0.25 μ g of fluorescence probes (Table 2-3), and 8 μ l of diluted cDNA. The reactions were performed in a Roche Lightcycler 480 system. The number of PCR cycles needed to reach the

fluorescence threshold (Ct) was determined in duplicate for each cDNA, averaged and then normalized against the reference gene GAPDH.

Table 2-3 Primers and Probes used in Taqman qPCR

Primers and Probes		sequence(5'-3')	Probe
PDGF-A	forward	acacgagcagtgcaagtgc	#77
	reverse	attccacctggccacct	
PDGF-B	forward	tgatctccaacgcctgct	#55
	reverse	tcatgttcaggccaactcg	
PDGFR-a	forward	agtggtgaccttcaatgg	#80
	reverse	ttgatttctccagcattgtg	
PDGFR-b	forward	cccttatcatcctcatcatgc	#29
	reverse	ccttccatcggatctcgtaa	

2.2.8 Gene cloning

PCR and subcloning were performed using standard protocols. Plasmid expressing IL18R α -GFP was constructed by amplifying the coding sequence of [plasmid] and cloning into a pcDNA3.1-based vector. GFP was cut from and inserted immediately downstream of the IL18R α coding sequence in the vector. Briefly, human IL18R α and GFP were amplified by PCR from templates respectively. Appropriate restriction enzyme sites were also introduced into the PCR products *via* the amplification primers.

The PCR products and backbone vectors were purified after running on an agarose gel. The purified PCR products and vector backbones were cut by the appropriate restriction enzymes in order that the PCR products could be inserted into the vector. The detailed protocol of restriction enzyme digestion, gel purification and ligation is shown below. In this study, GFP was first inserted into pcDNA3.1 utilizing *Bam*HI and *Hind*III sites; IL18R α was inserted into pcDNA3.1-GFP afterwards by using *Eco*R I and *Bgl* II sites.

Restriction enzyme digestion

A reaction volume of 10 μl was used for the digestion containing 1 μl buffer, 0.5-1.0 μg DNA, 1 μl enzyme and ddH_2O to 10 μl . The reaction was performed for 1 hour in a 37 $^\circ\text{C}$ water bath. The products were analysed and separated on a 0.8% agarose gel.

DNA gel extraction

Gel purification was used to ensure only DNA of the correct size was collected and to eliminate salts and enzymes from the digestion reaction. In this study Qiagen gel extraction kits were used and the protocol was according to the manufacturer's instructions. Briefly, target bands were cut under UV light and weighed. Sequentially, three gel volumes of QG buffer were added to the slices and heated at 50 $^\circ\text{C}$ for 10 minutes to melt the gel. Once gel dissolved, one gel volume of isopropanol was added into the tube. This solution was then aspirated and transferred into a QIAquick column which was spun 1.3×10^4 (g) for 1 minute. The column was spun once more after adding 500 μl QG buffer. Following centrifugation, the tube was then washed using 750 μl PE wash buffer and spun at 1.3×10^4 g for 1 minute. After that the DNA was eluted by ddH_2O and stored at -20. $^\circ\text{C}$.

Ligation

Ligation was used to insert the restriction-digested gene product into vector. In this study the T4 ligation system from Promega was used and the protocol was according to the manufacturer's description. A 1:3 ratio of insert DNA:vector with about 100 ng of vector was used in the ligation; a negative control reaction contained only the vector fragment. With the vector and insert the ligation system also included 0.5 μg ligase, 1 μl buffer and 1 μl ATP. The reaction tubes were incubated at 16 $^\circ\text{C}$ overnight. On the following day, the sample mixtures were used for transformation.

Transformation into *E.coli*

Transformation into *E.coli* was used in order to maintain stocks of the ligated plasmid. In this study, the subcloning competent DH5 α *E. coli* strain from Invitrogen was used; protocol was according to manufacturer's instruction. Firstly, about 1-5 μ l ligated plasmid solution were added to 50 μ l thawed DH5 α *E.coli* and swirled gently. The mixture was kept on ice for 15 minutes, followed by a 30 second 42 $^{\circ}$ C heat shock incubation. The mixture was then kept on ice for 5 minutes. Finally, after centrifugation, 50 μ l pellets were spread onto the surface of LB-Ampicillin plates. Colonies were picked next day and used to inoculate 10 ml cultures in order to harvest vector.

Plasmid mini-prep

The plasmids were extracted from *E. coli* for further experiments e.g. transfection. In this study, the plasmids were isolated by using a Qiagen mini-prep kit; the protocol followed according to the manufacturer's instructions. Briefly, the overnight cultured bacteria were spun and the pellet was re-suspended in 250 μ l P1 buffer. 250 μ l P2 buffer was added and mixed thoroughly. The mixture was then centrifuged for 10 minutes at 13,000 rpm. The supernatant was aspirated into a Qiaprep spin column and spun for 1 minute at 7500 rpm. The flow-through was discarded and the column was washed by adding 0.75 ml Buffer PE and centrifuged for 1 minute at 7500 rpm. Finally, the plasmid was eluted by adding 50 μ l EB buffer and stored at -20 $^{\circ}$ C for further experiments.

2.2.9 Gene Transfection

Transfection with Lipofectamine 2000

Transfection of various types of cells was carried out with Lipofectamine 2000 (Invitrogen, UK) according to the manufacturer's instructions. Essentially, cells were seeded at 1×10^5 per well in 24 well plates 24 hours before the transfection. Cells were then transfected using 1 μ g of vector and 2 μ l Lipofectamine 2000 reagent per well.

Electroporation

Several cell types in the experiments are difficult to transfect using Lipofectamine 2000; for these cells, transfection was carried out using an electroporator with appropriate settings. For 3T3 cells the setting is 500 V at low Voltage mode, capacitance 1600 μ F and the chamber gap is 4 mm; for dendritic cells, the conditions are 400 V at low Voltage mode, capacitance at 1600 μ F and chamber gap of 4mm. Immediately after electroporation, cells were incubated on ice for 5 minutes before transferring into full medium and culturing at incubator.

2.2.10 Immunocyto staining protocol

Immunocyto staining was used to visualize cellular protein expression and distribution. Briefly, cells were fixed with 4% paraformaldehyde in PBS at room temperature for 15 minutes, followed by rinsing in PBS 3 times. The concentration of antibody used was according to the description of the manufacturer. Essentially, the cultures were incubated with primary antibody at 4 °C overnight at appropriate concentration in PBS containing 5% BSA. On the following day, cultures were further incubated with fluorescence-conjugated second antibody for 2 hours at room temperature. Finally, the slices were mounted with Vectashield mounting medium (Vector Laboratories, USA) and observed under a fluorescence microscope.

2.2.11 Western blot assay

Buffers used in Western blot

TBST buffer

Tris-HCl buffered saline/Tween-20 (TBS/T) is the basic buffer used to wash western-blot membranes. TBS/T contains 50 mM Tris-HCl (pH 8.5), 150 mM NaCl, and 0.1% Tween-20.

Peroxidase-conjugated antibody was diluted in TBS/T containing 5% skimmed milk; this is also used to block the western-blot membranes after protein transfer.

SDS-PAGE buffer

SDS-PAGE buffer was composed of 250 mM glycine, 25 mM Tris base and 0.1% SDS. It was made as a 10 x stock and diluted by Milli-Q water before use.

Western-blot transfer buffer

Towbin buffer was used in this study as a western-blot transfer buffer. It contains 25mM Tris base, 192 mM glycine and 20% (v/v) methanol.

Western-blot stripping buffer

After probing, western-blot membranes can be stripped in a 3 hour incubation at room temperature and re-probed with other antibodies up to 2-3 times. The stripping buffer used in this study is: 200 mM glycine (pH 2.4), 0.1% Tween-20 and 100 mM β -Me (from <http://www.koko.gov.my/CocoaBioTech/Membrane%20Transfer9.html>).

Western blot protocol

Sample preparation

Freshly trypsinized cells or digested tissue were rinsed twice with cold PBS. The pellets were then re-suspended with 100 μ L RIPA buffer (Sigma, UK) and kept on ice for 5 minutes. The suspension was centrifuged at 14,000 \times g for 10 minutes at 4 $^{\circ}$ C to pellet cell debris. The supernatant was collected and 5 \times SDS-PAGE loading buffer added, followed by vortex mixing for 1 minute and heating at 95 $^{\circ}$ C for 5 minutes.

Running samples on a gel

For all analysis NuPAGE Novex 4-12% Bis-Tris gels (Invitrogen, UK) were used. Gels were cast using a Bio-Rad Mini protean II gel cassette. Gels were run at 200V for 60 minutes until the dye front came to the leading edge of the gel. Pre-stained protein marker (P7708; New England Biolabs) was also loaded to show the molecular weight of proteins.

Transfer membrane

Transfer of the gel contents onto a PVDF membrane is required prior to probing with antibody. First, the membrane is pre-wetted in transfer buffer. The transfer components are then assembled in the following order: case lid (front), sponge, waterman paper, membrane (to transfer samples onto), gel (containing samples), waterman paper, sponge,

case lid (back). The membrane transfer apparatus were placed into a Bio-Rad mini-gel box electrotransferer at 90 V for one hour.

Staining and detection

After transfer, the membrane was blocked in 5% skimmed milk in TBS/T for 1 hour at room temperature. The membrane was then washed vigorously for 5 minutes in TBS/T 3 times. Subsequently, the membrane was transferred to a 50 ml tube with 2-10 ml primary antibody solution in 2% BSA, TBS/T and put in a rotating mixer overnight at 4 °C. The membrane was then taken out of the tube and washed in TBS/T for 5 minutes 3 times. Primary antibody solution could be frozen at -18 °C and reused twice more. The membrane was then incubated in 10-20 ml peroxidase-conjugated secondary antibody solution in 5% skimmed milk, TBS/T for 2-3 hours at room temperature. Secondary antibodies were diluted in a ratio between 1:3000 and 1:8000.

After secondary antibody incubation, the membrane was washed in TBS/T for 5 minutes 3 times. It was then gently picked up using a pair of forceps and blotted onto a piece of tissue to absorb the excessive moisture. 2 ml developing solution (GE healthcare, UK) was applied on the membrane for 1 minute. The membrane was again gently blotted onto a piece of tissue and placed into a cassette. Horseradish peroxidase catalyses the developing reaction, which results in light generation. Light was absorbed on photographic film and developed in a Kodak X-OMAT 1000 processor machine.

Stripping and re-blot

If the sample needs to be re-probed, the membrane can be stripped and re-blotted. The membrane was incubated with stripping buffer for 20 minutes at room temperature on a shaking mixer and washed with TBST for 30 minutes. The membrane could then be blocked and re-blotted.

2.2.12 ELISA protocol

The levels of INF- γ in culture supernatants were measured by ELISA, using paired antibodies from e-Bioscience (e-Bioscience Biotechnology Ltd). The INF- γ levels (pg/ml)

were determined according to the standard curve. Briefly, anti- INF- γ monoclonal antibody was diluted to 10 $\mu\text{g}/\text{ml}$ in the coating buffer (0.1M NaHCO₃, pH 8.2) and 50 $\mu\text{L}/\text{well}$ applied to enhanced protein binding ELISA plates (Dynatech Immunlon) overnight at 4 $^{\circ}\text{C}$. The plates were washed twice with washing buffer (PBS/Tween 20 (0.05%)), and blocked with 100 $\mu\text{L}/\text{well}$ of 10% FCS in PBS for 1 hour at 37 $^{\circ}\text{C}$, then washed twice more with washing buffer. 50 μL of the supernatant from the cell culture and diluted cytokine were added to the plate and incubated at 37 $^{\circ}\text{C}$ for 3 hours. After washing four times with washing buffer, 50 μL of biotinylated anti-INF- γ detecting antibody (1 $\mu\text{g}/\text{ml}$) was added and incubated for 1-2 hours at 37 $^{\circ}\text{C}$. The plates were washed six times with washing buffer and 100 μL extravidin-peroxidase (2 $\mu\text{g}/\text{ml}$) added, followed by incubation for 1 hour at 37 $^{\circ}\text{C}$. The plates were washed six times and the colour developed by adding 100 $\mu\text{L}/\text{well}$ of TMB substrate for 10-30 minutes. The OD was read at 630 nm with a reference filter at 490 nm.

Calculation of results

Absorbance values for each set of triplicate standards and samples were averaged. (Note: triplicates should be within 20 percent of the mean value.) First, a standard curve was created by plotting the mean absorbance value for each standard concentration on the ordinate against the human IFN- γ concentration on the abscissa. A 4-parameter or 5-parameter curve fit was used to generate a best-fit curve through the points of the graph. To determine the concentration of samples, the absorbance value was firstly read on the ordinate and the corresponding value on the abscissa taken as the concentration.

2.2.13 EF stimulation

Transwell electrical stimulation

The transwell electric stimulation experiment set up was modified from a previous study (Lin et al., 2008). KG-1 cells were pre-treated with 20 ng/ml TNF- α one day before the experiment. Sequentially, 200 μl cell suspension (about 1×10^6 cells) was seeded into each top inner-well of the transwell plates (3 μm pore), (Greiner, Germany) and 500 μl

RPMI 1640 medium was added into the bottom wells. 10 ng/ml IL-18 was also added into each inner well. Various electric field strengths from 10V-30V were applied to the transwell plates by two electrically conductive agarose bridges (according to Figure 2-1), which were connected to a DC power supply.

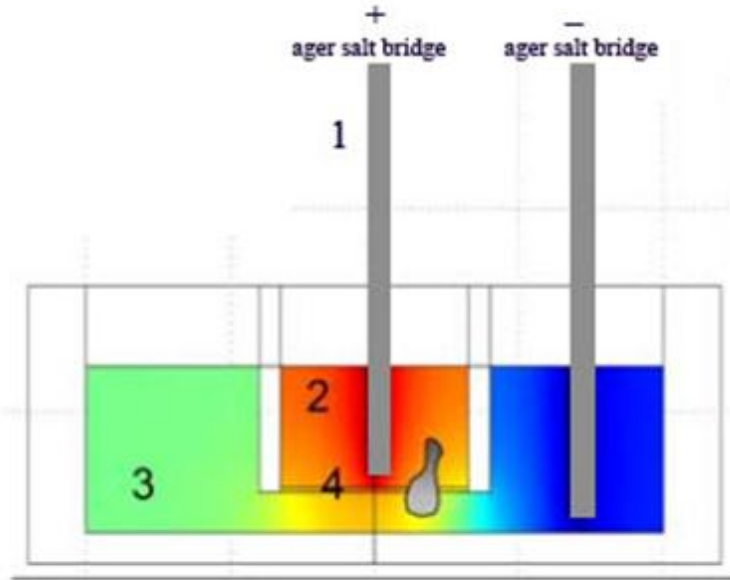


Figure 2-1 Transwell EF Stimulation Assay

Schematic illustrates the setup for a Transwell-based EF assay: 1. agar salt bridge electrode for carrying electric current; 2. top well; 3. bottom well; 4. membrane. The electric fields were applied across the Transwell (10 V-30V) by placing two agar salt bridges- which were connected to the DC power supply to the top well and bottom wells. Cells were allowed to migrate through the membrane from the top well to the bottom well in response to EF stimulation.

Electric stimulation lasted 4 hours and the voltage was checked every half hour to control current stability. For Western blot experiment, cells were harvested immediately after stimulation and washed in pre-cooled PBS twice and frozen down at -80 °C for further analysis. For ELISA experiments, cells were kept in culture overnight and then the supernatant collected.

EF stimulation of 2D culture

The experimental setup was based on previous work (Song et al., 2007a). The EF is applied to cells cultured in a custom-designed chamber via saline agar bridges connected to a DC power supply (Figure 2-). Essentially, glass cover slips were cut and glued by silicon to surround the electric chamber confining the cells. A 22×22 mm² no.1 glass cover slip was cut into two halves using a diamond pen. These two glass strips were glued at the bottom of a petri dish to form a 22×10 mm² square which was for cell seeding. After cell inoculation another 22×22 mm² glass cover slip was used to cover the cell-seeding area. Thus a sandwich-like EF chamber was formed with the petri dish at the bottom, cells in the middle and the cover slip on top. The depth and area of the chamber can be modified to suit different experimental requirements, i.e. for protein or gene analysis the cover glass can be upgraded to No.2 and culture area can be expanded to 50×40 mm². The cells inside the chamber can be maintained as a conventional cell culture in a 37 °C, 5% CO₂ incubator.

Application of EF to 3D culture

The processes of a normal cell from division and migration to proliferation are an accurately controlled series of events which inherently rely on spatial and temporal environmental factors. This makes the application of EFs to 3D cultures technically demanding. In this study the widely applied Matrigel method was used to make 3D environment for cell culture.

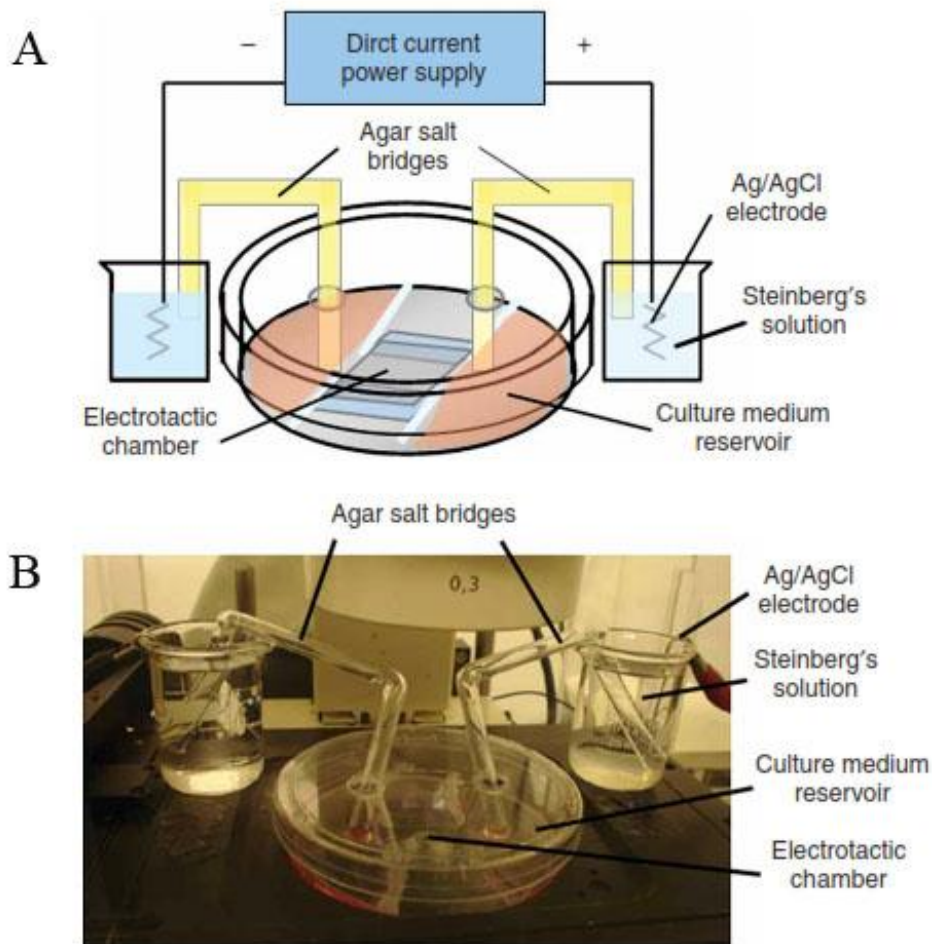


Figure 2-2 Electric field stimulation setup on the microscope stage

(A) Schematic drawing of the electric field (EF) setup. (B) A photograph of the actual setup of the EF application. Electric current from the DC power supply goes through the chamber via Ag/AgCl electrodes, Steinberg's solution and saline agar bridges.

The differences compared to EF treatment of a 2D culture include the resuspension of cells with Matrigel instead of medium just before inoculating the cells into the chamber. The gel should be covered with a glass cover slip before it sets solid, and the chamber should be kept moist to avoid gel shrinkage.

Another 3D culture method used in this study was mouse ear epidermis explant. In comparison with the 2D EF treatment setting, the epidermis tissues were put into the middle of the chamber instead of cells in culture. However there are two concerns about

this setup. The first is the dimension of the tissue as big and thick tissue would generate huge amount heat when applying an EF due to the Joule effect, which will inevitably damage the tissue and cell viability. Therefore in this study the mouse ear epidermis maximum area was $10 \times 10 \text{ mm}^2$. The second issue is the tissue should be kept relatively thin and flat to avoid “short-cut” effect, because current tends to flow through the path with the least resistance.

Electric field application

The electric field apparatus was set up as in Figure 2-2. Basically, a pair of agar bridges (2% agarose in Steinberg’s solution) connecting the media reservoirs in the chamber with Steinberg’s solution pools carried the current through the chamber; this was done to prevent possible damage to the cells due to the ion exchange between electrode and medium should they be directly connected. The pure silver electrodes were immersed in the Steinberg’s solution pools, which were connected to the power supply. Culture medium was changed every hour to avoid evaporation, as this would break the electrical connection. The electric field strength was verified at hourly intervals during the experiment to maintain steady stimulation. Data were pooled from at least two separate experiments and images were captured using a Delta Vision system.

2.2.14 *In vitro* wounding studies

Wound repopulation ability can be evaluated by using a monolayer scratch wound model. Cells were inoculated into an Electric Field chamber and cultured to confluence. The artificial wound was made by scratching along the surface of the tissue culture plastic with a standard 200 μL pipette tip. The cellular monolayer was washed once in phosphate-buffered saline, and re-fed with various media according the different experiment conditions. Chambers were incubated under a Zeiss Axiovert microscope with heating system. Images were collected every 5 or 10 minutes for 6 hours using Metamorph software, post-image analysis was carried out by using Image J.

Cell behaviour analyses

Cell tracking and behaviour quantification mainly followed the previous description (Zhao et al., 1999). Cell migration efficiency is evaluated and is calculated by the formula:

$$\text{Efficiency of cell migration} = \sum S_i \times (-)(\cos\theta_i) / T$$

S is the migration speed, θ is the angle between EF vector and cell translocation, i is the number of the cells traced and T is the total recording time.

2.2.15 PDGF and inhibitors treatment

In PDGF alone and PDGF combined with EF experiments, cells were washed and incubated with variable concentrations of PDGF 10 min before recording. In inhibitor treatment experiments, cells were treated with 10 ng/ml PDGF together with either 10 μ M Tyrphostin AG1296, 10 μ M Y27632 or 20 μ M LY294002 for 30 min before EF exposure and time-lapse recording.

2.2.16 Confocal imaging

The immunocytostaining slices were captured with a Leica SP5 confocal microscope with an Argon laser and analysed using Image J software. The fluorescence intensities across the cells were measured using a Line profile plugin. The colocalization of two fluorescence pixels were measured by the Intensity Correlation Analysis plugin.

2.2.17 Cell migration data analysis

Image stacks were collected by the DeltaVison system (API, USA) and analysed using Image J software. The analysis is described in detail in previous work (Zhao et al., 1999; Zhao et al., 1997). The displacement migration rate and direction were calculated by comparing the position at time= 0 and the final cell position. The trajectory migration rate was calculated by accumulating all the distance between images and divided by total time.

Directedness

A line was drawn between a fixed point and the centre of the cells of the last time point. The cosine of the angle between this line and the electric field vector (normally the EF vector is horizontal in the time lapse films) was used to calculate directionality. If the cosine of this angle equals 1 (angle is 0 degree) this indicates that the cells moved directly along the electric field vector towards the cathode and conversely if the cosine is -1 this indicates that the cells moved in the contrary direction. A value of 0 indicates random movement with respect to the cathode and anode. The cosine values were averaged to give an average directionality for the cell population.

Migration rate

There are two methods to calculate cell migration rate in this study: trajectory speed and displacement speed. The trajectory speed indicates the motility of the cells by accumulating all tracks of the cell migration. The result was calculated by accumulating all the distances travelled at each time point, which were divided by time. The displacement speed was measured using the same fixed coordinate method as the directionality. The distances and angles between the fixed point and the cells at each time point were used to calculate displacement speed. The displacement was calculated from the equation

$$C = a\sqrt{(a^2 + b^2 - 2ab\cos\theta)}$$

The displacement was then divided by the time interval between the whole image series.

2.2.18 Isolation and enrichment of lymph node dendritic cells

Dendritic cells were isolated as previously described by (Price et al., 1997) from auricular lymph nodes which drain the ear. In every animal group 5 mice were killed by CO₂ inhalation about 18 hours following intradermal injection or FITC painting. Lymph nodes were excised and pooled in pre-cooled PBS. Lymph node cell suspensions were prepared by mechanical disaggregation through a 70 µm cell strainer. Cells were washed with 10 ml RPMI-1640 full medium, centrifuged for 5 min at 300g and then re-suspended

in RPMI full medium. Viable cells were counted by exclusion of 0.5% trypan blue and the re-suspended at 5×10^5 /ml full RPMI medium. The DCs were enriched by density gradient centrifugation. 2 ml HistoDenz (Sigmaaldrich; 14.5% in full RPMI medium) was layered under 8 ml of DC suspension and centrifuged at 600g for 15 min. Interface cells (the low buoyant density fraction) were collected, washed once and re-suspended in full medium. The number of cells in the resulting cell suspension was counted using a haemocytometer and analyzed by flow cytometry.

2.2.19 FACS analysis

Cells were identified and analyzed for the presence of various cell markers by flow cytometry. Approximately 1×10^5 cells was re-suspended in FACS buffer and incubated on ice with fluorescence-conjugated primary antibody at an optimal concentration (10 μ g/ml) for about 30 min. Antibodies used in the experiment are listed in the table below. Cells were washed and centrifuged (300g, 5min) three times with 2 ml FACS buffer before analysis using the BD FACS calibur flow cytometer. Data were analyzed using Flowjo software. The markers used to identify dendritic cells are listed below in Table 2-4.

Table 2-4 Markers used to identify dendritic cells

Name	Catalogue no.	Manufacturer
Anti-mouse CD11C-FITC	11-0114	ebioscience
Anti-mouse MHC class II PE	12-5322	ebioscience
Anti-mouse Langerin PE	12-2075	ebioscience
Armenian Hamster IgG Isotype Control	11-4888	ebioscience
FITC		
Rat IgG2b K Isotype Control PE	53-4031	ebioscience
Rat IgG2a K Isotype Control PE	53-4321	ebioscience
Anti-mouse IL-18 R alpha Allophycocyanin	FAB1216A	R&D
MAb		

2.2.20 Statistical analysis

Data were assessed by ANOVA, followed by Tukey-Kramer multiple comparisons tests or the Kruskal-Wallis nonparametric test. This in turn was followed either by Dunn's multiple comparisons tests or by unpaired Student's t-test as appropriate. *P* value < 0.05 was regarded as significant; $p < 0.01$, very significant.

**CHAPTER 3 ELECTRIC FIELDS AND IL-18 MODULATE THE
DENDRITIC CELLS ACTIVITY *IN VITRO***

3.1 Introduction

This chapter explores the molecular mechanisms involved when an electric field applied across various types of dendritic cells, which resulted in an enhanced response to IL-18. The cytokine secretion and migration of dendritic cell were analyzed; and the intrinsic machinery i.e. the interactions between EF, IL-18 and IL18 receptors were investigated.

3.1.1 Dendritic cells

Dendritic cells are important antigen presenting cells which are central to the induction and regulation of most adaptive immune responses. The capacity for acquiring, processing and presenting peptides on MHC molecules are critical for their roles in antigen presentation. Unlike other antigen presenting cells such as macrophages and B cells, DCs are characterized as homing to the lymphoid organs for interaction with T lymphocytes. Thus the migratory capacity is an intrinsic feature distinguishing them from other antigen presenting cells (APCs). Many DCs encounter antigens at peripheral organs and become activated, thus migrate, with antigen in tow, into downstream lymph nodes. The pathways and mechanisms that govern how DCs migrate to lymph node, therefore, figure importantly in immune response and pathogen recognition.

3.1.2 Brief overview of DC research

Even though the skin dendritic cells were first described in 1868, the systematic and comprehensive studies were initiated in 1973 by Steinman and Cohn, when they identified mouse spleen DCs (Steinman and Cohn, 1973). Their serial work first established the concept that DC is a potent stimulator of primary immune response (Steinman et al., 1975; Steinman and Cohn, 1974; Steinman et al., 1979; Steinman et al., 1974).The consequent observations indicated they are widely distributed not only in lymphoid but also in non-lymphoid tissues (Hart and Fabre, 1981a, c; Hart et al., 1981). The later discoveries that they played an important role in heart and kidney

transplantation rejection generated further and extensive interests in DC research field (Hart and Fabre, 1981b, 1982; McKenzie et al., 1984).

3.1.3 DC isolation and identification

Due to the paucity of the DC population and the lack of markers, it was difficult to identify their residence and distinguish them from monocytes and macrophages. Now it is clear that DCs are widely distributed in most of the organs. Except those residing in lymphoid organs such as spleen and lymphoid nodes, DCs exert sentinel function mostly at peripheral nonlymphoid organs.

Epidermis Langerhans cells are the best characterized nonlymphoid DCs. Because it is feasible to enrich Langerhans cell population by eliminating keratinocytes, the enriched Langerhans cell suffice in most functional studies (Steinman, 1991). Several transplantation experiments confirmed there were a small group of DCs in heart (Codner et al., 1990; Fu et al., 1997; Hikawa et al., 2007). By depletion of macrophages, several other studies also revealed DCs' residence in lung (Benson et al., 2012; Lai et al., 2012), liver (Bamboate et al., 2009; Yu et al., 2012), and gut as well (Howe et al., 2009; Varol et al., 2009).

There are two distinct pathways of DC development which have been identified in mice – myeloid and lymphoid. *In vitro* evidence has shown that myeloid-committed precursors give rise to both granulocytes/monocytes and myeloid DCs under the influence of GM-CSF (Inaba et al., 1992; Scheicher et al., 1992). On the contrary, the development of lymphoid lineage DC is GM-CSF independent (Saunders et al., 1996).

Myeloid and lymphoid DCs differ in phenotype, localization and function (Banchereau et al., 2000). Both subsets have high expression levels of CD11c, MHC class II, and costimulatory molecules CD86 and CD40. To date, the most reliable marker to distinguish these two subset is CD8 α , which is expressed on the surface of lymphoid DC but is absent from the myeloid subset (Banchereau et al., 2000).

Similarly there are two main subsets of DC precursors circulating in the human blood: CD14⁺/CD11c⁺ monocytes and CD11c⁻IL3R α ⁺ precursor DCs (Figure 3-1). Those two subsets both are differentiated from CD34⁺ progenitor cells. The CD14⁺, CD11c⁺

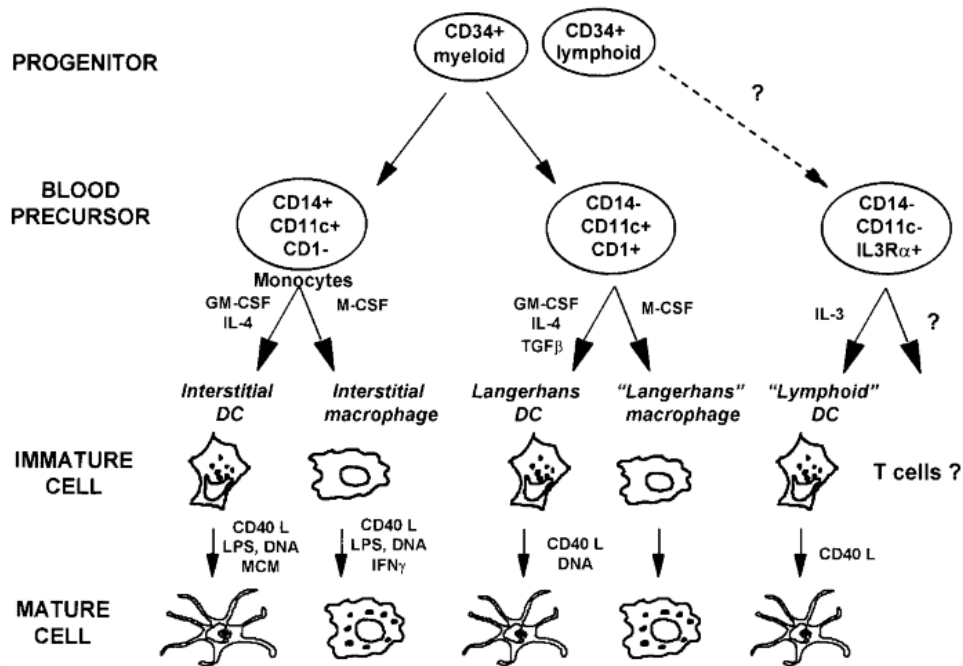


Figure 3-1 The subsets of dendritic cells

The schematic illustrates the hierarchical differentiation of CD34⁺ progenitor cells. The myeloid CD34⁺ progenitors differentiate monocytes (CD14⁺ CD11c⁺) that yield the immature DCs in response to GM-CSF and IL-4. Myeloid progenitors also can differentiate into CD11c⁺ CD14⁻ precursors, which gives rise to Langerhans cells when GM-CSF, IL-4 and TGF β exist. The CD14⁻ CD11c⁻ IL3R α ⁺ DC precursor may originate from lymphoid CD34⁺ progenitors and differentiate into lymphoid DC in response to IL-3 (Banchereau et al., 2000).

monocytes give rise to immature DCs under the influence of GM-CSF and IL-4 or TNF. Furthermore, those CD11c⁺ DCs can differentiate into LCs with TGF- β stimulation (Ito et al., 1999; Thomas and Lipsky, 1994). However, the CD11c⁻ IL3R α ⁺ precursors display many phenotypic differences expressing high level of CD13, CD14, CD33 and their differentiation pathway is not clear (Caux et al., 1996).

3.1.4 KG-1 cells as DC progenitors

Due to difficulties in the isolation and maintenance of homogenous human DC population with sensitivity to manipulation, several cell lines have been established to facilitate DC research. KG-1 is a cytokine-responsive, CD34⁺ myelomonocytic cell line derived from a patient with erythroleukemia undergoing myeloblastic relapse (Koeffler

and Golde, 1978). Following the discovery of the KG-1 cells, Koeffler discovered that with phorbol diesters treatment, KG-1 cells become adherent and develop pseudopodia with macrophage characteristics (Koeffler et al., 1981). Further study suggested that PKC activation by phorbol diesters is partially involved in KG-1 maturation process (Kiss et al., 1987).

Recently, KG-1 has been shown to have similar multipotent properties to Peripheral Blood Mononuclear Cells (PBMCs), possessing the capacity to differentiate into DC-like cells (DLCs) upon various treatments (Ackerman and Cresswell, 2003; Bharadwaj et al., 2005; Teobald et al., 2008). St Louis first reported the signaling pathway of KG-1 cells differentiating to DCs (St Louis et al., 1999). They found KG-1 matured into DLCs in response to GM-CSF plus TNF- α or PMA, which expressed MHC I⁺/MHC II⁺/CD83⁺/CD86⁺/CD14⁻ surface markers. Functional analysis also showed KG-1-derived DLCs could uptake fluid phase macromolecule and active resting T cells (St Louis et al., 1999).

Maturation of primary DC is characterized, except phenotypic extension of dendrites, by up-regulation of MHC and costimulatory and altered trafficking of MHC class II (Steinman, 1991). Ackerman and Cresswell further confirmed that during KG-1 maturation into DLCs, MHC I and MHC II molecules were relocated from Golgi apparatus to cell surface (Ackerman and Cresswell, 2003). The p38 MAP kinase activation was also found to be involved in KG-1 differentiation and antigen uptake and presentation (Bharadwaj et al., 2005).

3.1.5 KG-1 and IL-18

IL-18 was previously known as IFN- γ inducing factor, because it was originally identified by its strong capacity of IFN- γ induction in mice with endotoxin shock (Okamura et al., 1995). The immune response function of IL-18 was later determined by using KG-1 as a PMBC model *in vitro*. The results indicated that KG-1 was very sensitive to human IL-18 and responded to as low as 0.1 ng/ml which is as 100 times low as to mouse IL-18 (Konishi et al., 1997). Moreover, by using DMA/protein binding assay, NF- κ B was found to be the critical molecule transcriptionally mediating IL-18

induced IFN- γ expression in KG-1(Kojima et al., 1999). Another transcription factor T-box (T-bet) also can be upregulated in KG-1 with 4 hours of IL-18 stimulation. Inhibition of p38 MAPK suppresses this upregulation, indicating p38 MAPK is the upstream activator of IFN- γ expression (Bachmann et al., 2007b). This was further confirmed by a recent study by Koutoulaki, which demonstrated that: 1). IL-18 activated p38 MAPK through binding with IL18R α , and 2). this consequently recruited IL18R β therefore activated downstream signalling pathway (Koutoulaki et al., 2010a).

In addition, it was reported that IL-18 selectively upregulated intercellular adhesion molecule-1 (ICAM-1) expression in KG-1 cells, through an IFN- γ independent pathway (Kohka et al., 1998). This indicates that IL-18 might play potent roles in immunoregulation by mediating immune cells infiltration into the tissue.

3.1.6 In vitro culture of Langerhans cell

Epidermal Langerhans cells (LCs) are the most important antigen presenting cells of the skin immune system (Steinman, 1991). Due to the population scarcity and lack of appropriate isolation method, it was difficult to culture LCs until Schuler and Steinman reported the characteristics of freshly isolated LCs in 1985 (Schuler and Steinman, 1985b). The *in vitro* cultured LCs showed resemblance to spleen DCs in morphology and membrane markers, but displayed weak capacity to stimulate T cells which indicated LCs were immature (Schuler and Steinman, 1985b). The sequential studies disclosed that GM-CSF and IL-1 are essential for the maturation and function of LCs (Heufler et al., 1988; Witmer-Pack et al., 1987). During the maintenance of *in vitro* culture, the phenotype and function of LCs change significantly after 72 hours. The most striking changes have been found are 4 times increase of MHC class II molecules (Witmer-Pack et al., 1988). As a result, the LCs upregulate accessory cell functions for primary immune response.

3.1.7 Cytokines mediate LC migration

The migration capacity of LC is regarded as an indispensable feature for the antigen-specific sensitization of skin. By using Matrigel invasion assay, Kobayashi reported that

haptens treatment resulted in a significant stimulation of LC migration *in vitro*. Moreover, they also found that certain cytokines derived from dermal fibroblasts may promote and regulate the directional migration of LCs (Kobayashi et al., 1994). The GM-CSF that was thought to be important to maintain viability of LC *in vitro*, has been shown as a strong trigger for LC motility *in vitro* as well (Rupec et al., 1996). Another cytokine TNF- α was also considered to play an important role to mediate LC migration via membrane-associated MMP-2 and MMP-9 *in vitro* (Noirey et al., 2002).

In addition cytokine IL-18 was identified recently as a strong LC migration enhancer. In the skin, IL-18 is mainly secreted by keratinocytes and fibroblasts. Because its structure closely resembles to IL-1 β , which results in a hypothesis that IL-18 may initiate immune response of skin sensitization. Cumberbatch first disclosed that intradermal administration of IL-18 lead to a significant reduction in epidermal LC densities and a marked increase in lymph node DC number (Cumberbatch et al., 2001). Moreover, by using IL-18 knockout mice, it has been reported that IL-18 is the key mediator of LC migration acting upstream of IL-1 β and TNF- α (Antonopoulos et al., 2008).

3.1.8 EFs mediate immune cell migration

Physiological EFs have been proved to play important roles in inducing cell migration and division during wound healing (McCaig et al., 2009; Zhao, 2009). Recently, Lin reported that EFs induced human lymphocytes directional migration to cathode. Additionally, activation of Erk1/2 and Akt was required for the stimulation, which was shared with chemotaxis signalling pathway (Li et al., 2011). Based on the facts that: 1). LCs migrate directionally during wound healing; and 2) endogenous EFs are simultaneously generated when wound occurs, I hypothesize that EFs could potentially influence LCs' activities during wound healing.

This chapter explores the molecular mechanisms involved in EFs regulated cellular response of various types of DCs, resulting in an enhanced response to IL-18. The cytokine secretion and migration of DCs shall be analyzed, and the intrinsic machinery i.e. the interactions between EF, IL-18 and IL-18 receptors shall be investigated.

3.2 Results

3.2.1 EFs increase IFN- γ expression via activation of p38 MAPK in KG-1 in vitro

3.2.1.1 EFs increase IFN- γ expression in a dose-dependent pattern

Although IL-18 could trigger KG-1 to express IFN- γ , the dynamic interaction between IL-18 input and IFN- γ output on KG-1 is not clear yet. To establish the reaction curve, ELISA was used to measure IFN- γ production and standard curve was generated from Four Parameter Logistic Equation ($R=0.9983$) (Figure 3-2 A).

IL-18 was titrated to quantitatively illustrate its ability in inducing IFN- γ expression by KG-1 (Figure 3-2 B). In order to exclude the perturbation of asynergic expression level of the IL18R α , two groups of KG-1 cells: 50 ng/ml TNF- α overnight pretreated group and naïve KG-1 (no TNF- α treated) group were used for a comparative study. IL-18 induced IFN- γ expressions were significantly higher in TNF- α treated group than non-treated group. KG-1 cells not exposed to TNF- α , but otherwise subject to identical conditions, expressed very low amount of IFN- γ . In addition, TNF- α treatment also reduced the IL-18 requirement to trigger IFN- γ expression 5 times less compared with non-treated group, since 2 ng/ml IL-18 treatment induced detectable IFN- γ in treated group, in contrast 10 ng/ml IL-18 was needed to achieve the same in non-treated group. Furthermore, TNF- α treated group showed prominent dose dependent pattern towards IL-18 stimulation.

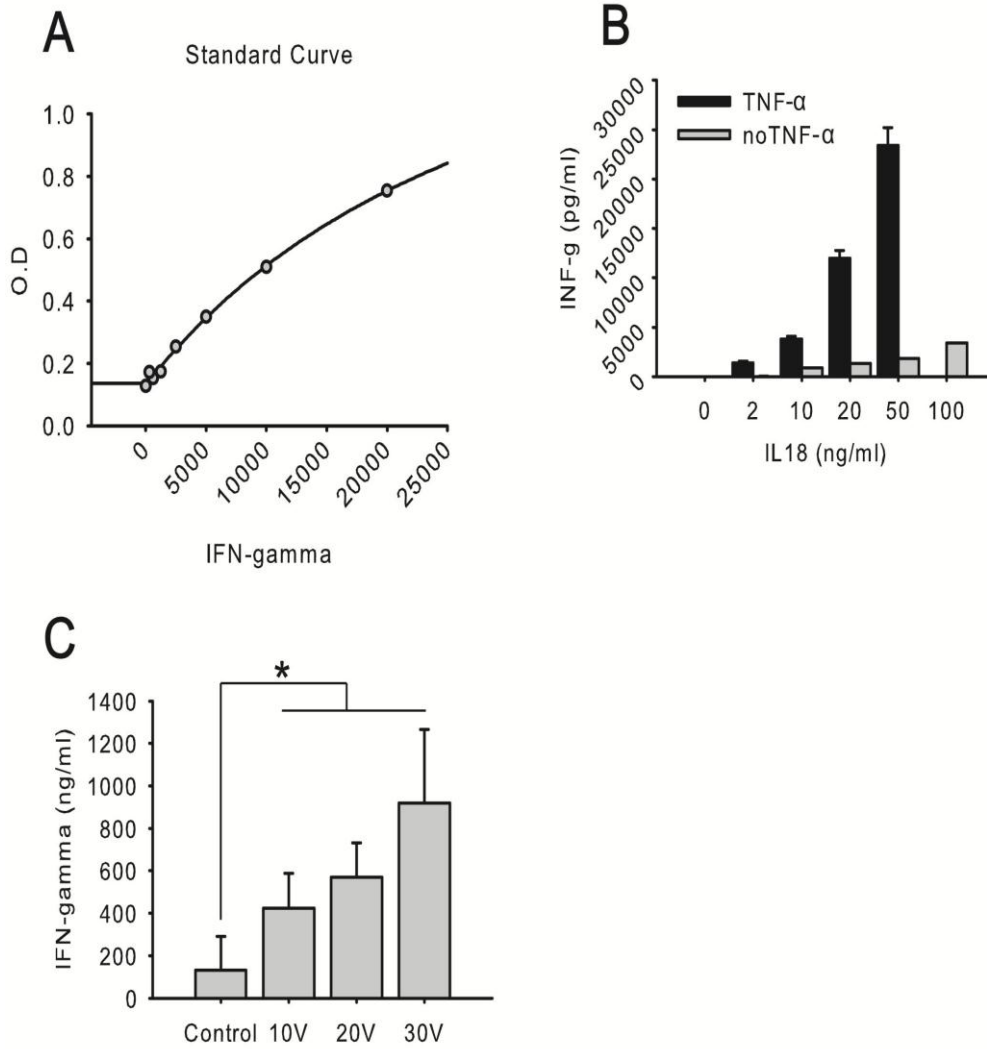


Figure 3-2 EFs increase IFN-gamma expression

A. Standard cure of IFN- γ concentration ELISA read out

B. Titration of IL18. IL18 was titrated to illustrate its ability to induce INF- γ expression by KG-1 cells. KG-1 cells were primed with or without 20 ng/ml TNF- α for overnight and washed with fresh medium next day before stimulated with 0-100 ng/ml IL-18 in 2×10^6 /ml density in 24 well plate. Data are presented as mean \pm SD

Based on the results from Figure 3-1 B, 50 ng/ml TNF- α pretreatment plus 20 ng/ml IL-18 were used for the EF stimulation experiments. Application of EFs ranged from 10V-30V 3 hours across cultured KG-1 cells in the trans-well chamber resulted in a significant increase of IFN- γ expression in a dose-dependent manner (Figure 3-2 C). During the experiments, the temperature of medium were measured and kept at 37 °C in order to eliminate the influence by electric current-generated heat. Culture media were harvested 24 hours later of EF exposure and the amount of IFN- γ were quantified. Low (10V) and high (30V) EFs stimulation increased IFN- γ expression to 423 and 920 ng/ml which were 3 and 7 times higher than control, respectively.

3.2.1.2 Phosphorylation of p38 MAPK were increased in KG-1 when exposed to EFs

After EFs exposure, KG-1 cell were lysed and cytosolic protein were analyzed by Western blot (Figure 3-3). Both IL18R α and IL18R β were found no fluctuating in either IL-18 only or EF & IL-18 treatment in comparison with control group (Figure 3-3 A, B). Interestingly, phosphorylated p38 MAPK was significantly increased in the group exposed to EF & IL-18 treatment (Figure 3-3 C, D). With IL-18 induction, phosphorylation of p38 was significantly upregulated as well, which is in consistent with previous study (Bachmann et al., 2007b; Koutoulaki et al., 2010b). However, EF & IL-18 treatment even showed stronger induction on phosphorylation of p38 comparing with IL-18 stimulation alone, indicating EF & IL-18 have synergistic influence on p38 phosphorylation. Three independent experiments were carried and representative images were shown.

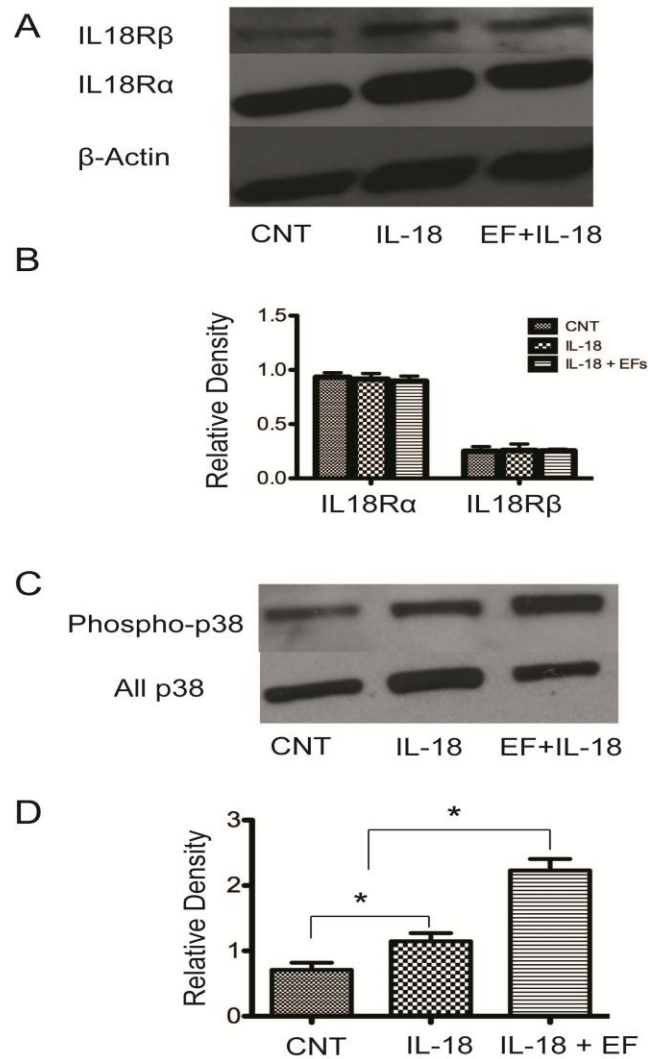


Figure 3-3 Phosphorylation of p38 MAPK were increased in KG-1 when exposed to EFs

A. KG-1 cells stimulated with IL-18 and EFs plus IL-18 were harvested immediately after exposure and the expression of IL18R α and β were analyzed by Western blot, β -actin was taken as internal control.

B. The relative grey density of Western blot bands of IL18R α and IL18R β were analyzed by ImageJ software, where β -actin as internal control. Data are presented as mean \pm SD,

C. Both phospho-p38 and total p38 were detected by Western blot using the same samples as (A)

D. The phosphorylation levels of p38 were relatively quantified by using the ratio of p-p38 and total p38. Data are presented as mean \pm SD, *: $P < 0.05$

3.2.2 IL18R α polarized to anode when exposed to EFs in 2D culture

Application of 200 mV/mm EFs across cultured KG-1 for 15 minutes in EF stimulation chamber resulted in IL18R α other than IL18R β polarizing towards the anode. Immediately after EF stimulation, KG-1 cells were directly fixed *in situ* and stained with IL18R α and IL18R β antibodies which were targeted with secondary antibodies conjugated to FITC and TRITC, respectively. The distributions of IL18Rs were captured under confocal microscope (Figure 3-4).

On KG-1 cells not exposed to EFs, but otherwise subject to identical conditions, both IL18R α and IL18R β distributed evenly across the cell membrane. On the contrary, with EF stimulation, IL18R α was asymmetrically redistributed to the cell membrane facing the anode. However, EF did not show such effects on IL18R β .

3.2.3 EFs increase KG-1 sensitivity towards IL-18 in 3D culture

IL-18 has been previously reported as a chemoattractant to induce T cells and DCs morphological polarization and directional migration (Gutzmer et al., 2003; Kaser et al., 2004; Komai-Koma et al., 2003). In 3D culture, EF stimulation increased the response of KG-1 cells to IL-18 (Figure 3-5). KG-1 cells were pretreated with TNF- α overnight to induce IL18R α expression and then the cells were mixed with Matrigel and seeded into the electrical chamber. After Matrigel solidified, the cells were treated with either 250 ng/ml of IL-18 gradient diffused from Matrigel premix (Figure 3-5A, high side of triangle indicates high concentration of IL-18) or 200 mV/mm of EFs (Figure 3-5B, anode to the right), or a combination of both (Figure 3-5C). The cell migration were tracked, migration directedness and speeds were analyzed with Image J software. I found that application of a 200 mV/mm EF across KG-1 cells in 3D resulted in a significant increase of migration towards IL-18 chemoattractant gradient (Figure 3-5 C, D).

During the 90 minutes experiment, in the presence of both EF and IL-18, KG-1 cells showed constantly directional migration to the source of IL-18. In contrast, the orientation of KG-1 cells fluctuated in either IL-18 only group or EF group (Figure 3-5 A, B). Thus neither EF stimulation nor IL-18 alone is sufficient to induce KG-1 directional migration in 3D culture. The combination of EF and IL-18 significantly

increased KG-1 cell migration speed and directedness. Interestingly, if dividing the 90 minutes experimental duration into two 45 minutes halves, the cell migration speed only increased in the second half, suggesting a potential build-up of signaling might be required before directional migration taking place (Figure 3-5 D).

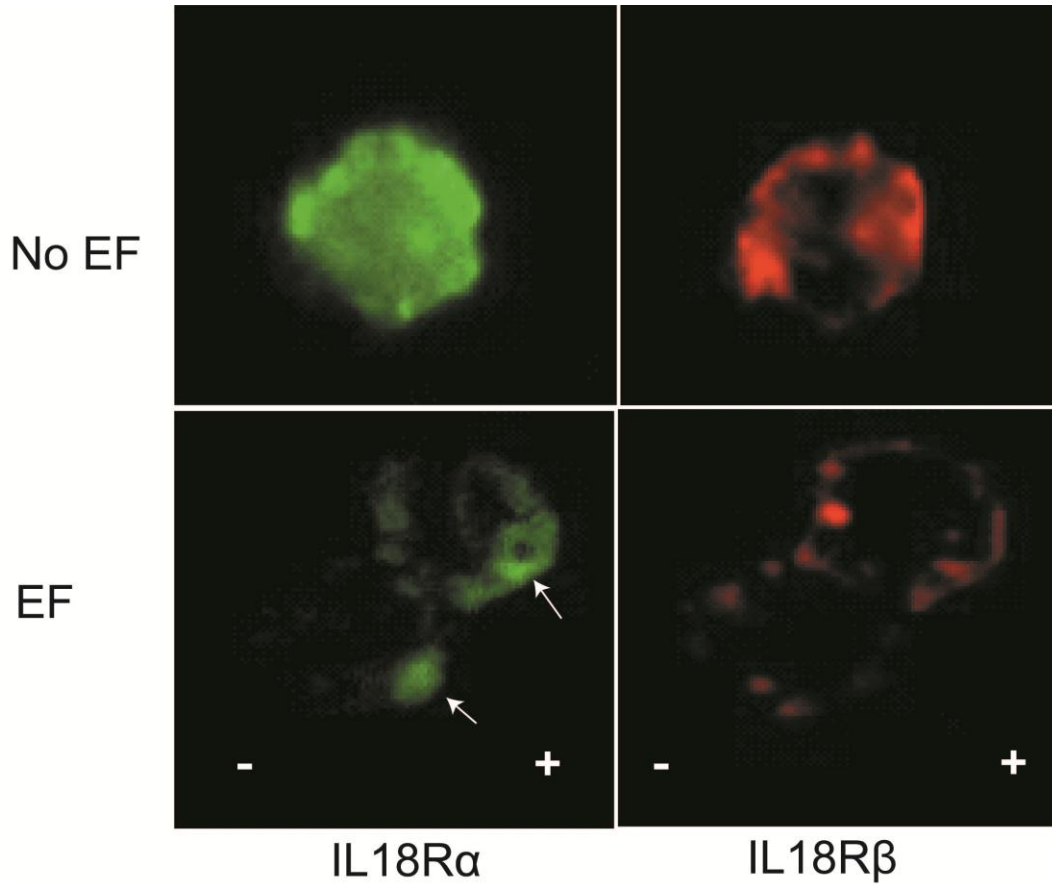


Figure 3-4 IL18R α polarized to anode when exposed to EFs in 2D culture.

Immunocytochemistry staining of KG-1 with IL18R α and IL18R β . The cells with/without exposure to EFs 15 minutes were fixed and stained with anti-IL18R α and IL18R β , which were targeted with the second antibodies conjugated with FITC and TRITC, respectively. In control group, both IL18R- α and - β are evenly spread around the cell membrane; with EFs treatment IL18R α shows polarization to anode (arrow), IL18R β shows no difference to control group. +: anode,-cathode.

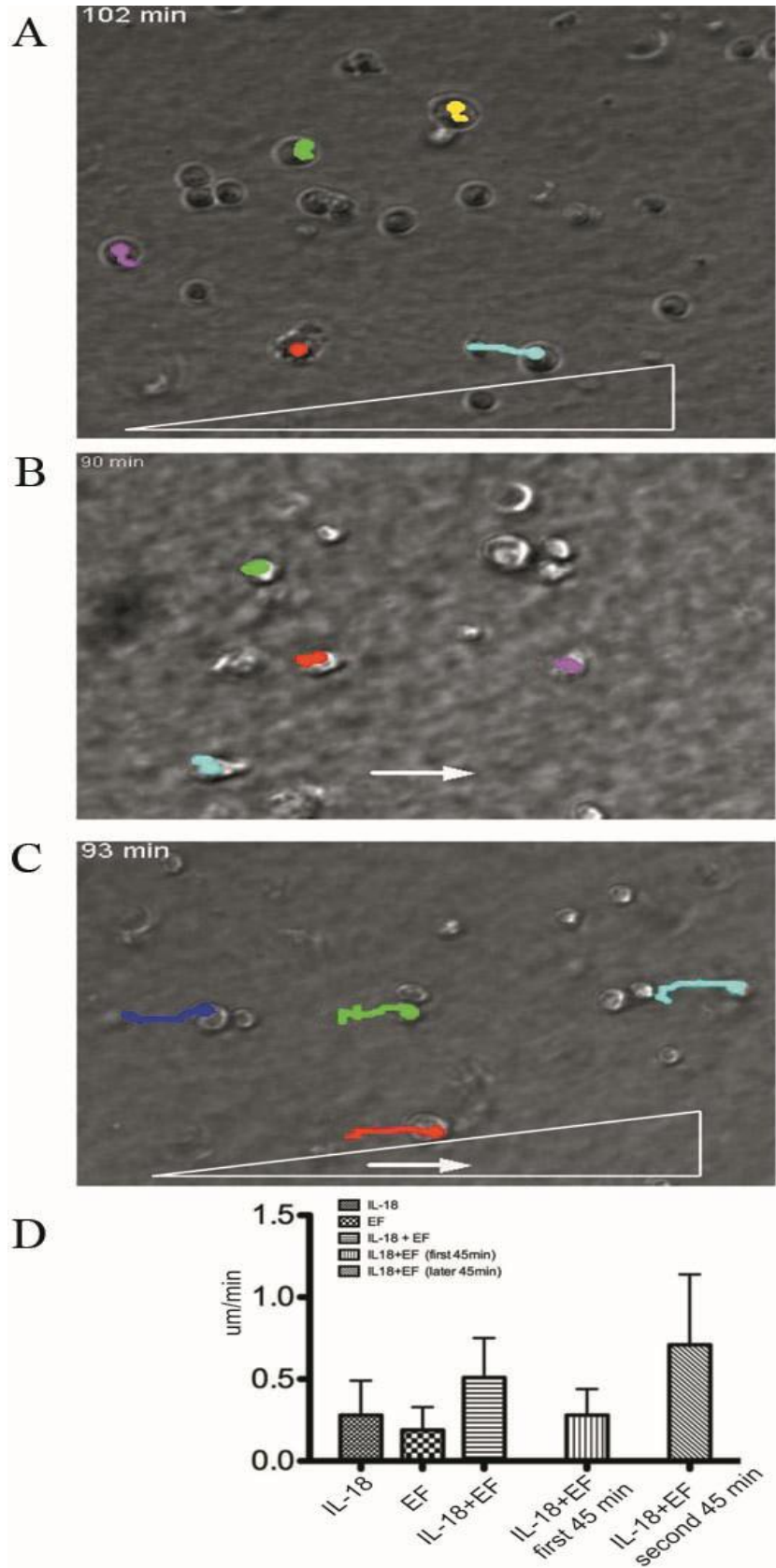


Figure 3-5 EFs increase KG-1 sensitivity towards IL-18 in 3D culture

A. KG-1 cells were mixed with Matrigel and then seeded into EF chamber. IL-18 (200 μ l, 50 ng/ml) was injected at the right side of the chamber and the IL-18 gradient across the chamber was established by diffusion. (The high side of triangle indicates the high IL-18 concentration). The cells were exposed to IL-18 gradient for 2 hours and the cells migration were tracked and analyzed. Cells display slow migration. B. A 200 mV/mm EFs were applied across the 3D Matrigel cultured KG-1 cells. (Arrow indicates the anode) The cells migration were tracked and analyzed. Cells exhibited slow migration. C. KG-1 cells were exposed to IL-18 and EFs simultaneously in 3D culture, the concentration of IL-18 is the same as (A) and the strength of EFs was the same as (B). Cells show directional migration towards high IL-18 concentration side (the anode). D. Migration speed of KG-1 cells in the condition of (A, B, C) were analyzed accordingly. In addition, migration speed of cells treated with IL-18 plus EFs were split into two halves: the first 45 and the following 45 minutes. Data are presented as mean \pm SD, *: $P < 0.05$.

3.2.4 Neither IL-18 nor EFs alone is able to polarize IL18Rs of KG-1 in 3D culture

Immediately after either IL-18, EFs or both stimulations on KG-1 cells in 3D culture, cells were fixed and stained with IL-18 α and IL-18 β antibodies. Fluorescence coupled secondary antibodies were used to visualize the distribution of receptor molecules on the cell membrane under the confocal microscope (Figure 3-6 and Figure 3-7). Despite the application of 250 ng/ml IL-18 at right side (high side of triangle indicates high IL-18 concentration), both IL18R α and IL18R β are evenly distributed around the cell membrane (Figure 3-6A). The similar results are found in the KG-1 cells exposed to 200 mV/mm EFs alone (Figure 3-6B).

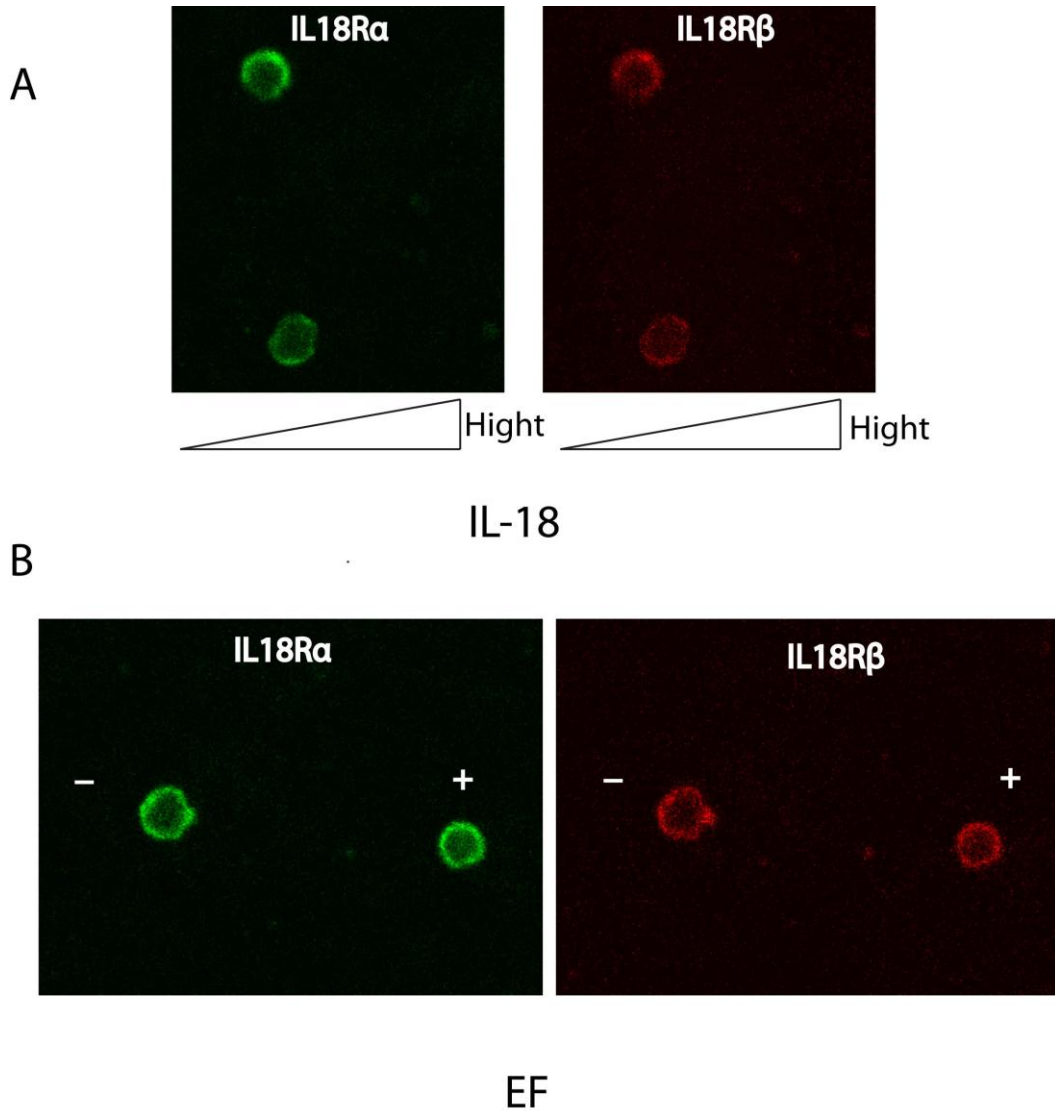


Figure 3-6 Neither IL-18 nor EFs alone is able to polarize IL18Rs of KG-1 in 3D culture

KG-1 cells exposed with IL-18 gradient (A) and EFs (B) for 2 hours, and immediately after exposure cells were fixed and stained with anti-IL18R α (green) and anti-IL18R β (red). Triangle indicates the gradient of IL-18 and the high side means the high IL-18 concentration. +: anode, -, cathode.

3.2.5 EF increases sensitivity of KG-1 in response to IL-18 in 3D culture

Chemotaxis is postulated to occur through modulation of re-polarization of membrane receptors. Based on analysis of two distinct stages of cell migration in response to IL-18 as a chemoattractant in parallel with the electrotaxis (Figure 3-5D), KG-1 cells were fixed and IL18Rs were stained at two counterpart time points: 15 minutes and 60 minutes to determine if redistribution of receptors occurred, respectively. The distribution of IL18R α and IL18R β were thus observed and analyzed with confocal microscope (Figure 3-7).

Figure 3-7 A illustrates that in the first 15 minutes only IL18R α is polarized to IL-18 gradient (high concentration toward left) in the presence of 200 mV/mm EF treatment (anode to the left). In the contrary, the IL18R β distributes evenly around the cell membrane. The fluorescence intensity across the cells (dashed line) is calculated and the intensity pattern is extracted below. Clearly higher fluorescence intensity value of IL18R α shows at the left side of the cell membrane towards the anode of EF and source of IL-18 gradient. But, this effect does not show on IL18R β . At the 60 minutes time point, both IL18R α and IL18R β are asymmetrically redistributed towards the upstream of IL-18 gradient in EF. The lineal scanning profiles of fluorescence intensity also confirms that both IL18R α and IL18R β signalling are polarized toward the left side of the cell membrane where the high IL-18 concentration and anodal of EF reside (Figure 3-7B).

The interaction of IL18R α and IL18R β has been described as the prerequisite of IL-18 signalling transduction (Koutoulaki et al., 2010b). The co-localization of both receptor subunits is widely used to indicate the probability of their molecular interaction and initiation of signalling. Figure 3-7 C and D depict the co-localization of IL18R α and IL18R β at 15 minutes and 60 minutes during the experiments. Although at both time points, the co-localization of IL18Rs (indicates as yellow) tend to accumulate at anodal side, the stronger yellow signal appears at the 60 minutes implying a stronger co-localization (Figure 3-7 C). The co-localization can be quantified by using the specific software (Bolte and Cordelieres, 2006), and the Pearson's correlation index is used for quantification. After 60 minutes exposure to EF, the Pearson's correlation index of IL18Rs was increased to 0.58 which was significantly higher than the one at 15 minutes (Figure 3-7 D).

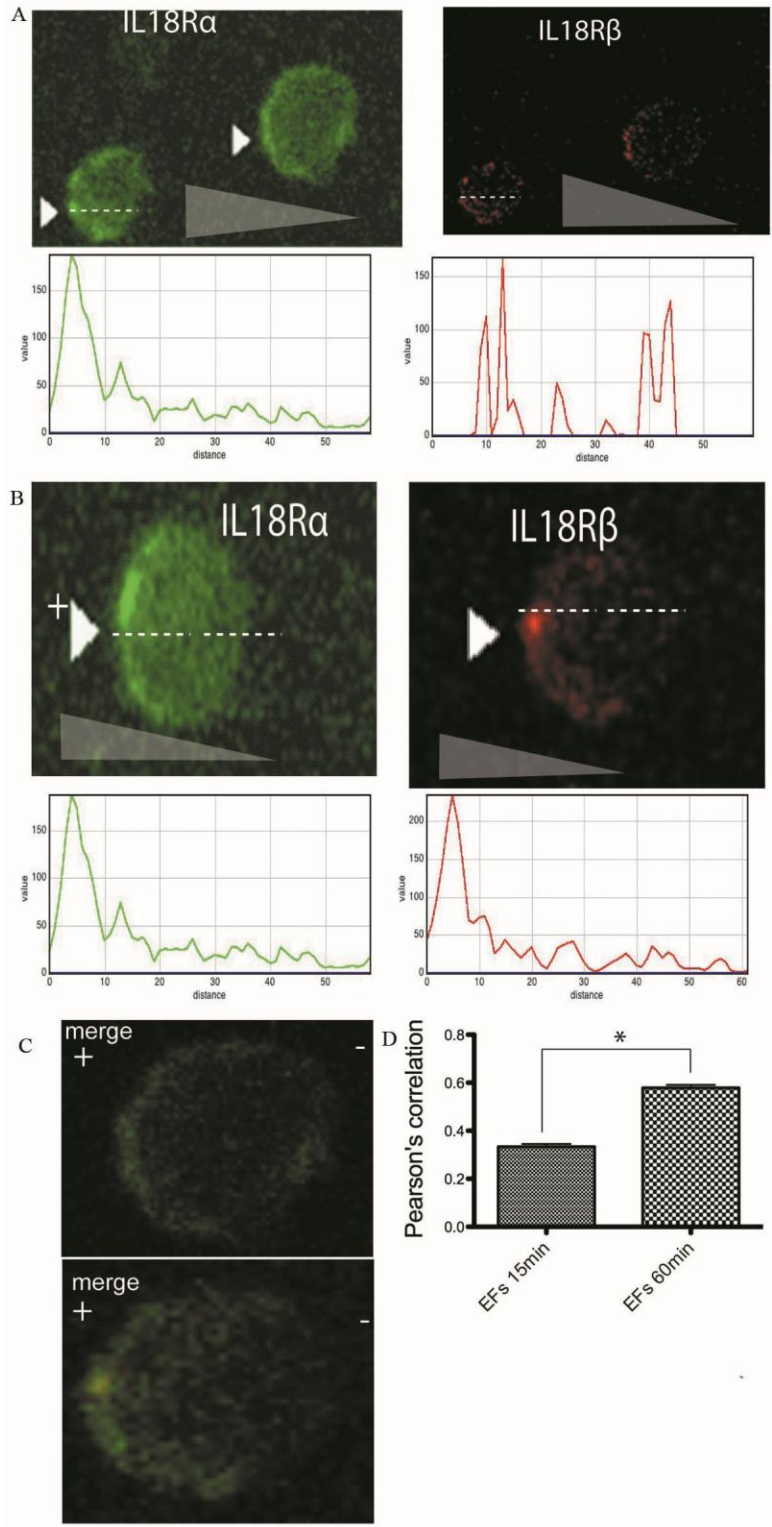


Figure 3-7 EF increases sensitivity of KG-1 to IL-18 in 3D culture

A-B. IL18R α and IL18R β fluorescence intensity across the KG-1 exposed to EFs plus IL-18 at 15 minutes and 60 minutes, respectively. Dash lines across the cells on confocal image indicate pixels measured in each graph below. At first 15 minutes exposure, IL18R α shows accumulation at anodal side, but IL18R β does display this effect (A); after 60 minutes, both IL18R α and IL18R β exhibit anodal polarization (B).

C-D. Co-localization of IL18R- α and- β , the images (C) are merged from A (up), and B (bottom); (D) and the colocalization were quantified by using ImageJ. Data are presented as mean \pm SD, *: $P < 0.05$.

3.2.6 IL18R α -GFP vector construction and expression

The fluorescence labeling techniques with GFP or other fluorescence proteins immensely facilitate the research on molecular dynamic at single cell level. I thus cloned the human IL18R α and conjugated with GFP in order to visualize the IL18R α movement under the EF treatment in real time.

The IL18R α -GFP vector was designed as shown in Figure 3-8 A. The DNA fragment IL18R α -GFP was inserted into pcDNA-3.1 plasmid and the positive clones were sequenced. The IL18R α -GFP vector was then transfected with Cos7 cells using lipofectamine 2000 (Invitrogen). The transfection efficiency was approximately 20% (Figure 3-8 B, a-b). Sequentially, the transfected cells were split at 1:5 ratio into new flasks with the medium containing 50 μ g/ml G418. Two weeks later, the G418 resistant clones were formed (Figure 3-8 B, c-d), and the clones were picked up, expanded and harvested. The human IL18R α -GFP fusion protein of stable transfected cell line was identified by Western Blot (Figure 3-8 C).

3.2.7 Dynamic movement of IL18R α when exposed to EFs.

The dynamic movement of IL18R α in the presence of EFs can be analyzed with real time recording using the stably transfected IL18Ra-GFP cell line. With the EFs (200mV/mm) stimulation, GFP signals moved to the anodal side, which indicates asymmetrical distribution of IL18R α activity. The prominent effects started from 450

seconds onwards and IL18R α was constantly accumulated at anodal side of the cell membrane in the presence of EF. When EF polarity was reversed, IL18R α started redistribute to the opposite side of the cell membrane to accommodate the new anodal orientation. Where IL18R α -GFP expressing cells were not exposed to EFs, IL18R α randomly fluctuated along the movement of cell lamellipodia. This demonstrates that EFs induce IL18R α moving towards the anode

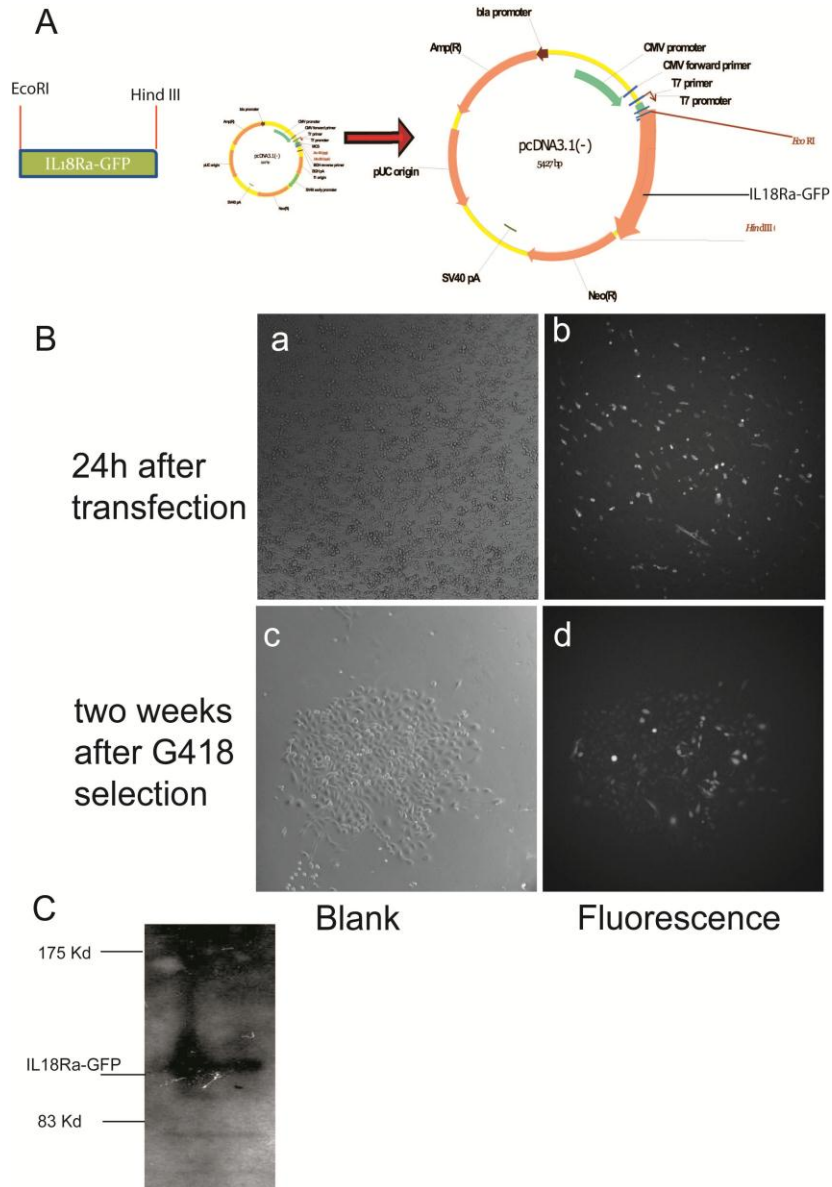


Figure 3-8 Vector pcDNA3.1-IL18Ra-GFP construction and expression

A. Schematics illustrate the process of vector construction of pcDNA3.1-IL18R α -GFP. The human IL18R α were cloned and tagged with GFP, and then inserted into pcDNA3.1 plasmid with indicated restriction enzyme sites.

B. The Cos-7 cells were transiently transfected with IL18R α -GFP plasmid (a, b) and the transfected cells formed G418 resistant clones which expressed IL18R α -GFP after 2 weeks 50 μ g/ml G418 selection (c, d).

C. Western blot confirms IL18R α -GFP expression.

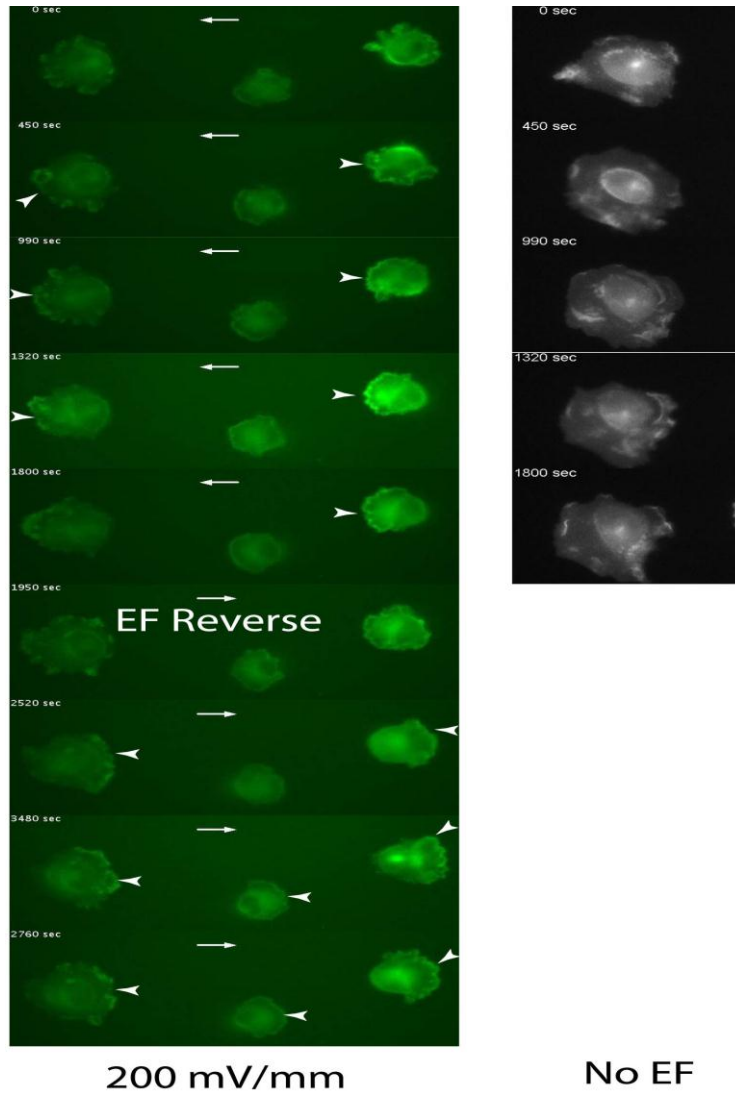


Figure 3-9 Dynamic movement of IL18Ra when exposed to EFs

IL18R α -GFP transfected Cos-7 cells were stimulated with 200 mV/mm EFs (left) or no EF control (Right). During the EFs exposure, the direction of EF was reversed in the middle of the recording. Arrows indicate the direction of EFs and point to anode; arrow heads indicate the distribution of IL18Ra-GFP molecules.

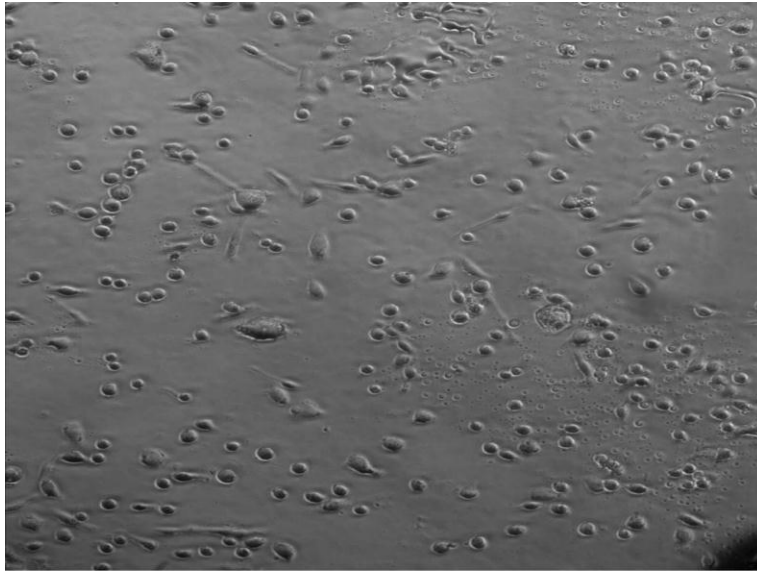
3.2.8 Differentiation of KG-1 into dendritic cells

The cell line KG-1 has been used as an *in vitro* model for human myeloid dendritic study such as differentiation, immune responses, antigen presenting (Bachmann et al., 2007b; Koutoulaki et al., 2010a; Poleganov et al., 2008). It has been reported that several chemicals and cytokines can induce KG-1 differentiation and/or maturation (Teobald et al., 2008). KG-1 cells were cultured with 20 ng/ml TNF- α plus 10 ng/ml PMA for 3 or 7 days to induce their differentiation and/or maturation. Undifferentiated KG-1 cells show rounded morphology and grow in suspension with little evidence of adhesion (Teobald et al., 2008). TNF- α and PMA induced a proportion of KG-1 cells to differentiate, with approximately 20% adhering to the petri dish in 3 days (Figure 3-9 A). Whilst continuous culturing for 7 days with TNF- α and PMA, cells exhibited obvious morphological changes with bigger size and more polarized protrusions (Figure 3-9 B). These observations are in agreement with the previous studies (Koeffler et al., 1981; Teobald et al., 2008).

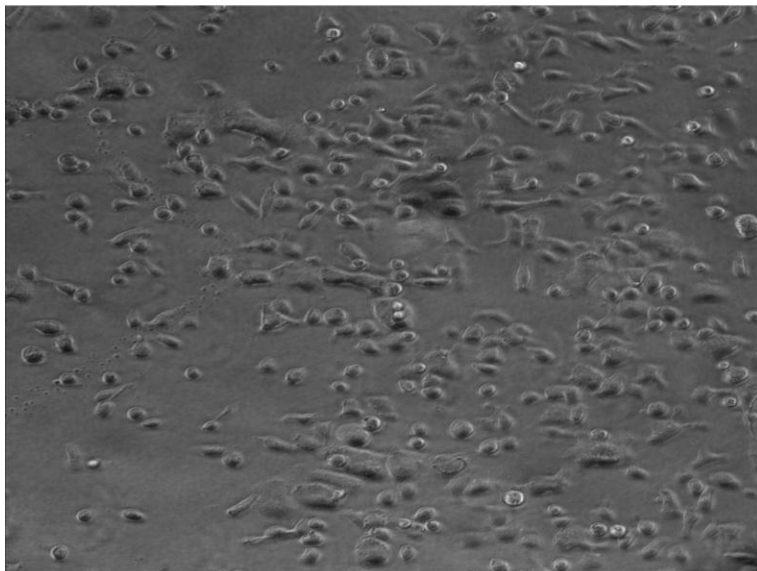
3.2.9 EFs and IL-18 increase the motility of KG-1-derived DCs

Once KG-1 cell differentiates into DC, it gains the capacity to migrate, along with the protrusion of pseudopods and polarization in morphology. After 7 days induction and differentiation, KG-1-derived DC stably showed typical characteristic of dendritic cells, therefore I used the cells from this time point for the following migration studies.

The KG-1-derived DCs were consistently treated in the following 3 conditions – 200 mV/mm EFs only, 100 ng/ml IL-18 only, or EFs plus IL-18, respectively for 2 hours. The cell behaviours were tracked (Figure 3-11A), and migration speeds were analysed afterwards (Figure 3-11B). The IL-18 and EFs increased cells migration speed up to 3.71 ± 1.3 and 3.29 ± 0.86 $\mu\text{m}/\text{min}$ from the control cells of 2.41 ± 0.54 $\mu\text{m}/\text{min}$, respectively. Moreover, the combined treatments of EFs (200 mV/mm) and IL-18 (100 ng/ml) significantly elevated cell migration speed to 5.76 ± 1.56 $\mu\text{m}/\text{min}$ compared with either treatment alone or control group (ng/ml) significantly elevated cell migration speed to 5.76 ± 1.56 $\mu\text{m}/\text{min}$ compared with either treatment alone or control group.



TNF α + PMA 3 days



TNF α + PMA 7 days

Figure 3-10 Differentiation of KG-1 into dendritic cells

KG-1 cells were induced by TNF- α (20 ng/ml) and PMA (10 ng/ml) for 10 days and then showed dendritic cell phenotype. Photomicrographs show the phenotype of KG-1-derived DCs at day 3 (up) and day 7 (bottom).

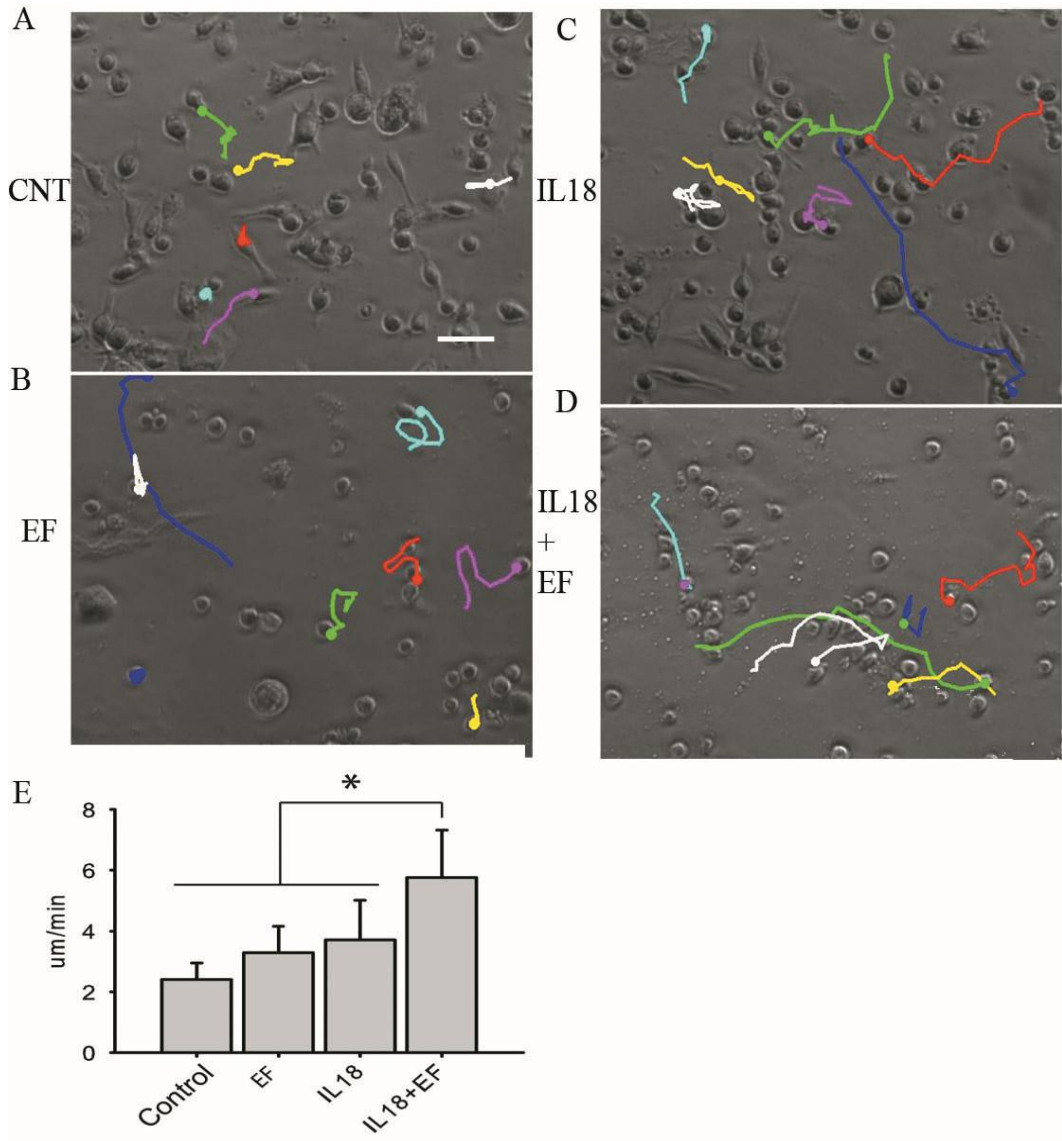


Figure 3-11 EFs and IL-18 coordinatively increase the motility of KG-1-derived DCs.

KG-1-derived DCs were stimulated with EFs (200 mV/ml), IL-18 (100 ng/ml) and IL-18 plus EFs, respectively, for 2 hours. The cells movement were tracked (A) and the migration speed in each situation were analysed (B). Data are presented as mean \pm SD, *: $P < 0.05$.

3.2.10 High strength EFs trigger the directional migration of KG-1-derived DCs towards anode in 2D

Although DCs showed higher motility under the combined treatments of EFs (200 mV/mm) and IL-18, the migration directedness appeared to be random without persistence in any particular direction. Higher voltage EFs were used to determine if these could induce directional migration on DCs like other cells types.

Briefly, the DCs were treated with 3 conditions: IL-18 (100 ng/ml), 200 mV/mm EF plus IL-18 (100 ng/ml) or 400 mV/mm EF plus IL-18 (100 ng/ml), respectively. With the treatment of IL-18 and 200 mV/mm plus IL-18, the DCs exhibited migration directedness of -0.15 ± 0.05 and -0.26 ± 0.09 , respectively. The minus data indicates the migration direction is toward the anode of the EF. However, when EF raised to 400 mV/mm, DCs showed strong directional migration towards anode with the index as -0.59 ± 0.15 , which was significantly higher than the IL-18 only or 200 mV/mm Plus IL-18 groups (Figure 3-12 D, E). This implies that the EF and IL-18 compete on the modulation of elements of the cytoskeleton through which cells migrate

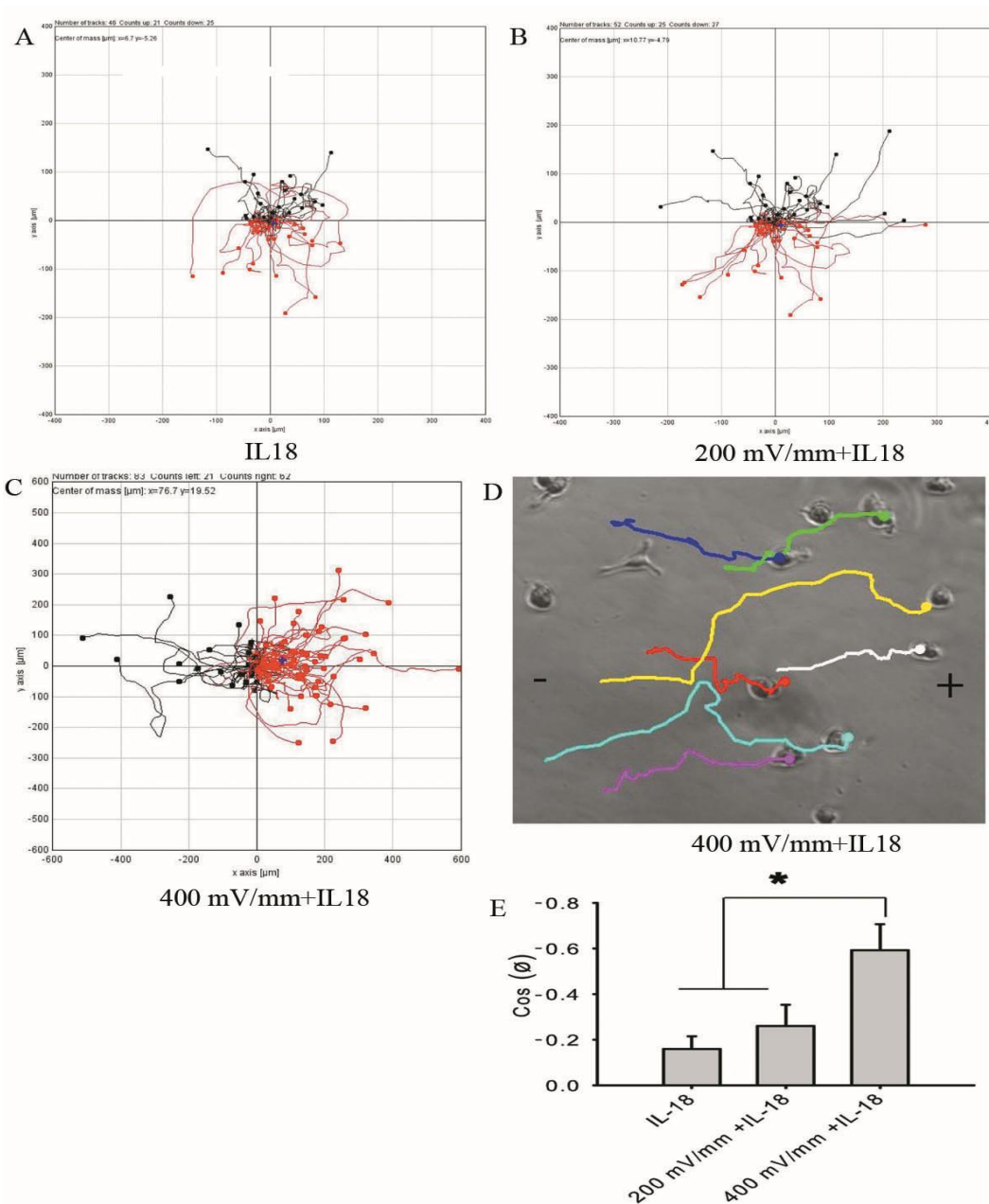


Figure 3-12 High strength EFs induce KG-1-derived DC directional migration

KG-1-derived DCs were stimulated with IL-18 (100 ng/ml) and IL-18 plus EFs (200 mV/mm or 400 mV/mm), respectively for 2 hours. The cells movement were tracked and analysed afterwards.

A-C. composite drawings where each cell is superimposed to origin point and the cell migration path traced over 2 hours

D. cells exposed to 400 mV/mm plus IL18 were traced over 2 hours

E. The migration directionality in each situation was analysed. Cos(θ) indicates the degree of directionality, 0 means random migration, 1 means directional migration, - means direction towards anode. Data are presented as mean \pm SD, *: $P < 0.05$.

3.2.11 EFs promote DC chemotaxis in response to IL-18 in 3D culture

IL-18 is an important proinflammatory cytokines and also induces various cells migration as a chemoattractant (Bachmann et al., 2007b; Ohtsuki et al., 1997). To further confirm whether KG-1-derived DCs have chemotaxis response to IL-18 and whether EFs have any influence on the process, I applied similar 3D culture method used in KG-1 cells to test the KG-1-derived DCs. DCs displayed an evident response to IL-18 and migrated towards the IL-18 source on the left (Figure 3-13 A). The EFs enhanced this response with increased migration speed of DC from 2.7 ± 0.17 $\mu\text{m}/\text{min}$ up to 6.3 $\mu\text{m}/\text{min}$ (Figure 3-13 B, C).

3.2.12 IL18R α is essential for EF-induced directional migration of KG-1-Derived DC

To further confirm if IL18Rs is required for EF induced directional migration of DC, the IL18R α over-expressing cells were used for the following experiments. KG-1 cells were firstly induced only by PMA for 3 days to become DC like cells. Because cells had not been treated with TNF- α , the KG-1-derived DCs kept low IL18R α expressing level (Koutoulaki et al., 2010b). Before the PMA induction, the KG-1 cells were electroporated with IL18R α -GFP plasmid. The GFP positive cells were then sorted and mixed with non-transfected cells without TNF- α treatment for induction. After PMA induction, the DCs were applied with 200 mV/mm EFs and the cells behaviours were recorded by fluorescence microscope.

The further analysis of cell migration exposure to EFs is illustrated as Figure 3-14. The GFP positive cells showed directional migration response to EFs toward anode and did U-turn with the polarity reversal of EFs (Figure 3-14 A). The GFP negative cells displayed very less movement and no response to the EF reversal compared with GFP positive cells (Figure 3-14 B). In combination with the previous data that EF triggered asymmetric redistribution of IL18Rs (Figure 3-4, 3-7 and 3-9), this observation strongly implies that IL18Rs are the key regulator controlling the electrotaxis of DCs.

3.2.13 IL-18 increases co-localization of IL18R α and actin

The cytoskeleton elements, particularly actin plays an essential role in mediating cell motility. To further investigate the potential involvement of actin during DC migration, the IL18R α and actin filament expression were visualized using immunocytochemical staining. Rhodamin phalloidin binds and stabilizes microfilaments and an anti-IL18R α antibody indicates the distribution of the IL18R α .

The DCs were incubated with 100 ng/ml IL-18 for 15 minutes, and sequentially fixed and stained. The none-treated cells were taken as control. The yellow pixel in the merged pictures indicates the co-localization of the actin and IL18R α , and the co-localization index was calculated using the software (Bolte and Cordelieres, 2006) .Figure 3-15 illustrates that the co-localization of IL18R α and actin increases significantly in IL-18 treated group to 0.37 ± 0.029 in comparison with the control group which is 0.087 ± 0.014 . This finding indicates that IL-18 may potentially contribute toward the activation in the leading edge of the migrating DCs.

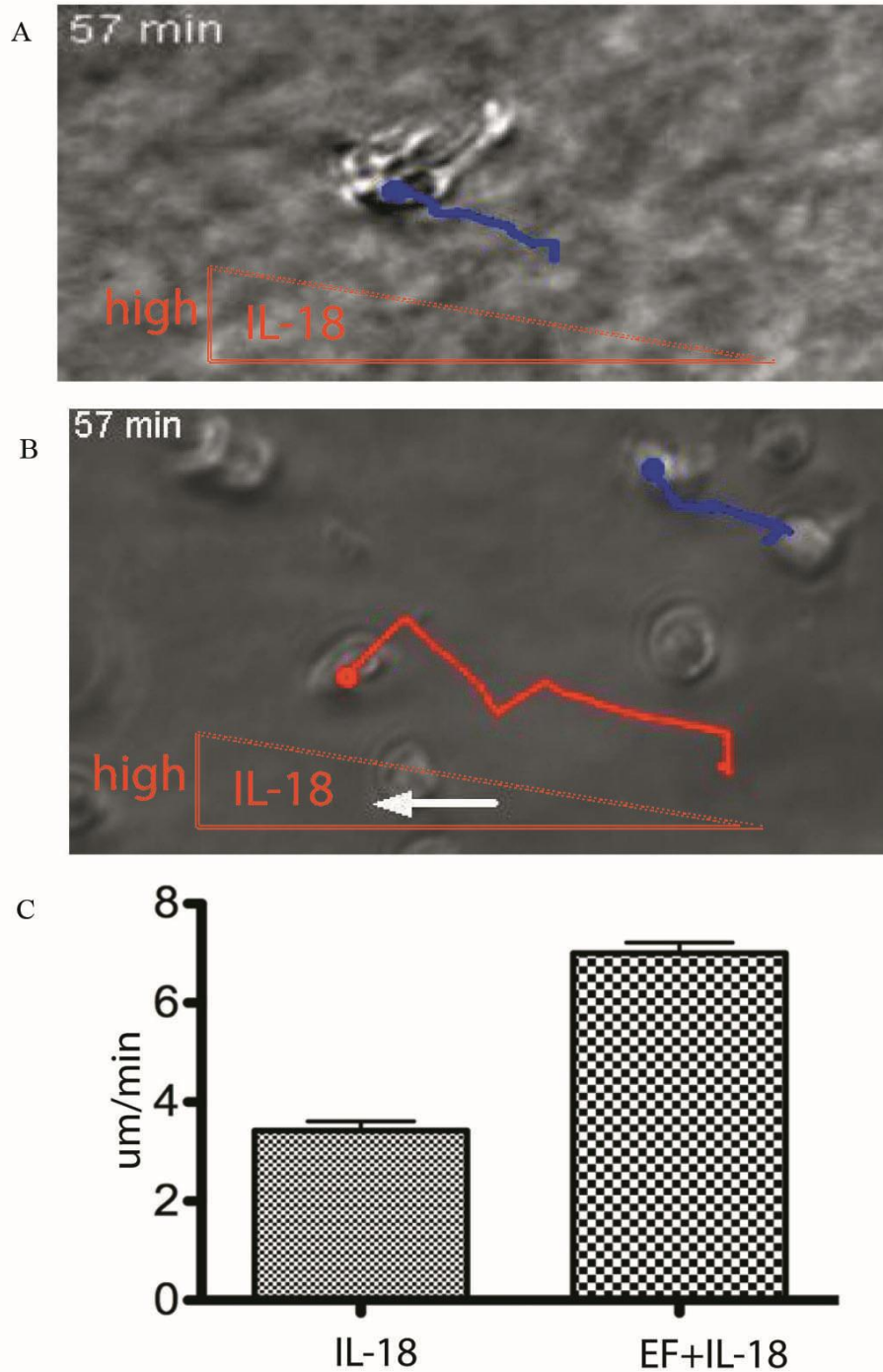


Figure 3-13 EFs promote KG-1-derived DC chemotaxis in response to IL-18

KG-1-derived DCs were mixed with Matrigel and seeded into EF chamber with IL18 (200 μl , 50 ng/ml) injected at right side. Cell then were traced without EF stimulation (A) or with 200 mV/mm EFs exposure (B) over 2 hours, the migration speed corresponds to each situation were analysed using Image J (C). Arrow indicates the direction of EFs towards anode, triangle indicate IL-18 gradient and the high side means the high IL-18 concentration. Data are presented as mean \pm SD, *: $P < 0.05$.

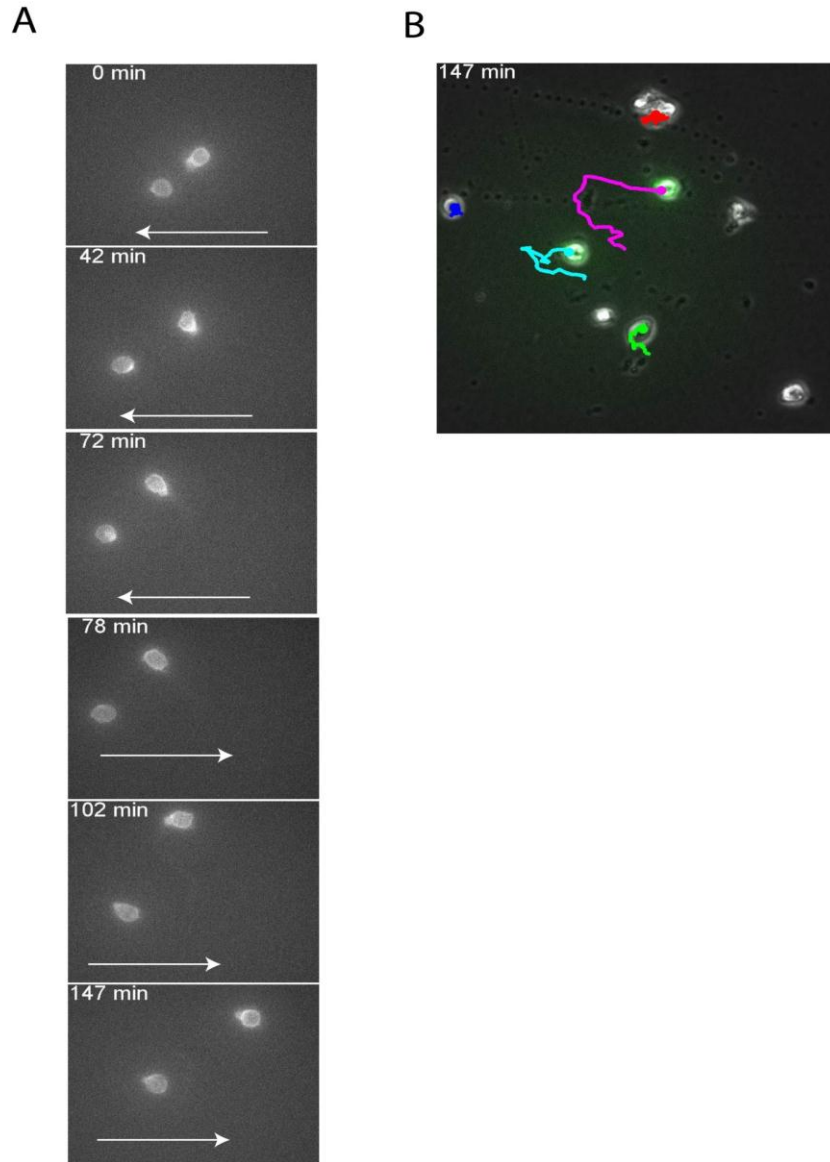


Figure 3-14 IL18Ra is essential for EF-induced directional migration of DC

The IL18R α -GFP over expressed KG-1-derived DCs were mixed with control KG-1-Derived DCs and then exposed to EFs stimulation for 3 hours with EF direction reversed at the middle of the process. A. fluorescence images shows the movement of IL18R α -GFP over expressed cells; B both control cells and IL18R α -GFP over expressed cells were tracked during EFs stimulation and the image was merged from bright field and fluorescence. Arrow indicates the direction of EFs towards anode.

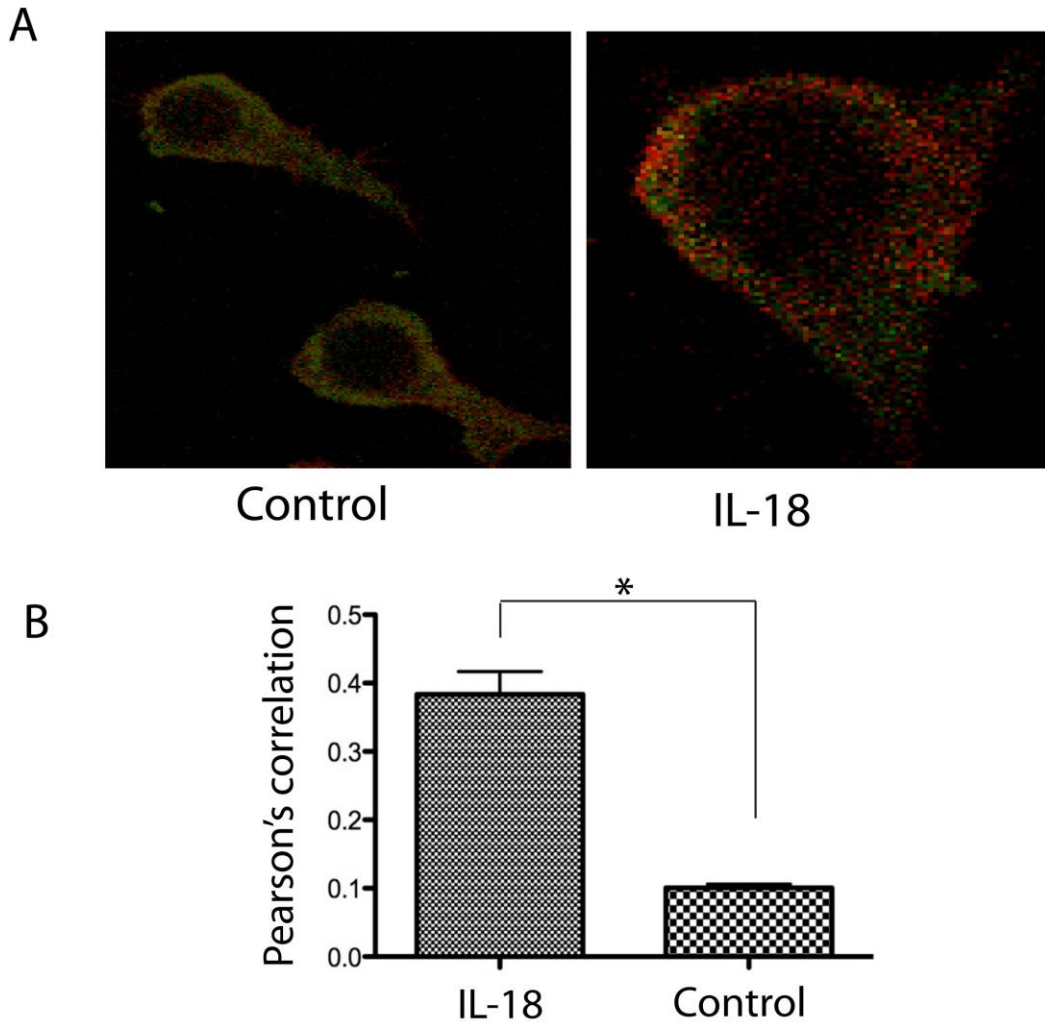


Figure 3-15 The co-localization of IL18R α and actin increased by IL-18 in DC.

A. Immunocytochemistry staining of KG-1-derived DCs. Cells treated with IL-18 (50 ng/ml) or maintained in normal medium for 15 minutes and then stained with Rhodamine phalloidin (red) and anti-IL18R α (green). The fluorescence images were captured using confocal microscope.

B The colocalization of IL18R α and actin in (A) were analysed using ImageJ. Data are presented as mean \pm SD, *: $P < 0.05$.

3.3 Discussion

In this chapter, I tested the hypothesis that EF stimulation triggered the directional migration of KG-1 cells and KG-1 derived DCs via IL18Rs signalling. I demonstrated that EFs promoted activation and asymmetrical distribution of IL18R α which potentially triggered downstream signalling cascade activation and induced directional migration.

3.3.1 Immune response of the cells in EFs

Previous studies have proved that disruption of an epithelial layer instantaneously generates endogenous electric fields, which have been shown to be important in wound healing. Despite numerous documentations that immuno-modulation is one of the key factors regulating wound healing profile, there is little study exploring the involvement of EFs in immune response during wound healing. The CD34⁺ myelomonocytic cell line KG-1 possesses similar characteristic of PBMC, and is used as a typical cellular model for cytokine response research. Particularly, it dose-dependently responds to cytokine IL-18 to produce IFN- γ (Koutoulaki et al., 2010a). Based on this model I designed the experiments to measure the change of the IFN- γ expression level when applied EFs on KG-1, upon which our hypothesis was tested.

I found that the response of KG-1 to IL18 can be enhanced by the application of EFs. The IFN- γ expression is lifted up to 3 and 7 times by 10V and 30V in transwell EFs stimulation, respectively. Apparently, the signalling cascade of IL-18 induced IFN- γ expression starts from the binding of IL-18 to the IL18 receptors. However, I found that application of EFs did not alter the expression of IL18 receptors, but increased the phosphorylation of p38 MAPK. It is in agreement with the previous report that application of EFs to HeLa S3 cells induced phosphorylation of MAPKs, including p38, JNK and ERK, and their upstream kinases (Morotomi-Yano et al., 2011). The human blood T cells exposure to EFs also showed increased activation of Erk1/2 (a member of MAPK family) and Akt. In addition Zhao described that inhibition of MAPK resulted in a reduced EF-directed cathodal migration of cornea epithelial cells (Zhao et al., 1999). Nevertheless, Rajnicek found that inhibitors of PI3K, MEK1/2 or p38 MAPK did not affect EF-induced growth cone turning (Rajnicek et al., 2006b). This may be due to the

fact that the response to EF stimulation is cell type specific, and that different cell types require different signalling pathway to respond to EFs.

The shared activation of Erk1/2 and Akt signalling pathway by electrotaxis and chemotaxis suggests that cells may have common intracellular machinery to respond to extracellular stimuli. However, the kinetics of MAPK and Akt signalling distinguishes the electrotactic response from chemotaxis, because even though the phosphorylation of p38 MAPK was significant after one hour of EF treatment, this was not appreciable by western blot in the first 10 minutes of EF treatment, which forms sharp contrast with the rapid action of chemotaxis in seconds (Wang et al., 1998). The mechanism behind this is still largely unknown, but one potential explanation is that electrotaxis need to build up asymmetric distribution of receptor gradient(s) across the cell membrane, sometimes involving several signalling cascades simultaneously, which can be more time consuming than a simple ligand – receptor binding reaction in chemotaxis.

3.3.2 IL-18 regulates immune cells function via activation of p38

IL-18 is an important regulator of several type of cells during infection or inflammatory. The MAPK activation appears to play a prominent role in IL-18 signaling, being involved in transcription and translation of IL-18-induced IFN- γ mRNA and IL-18-induced cytolytic effects. Kalina et al reported that IL-18 strongly enhanced tyrosine phosphorylation of STAT3 and of the p38MAPK in NK cells. Additionally, the stimulatory effect of IL-18 on IFN- γ protein production was counteracted by inhibition of MAPK (Kalina et al., 2000). Furthermore, inhibition of p38 MAPK, blocked enhanced regulate stem cell factor expression, indicating that p38 MAPK activity is required for IL-18-enhanced SCF production in murine melanoma cells.

On the other hand It has been demonstrated that IL-18 secretion was through p38 and ERK1/2 MAPK pathways. Niyonsaba et al reported that the inhibition of p38 and ERK1/2, but not JNK, almost completely nullified IL-18 secretion in primary cultured human keratinocytes (Niyonsaba et al., 2005). In addition, the synthesis of pro-IL-1beta and pro-IL-18 in THP-1 macrophages was also reported by the activation of p38 MAPK and NF- κ B (He et al., 2012).

3.3.3 Receptor polarization in EFs

It is still very controversial how the cells sense an EF in the first place. In this study, IL18R α was redistributed to one side of the KG-1 cell membrane facing anode in the EFs, upon which the chemotaxis to IL-18 was enhanced. This has been further confirmed by *in situ* ICC staining in 3D culture. The real-time fluorescence imaging of the IL18R α over-expressed cells revealed the dynamics of IL18R α in the presence of EFs stimulation. The real time movies evidently showed the polarization of IL18R- α can be controlled by the direction of EFs in less than 5 minutes, and that over expression of IL18R α triggered the electrotaxis of DCs in comparison with low IL18R α expressing cells, suggesting that IL18Rs may be one of the upstream regulators to initiate EF-induced responses of immune cells.

One potential mechanism of electrotaxis is that cell surface receptors can be re-distributed by the applied EFs therefore trigger downstream signalling for directional sensing, and allowing cells to move along the EF vector directionally. A broad spectrum of EF-induced activities involves membrane receptors activation upon EF exposure. Pu reported that EF-enhanced directional migration correlates well with the expression level of EGF receptor (EGFR/ErbB1) (Pu et al., 2007). In addition, Zhao proposed that EGFR asymmetric redistribution triggered electrotactic response of cornea epithelial cells (CECs) (Zhao et al., 1997). Moreover, EF-directed reorientation, elongation and migration of endothelial cells have been reported to require VEGF receptor activation (Zhao et al., 2004). Our finding is consistent with the previous studies that EF-enhanced response of KG-1 cells to IL-18 requires the activation of IL18R α (Koutoulaki et al., 2010b). However, I showed that the polarization of the receptors rather than the up-regulation of expression is the key to trigger the downstream pathway.

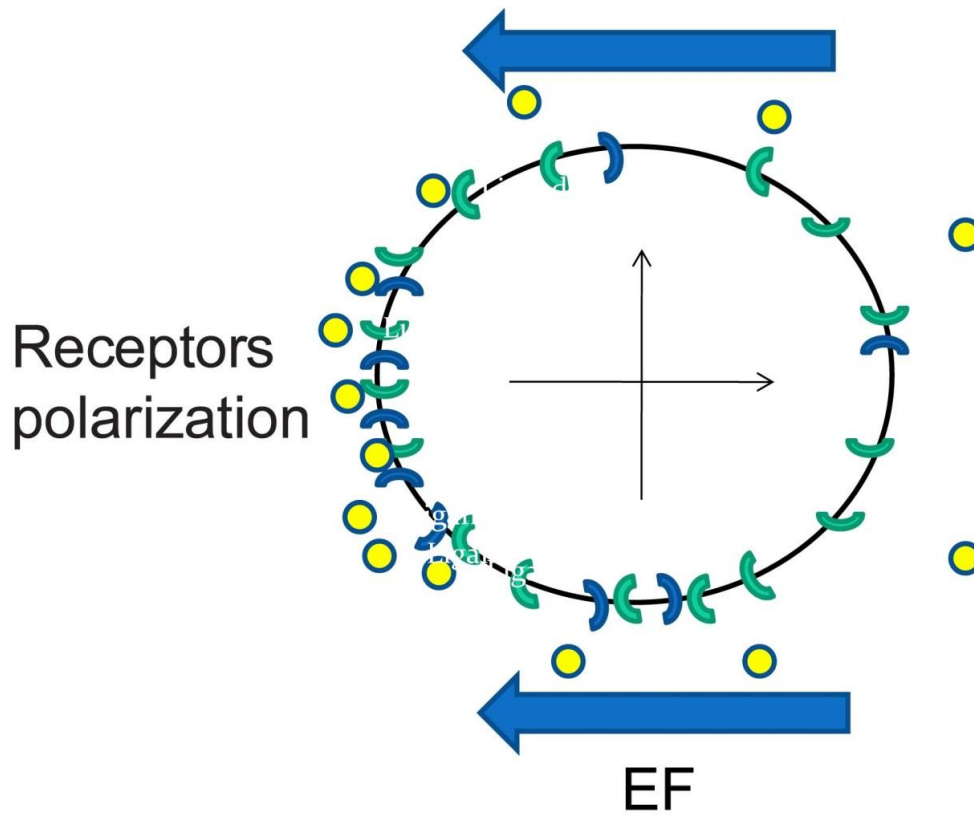
I demonstrated that IL18Rs are crucial for sensing EFs. In this study, I discovered that DCs overexpressing IL18R α were responsive to EFs stimulation whilst the cells with less IL18R α expression showed abolished effects. This suggests that potential signalling molecules might be intrinsically activated downstream of IL18Rs which interpret electric signals to downstream cellular response. In breast cancer cells, inhibition of ErBB1

completely abolished the directional response of MTLn3 cell (a breast cancer cell line) to EF stimulation. This was confirmed by transfecting low metastatic clone MTC cells with human ErbB1, which significantly increased the electrotactic response (Pu et al., 2007). Rajnicek proposed that Rho/Cdc42 is a molecular switch to control electrotaxis in CECs. They found that attenuation of cdc42 signalling abolished electrotaxis and enhanced contact guidance whereas inhibiting Rho signalling enhanced electrotaxis (Rajnicek et al., 2007a).

Deciphering the sensing mechanisms and the signalling cascade of electrotaxis is very important conceding embryo development, wound healing and cancer invasion. Using those “molecular switches” allows us to control cells physiological homeostasis while at the same time guiding their directional migration.

3.3.4 The role of actin in electrotaxis

It is clear that microfilaments are essential in cell migration. The actin cytoskeleton plays a major role in coordinating protrusions such as lamellipodia and filopodia, and forming adhesions, and finally retracting the tail (Le Clainche and Carlier, 2008). I showed in this study that IL-18 induced the increase of colocalization of IL18R α and actin. One of the explanations of the electrotaxis of KG-1-derived DCs towards IL-18 is to be through the polarized colocalization of IL18R α and actin. Komai-Koma et al first identified that IL-18 induced chemotaxis of T cells is via actin filaments assembly (Komai-Koma et al., 2003). Asymmetric redistribution of actin was observed in EF-induced directional migration of CECs (Zhao et al., 1997). Based on previous results, I propose in this study that asymmetric actin assembly is involved in the regulation of electrotaxis through EF-induced polarization of IL18R α .



 Receptor

 Ligand

Figure 3-16 Receptor polarization in EFs

Schematic illustrates the model of EF-induced cell response. The receptors on the cell membrane are polarized to the anodal side by EFs induction. The accumulation of the receptors locally enhances the ligand-receptor interaction thus triggers the downstream signalling pathways.

3.3.5 Advantage of 3D model

I adopted 3D culture system in this study to evaluate the interactions between electrotaxis and chemotaxis of KG-1 and KG-1-derived DCs. 3D culture system

apparently has several advantages compared to the conventional 2D model. Firstly, even though 2D culture technique is routinely and widely used, the cell culture in 2D is arguably primitive and does not reproduce the physiological environment *in vivo*. The 3D culture model can overcome these shortcomings and clearly shows more physiological relevance. Secondly, the diffusion of IL-18 can be controlled in 3D culture to easily form a chemical gradient, thus the cellular response to IL-18 can be measured accordingly. In the contrary, it is difficult to generate an IL-18 gradient in 2D culture (doable with coated slides through bio-engineering; however it is highly technically demanding). Thirdly, EFs application in 3D culture can avoid the disturbance induced by micro movement of medium.

3.4 Summary

Chemotaxis and Electrotaxis have been compared and argued of their influence in the regulation of wound healing. Chemotaxis enables immune cells to reach sites of infection, regulate tissue homeostasis and is crucial for forming embryonic patterns (Jin et al., 2008). Electrotaxis, on the other hand, has been shown to have overriding effects in directing cell migration during wound healing and embryo development (Zhao, 2009).

In this study, I summarized the data with a model of directional sensing and migration in electric fields. I propose that the EF-induced re-distribution of the receptors on the cell surface results in a shift of membrane receptors between the cathode-facing and the anode-facing membrane the cell (Figure 3-16). There would be a higher probability to overcome the threshold of signal transduction at the higher density receptor side. The downstream signalling cascade therefore can be ignited. Although further evidence is required to clarify the electrotactic ability involving various specific membrane receptors on difference cell types, the possible differential membrane receptor distribution across the cell axis induced by the EFs is expected to initiate the cells to sense the EF.

More importantly, those data imply that the applied EFs can direct the orientation and migration of cells in the presence of a chemoattractant gradient enabled by the receptor polarization mechanism. Thus, in the possible physiological situations wherein co-existing chemoattractant gradients and EFs are presented such as during the wound

healing, electrotaxis of relevant cell types may preferentially guide the migration of cells over chemotaxis that will either facilitate or delay the normal physiological processes depending on the configurations of the co-existing chemical and EFs. Furthermore, such preferential migration toward the EFs may permit novel non-invasive clinical applications for guiding and manipulating cell trafficking and positioning in tissues electrically without the distraction from the chemotactic guiding cues.

The existence of various chemoattractants in tissues supports the fundamental hypothesis that electrotaxis can be initiated by receptor shifting on the cell membrane and activated by the chemoattractants. More complications arise by considering polarization of multiple cell membrane receptors and multiple cytokines/chemoattractants (presumably at different rates and toward different directions relative to the EFs) and the downstream signalling processes and interplays triggered by these receptors. Despite these limitations and complications, the present studies discussed that EF-induced membrane receptor polarization amplifies the downstream signal cascade, provides interesting predictions that are consistent with previously reported experimental results, and further suggests the possibility of preferential orientation and migration of immune cells toward the EFs in coordination with chemoattractant gradients.

In short, the mechanism of EF-induced KG-1/DC sensitization in responding to cytokines and its impact on DC migration were demonstrated for the first time in this study. The results indicated that physiological and pathological EFs can be potentially involved in the important host immune responses. The finding may have an implication of clinical therapy for cancer treatment and help to develop more effective vaccination protocols.

**CHAPTER 4 ELECTRIC FIELDS CONTROL DIRECTIONAL
MIGRATION OF LANGERHANS CELL *IN VITRO* AND *IN VIVO***

4.1 Introduction

In this study I investigated the migratory activity of Langerhans cells exposed to a physiological electric field (EF) and IL-18 gradient *in vitro* and *in vivo*. Firstly the concurrent effects of both guidance cues on 3D cultured Langerhans cells were studied; and the migration rate and directionality were analysed. Additionally, this was tested on mouse epidermis tissue culture to further explore the Langerhans cell migration with these two treatments. Finally, the IL-18 and EFs were applied intradermally on mice and the migratory Langerhans cells were isolated, sorted and analysed.

4.1.1 Langerhans cells

Langerhans cells (LCs) – a specialized subset of dendritic cells (DCs), has been considered to be the major sensitizing cells in the skin by initiating productive immunity in naïve resting T cells. Epidermal LCs account for 3-5% of all nucleated cells in epidermis of mice and human. They are arranged in a network close to keratinocytes (Figure 4-1).

LC is derived from bone marrow stem cells, which was found in mice and human (Frelinger et al., 1979; Volc-Platzer et al., 1984). LC precursors acquire immunologically important molecules/markers such as CD45, MHC class II and Langerin. Myeloid monocyte cultures induced with GM-CSF, IL-4 and TGF- β can differentiate into dendritic cells / LCs (Geissmann et al., 1998). LCs express typical myeloid antigens such as CD11b and CD33. However, LCs also express CD8- α , a marker of lymphoid descendants, when they achieve maturation (Anjuere et al., 2000).

Migration and transport of antigen from the skin to the lymph nodes is an important feature of LCs. This may lead to immunity or tolerance, depending on the circumstances under which migration takes place (Romani et al., 2003). However, there is still no effective and mature method to isolate and primary culture LCs *in vitro*. Moreover, there are two status of LCs residing in the epidermis: resting and inflammatory. They are interconvertible in response to stimuli, which also raise the bar of difficulty to study the pure group LCs *in vivo*.

4.1.2 How to study LCs

Histochemistry is the most widely used method for studying distribution of LC in the skin. Skin offers a simple and helpful experiment model, because it can be easily separated into epidermis and dermis sheets using simple chemicals (EDTA) or enzymes. Such sheets allow an en face view onto the epidermis that facilitates quantification and investigation.

The classic method to obtain LC is to enzymatically separate epidermis and dermis and to digest epidermis into single cell suspension. Trypsin and dispase are commonly used for this purpose. Because the resulting suspension only contains about 1-3% LCs, so that the enrichment method becomes crucial for *in vitro* LC study. However, the LC obtained by trypsinization is immature, yet they do not fully represent LCs *in situ* (Schuler and Steinman, 1985a). Secondly cell surface markers may be sensitive to trypsin treatment, so freshly isolated LC should be carefully tested.

Skin explant is a very useful alternative approach to the trypsinization method. Enriched LCs would emigrate from the epidermal sheet explant culture over a period of 2-4 days. This method has been successfully established in human and mouse skin models (Romani et al., 2003). In contrast to the trypsinization method, the LCs isolated from skin explants are mature by all established phenotypical, morphological and functional criteria (Romani et al., 2003).

In terms of the application of specific marker for LC, Langerin and other common DC markers such as Cd11c, MHC class II largely facilitates the *in vivo* research. The GFP conjugated Langerhans cells and *in vivo* imaging technology allow visualization of LC behaviours in real time. Animal *in vivo* experiments especially using the genetic modified mice have largely expanded our understanding of the functions of LCs in both physiological and pathological situations.

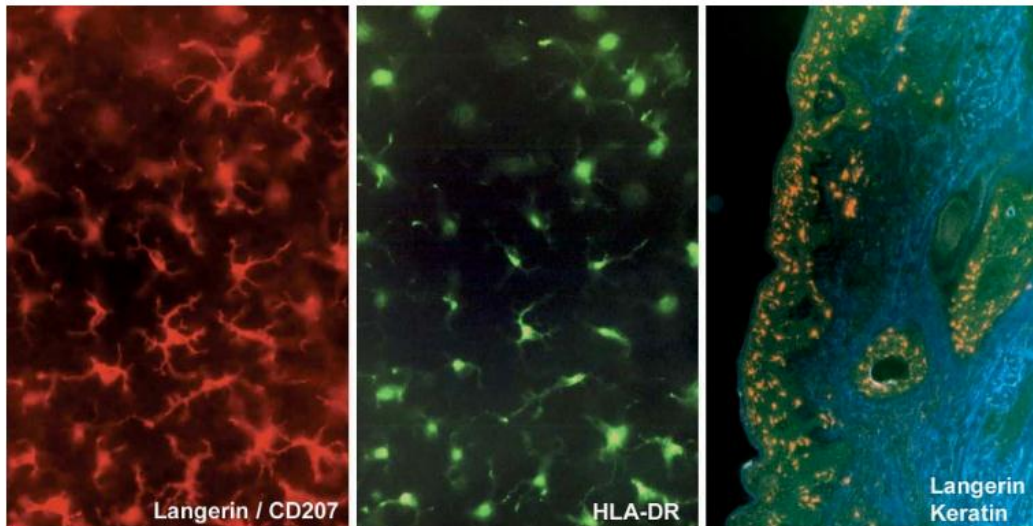


Figure 4-1 IHC staining of LC

Left and middle: the typical network of LC on epidermal sheet. The epidermal sheet was double -labelled with MHC class II (green fluorescence) and Langerin (red fluorescence). Right panel: Section of human skin immunolabeled with antibody against Langerin (red/yellow fluorescence) shows exclusive localization of Langerhans cells in the epidermis (Romani et al., 2003).

4.1.3 LC migration *in vitro*

After the discovery of LC, the characterisation of its physiological function has lagged mainly due to the hurdles in isolation and culture techniques. Schuler and Steinman first investigated the function of LCs in comparison with spleen DCs *in vitro* (Schuler and Steinman, 1985b). They used trypsinazition method to isolate LCs and found LCs were similar to spleen DCs morphologically and functionally. Both cell types are non-adherent, appearing buoyant density, and express high level of MHC class II. However, during the *in vitro* culture, the immunostimulatory ability of LCs became stronger than spleen DCs with challenged with GM-CSF. Several studies confirmed that freshly isolated LCs lack of immunostimulatory and migratory ability, but with GM-CSF and IL-4 treatments they became mature (Gerberick et al., 1991; Picut et al., 1988).

Apart from immunostimulatory capacity, migratory ability was also investigated as another intrinsic feature of LCs. Kobayashi applied a reconstituted basement

membrane invasion assay to evaluate the migratory capacity of LCs *in vitro* (Kobayashi et al., 1995). They showed that hapten could trigger LC migration. Additionally, Saitoh showed that anti-IL-6 and anti-TNF- α antibodies inhibited donor LC migration into epidermis, which indicates that IL-6 and TNF- α are responsive to promote LC migration (Flint et al., 1998). Moreover, Noirey reported that TNF- α initiated pro-MMP2 and pro-MMP-9 production followed by cell migration in a dose dependent manner in explant culture (Perez et al., 2005). Furthermore they proved that antigens (rBet v1) induced synthesis of TNF- α and TNF R II in LCs which implied an autocrine loop in antigen-induced LC migration (Noirey et al., 2002).

However, the mechanism of LC migration *in vitro* is still an enigma. It is largely unknown why the matured LCs gain the capacity to migrate, how these cytokines mediate the LC migration, and which signalling cascade(s) are involved.

4.1.4 LC migration *in vivo*

The physiologic roles of LC were characterised by a series of *in vivo* studies on contact hypersensitivity. The typical contact hypersensitivity study is to sensitize mouse by painting ears with irritants such as oxazolone and trinitrochlorobenzene. The intensity of contact hypersensitivity is indicated by ear swelling. The correlation between the density of LC in epidermal and ear swelling index reveals that LCs play the main role in the induction of contact hypersensitivity. Additionally, pre-treatment with neutralizing IL-1 β antibody, but not TNF- α and GM-CSF, significantly prevented the allergen-induced contact hypersensitivity of mouse ear (Rambukkana et al., 1996). This suggests that IL-1 β is crucial for initiation of LC migration in contact hypersensitivity. The following studies not only confirmed this result but also identified the signalling pathway of allergen-induced LC migration. Antonopoulos investigated contact hypersensitivity using IL-1 β and IL-18 knockout mice and found that IL-18 plays a central role in cutaneous immune response by mediating LC migration in contact hypersensitivity, acting upstream of IL-1 β and TNF- α (Antonopoulos et al., 2008). Additionally, IL-4 was found to prevent LC migration through downregulating TNF RII (Takayama et al., 1999). Moreover, enhanced LC migration was found in IL-10 knockout mice due to its reversed regulation on TNFR II expression (Wang et al., 1999b). Except IL-18-IL1 β pathway, there are a variety of other cytokines which were found also important for DC migration *in vivo* (Wang et

al., 1999a). Sato reported that deficiency of CCR 2 (CC chemokine receptor) led to reduced LC migrated to Lymph node.

Another focused area of LC migration is cell-skeleton molecules. Price found that alpha 6 integrin plays an important role to induce LC migration into lymph node by regulating access across the epidermal basement membrane (Price et al., 1997). Ross reported that beta and gamma actin isoforms were upregulated during LC maturation, suggesting actin filaments also are required for LC motility (Ross et al., 1999). In addition, the actin-binding protein fascin was reported to be involved in the formation of dendritic process of motile LCs (Ross et al., 1998). In short, the *in vivo* research of LC migration is summarized in Figure 4-2.

Based on the fact that the cytokine secretion and electric fields are co-occurring in the skin tissue during the wound healing or inflammation, I hypothesise that physiological EFs could potentially play a role in IL-18 mediated LC migration *in vivo* and *in vitro*.

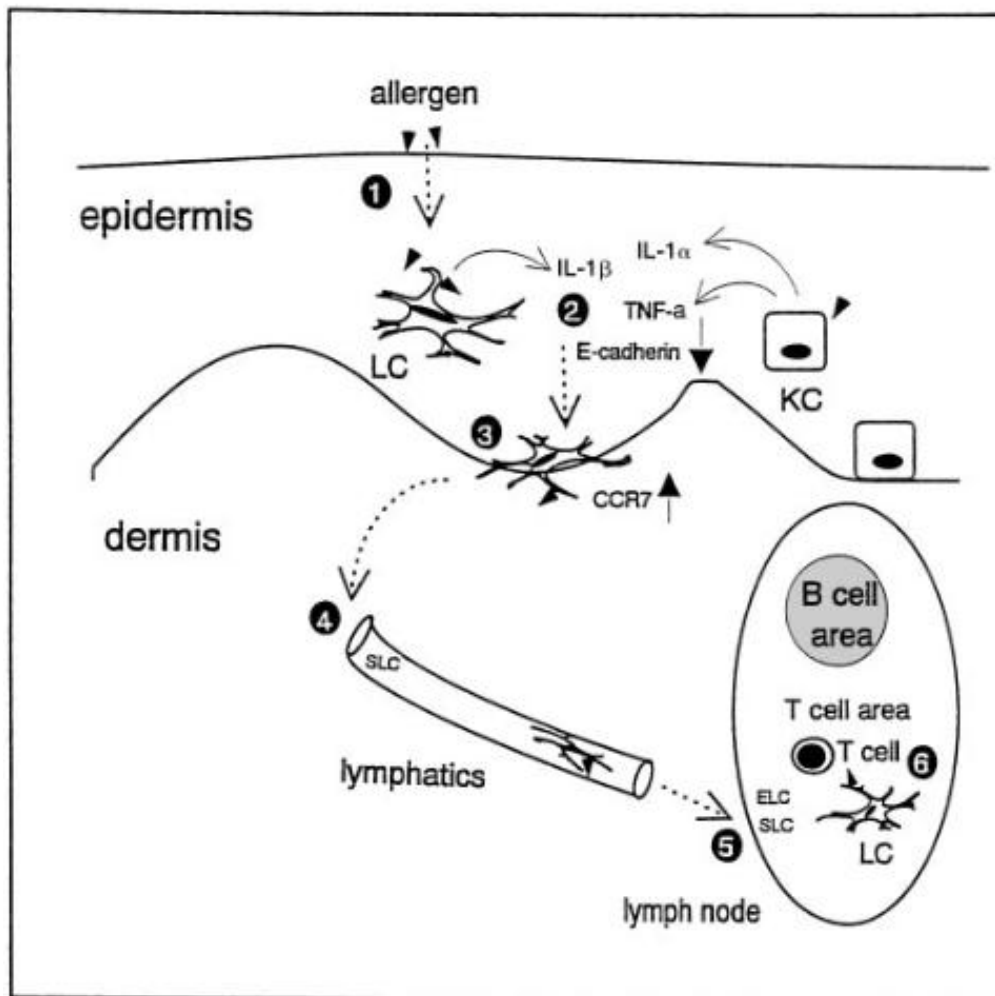


Figure 4-2 Schematic of LC migration

The migratory steps of LCs and major contribution of cytokines are highlighted (Wang et al., 1999a): (1) contact allergens penetrate the epidermis. (2) LCs are activated by IL-1 β and TNF- α . (3) downregulation of E-cadherin leads to LC migration from epidermis to dermis. (4) upregulation of CCR7 on LCs and its ligand SLC attract LCs into lymphatic. (5). ELC another ligand of CCR7 attracts LCs into T cell area. (6). LC present processed antigen to naïve T cells (Ross et al., 1998).

4.2 Result

In this chapter EF-modulated migration of LC in response to IL-18 was assessed *in vitro* and *in vivo*. This allows us to comparatively and systematically study the potential roles of electric signals in immune defence of the skin.

4.2.1 EFs induce LC migration in response to IL-18 in 3D culture

Primary mouse epidermal LCs were isolated using dispase enzyme and cultured in RPMI complete medium containing 50 ng/ml GM-CSF and 20 ng/ml IL-4. Cell were maintained in 5% CO₂, 37 °C incubator for 2 days before EF stimulation. LCs were mixed with Matrigel and then seeded into EF chamber for EF stimulation. The EF experiments were divided into 4 groups: control, IL-18 only, EFs only and IL18+EFs. IL18 was injected locally on one side of the chamber 10 minutes before EFs to generate an IL-18 gradient across the gel. The EF strength was 200 mV/mm and the whole exposure lasted about 2 hours.

Upon EFs application to 3D cultured LCs, there was no significant change on migration speed and directionality (Figure 4-3, A (a-b) and C (a-b)). The migration speed of control and EF stimulation group were 0.77 ± 0.32 $\mu\text{m}/\text{min}$ and 0.97 ± 0.34 $\mu\text{m}/\text{min}$, respectively, and the directionality index were -0.26 ± 0.067 and -0.17 ± 0.081 , respectively. In the absence of EFs, the diffused IL-18 slightly upregulated migration speed of LC up to 1.74 ± 0.65 $\mu\text{m}/\text{min}$ and the directionality is up to -0.43 ± 0.052 (Figure 4-3, A (c), B (c), C and D). Interestingly, EFs significantly increased the migration speed of IL-18 treated LCs to 3.33 $\mu\text{m}/\text{min}$ compared with control, EF only and IL-18 only groups. EFs also promoted the directedness of LCs to -0.68 ± 0.05 , indicating that in the presence of EFs LCs sensed IL-18 and moved more persistently toward the IL-18 source.

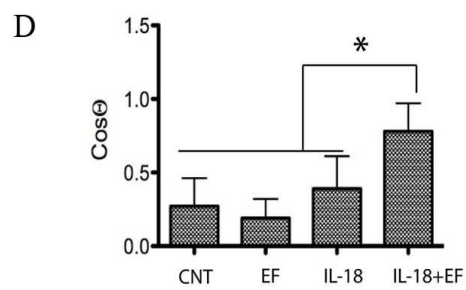
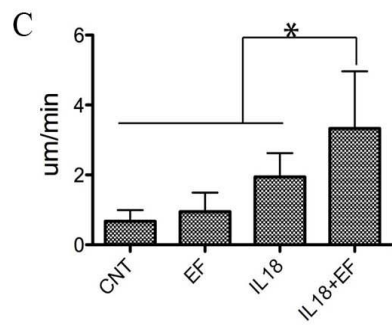
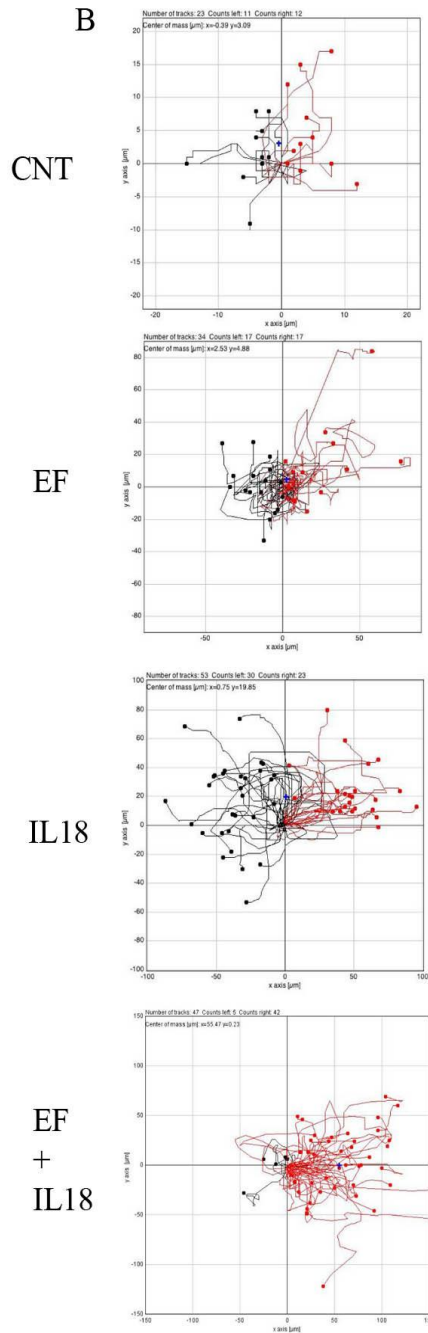
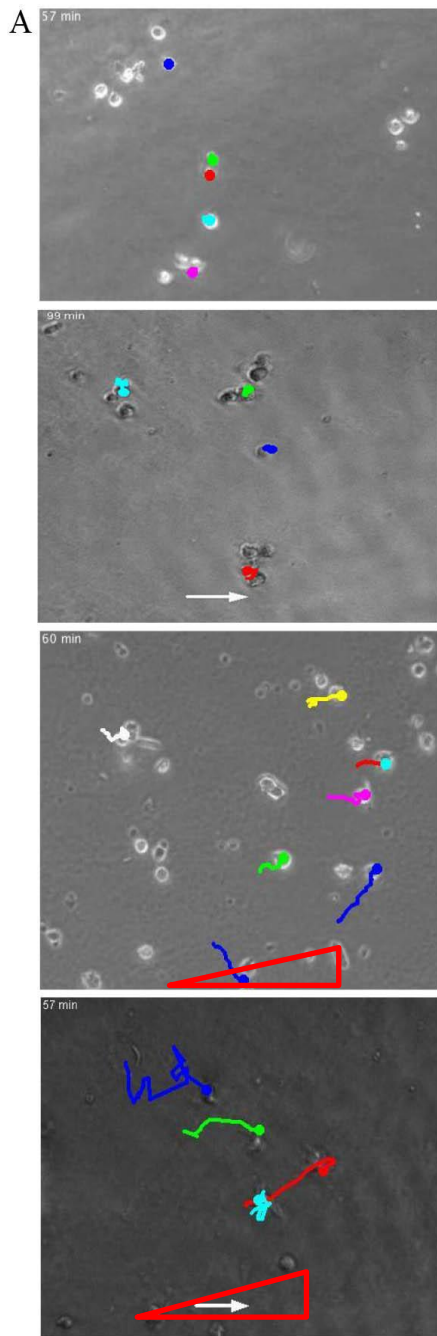


Figure 4-3 EFs enhance LCs migration towards IL18

Primary cultured mouse LCs were mixed with Matrigel and then seeded into EF chamber. IL-18 (200 μ l, 50 ng/ml) was injected at the right side of the chamber and the IL-18 gradient across the chamber was established by diffusion (A (c) and (d)). (the high side of triangle indicates the high IL-18 concentration).

A. The cells were exposed to 200 mV/mm EFs (A (b)), IL-18 gradient (A (c)), and EFs plus IL-18 (A (d)), respectively, for 2 hours and the cell migration were tracked and analysed. The control group cells were without any treatment.

B. Composite drawings where each cell is superimposed to origin point and the cell migration path traced in each situation corresponding to A.

C. Migration speed of LCs in each condition of (A) were analyzed accordingly.

D. Migration directedness of LCs in each condition of (A) was analyzed accordingly. Data are presented as mean \pm SD, *: $P < 0.05$

4.2.2 EF induce LC migration *ex vivo*

Mouse ear skins were split and the epidermis halves were prepared in explant culture as described in Chapter 2. After 18 hours of explant culture, the epidermis sheets were transferred into EF chamber treated with IL-18 (200 ng/ml), 200 mV/mm EFs and IL-18 plus EFs, respectively. The experiments lasted from 60 minutes to 2 hours, the emigrated LC were counted. It was evident that only in the treatment of EFs plus IL-18, a large quantity of LC emigrated out from the epidermis; on the contrary, neither EFs nor IL-18 alone induced LC migration out of the epidermis (Figure 4-4 and supplemental movies). However, it took about 40 minutes to induce LC migrate under EFs plus IL-18 exposure according to the time lapse recording data, indicating cells need to be sensitized or activated first to build up the essential regulating force(s) before electrotaxis can be initiated.

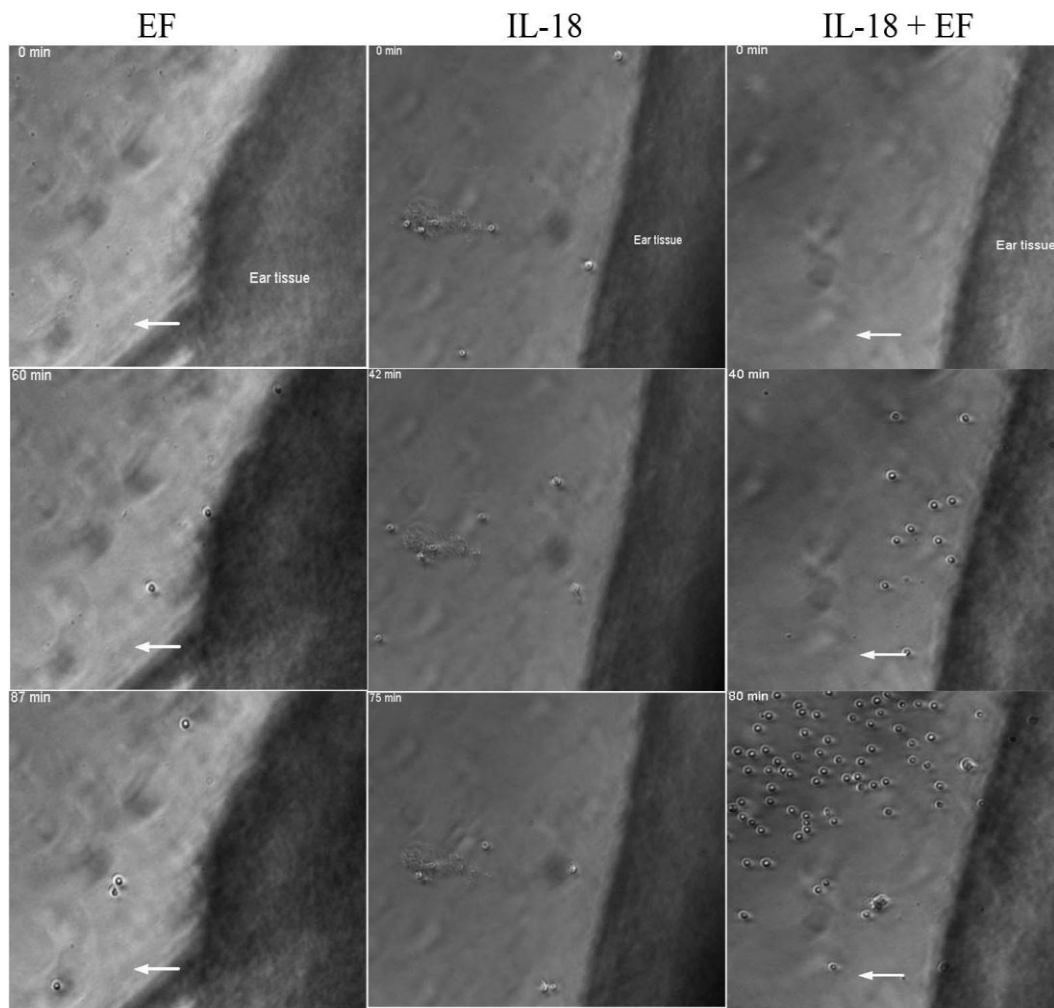


Figure 4-4 EFs and IL-18 promote LC emigrate from epidermis explants.

Mouse ear epidermis was cultured in EF chambers and stimulated with EFs (200 mV/mm), IL-18 (200 ng/ml) and EFs plus IL-18 for 2 hours, respectively. Arrows indicate the direction of EFs and the ear tissue are labelled. Compared with the limited emigration of cells out of the epidermis when treated with either EF (left) or IL-18 alone (middle), a combination of both treatments synergistically enhanced the emigration of LCs (right).

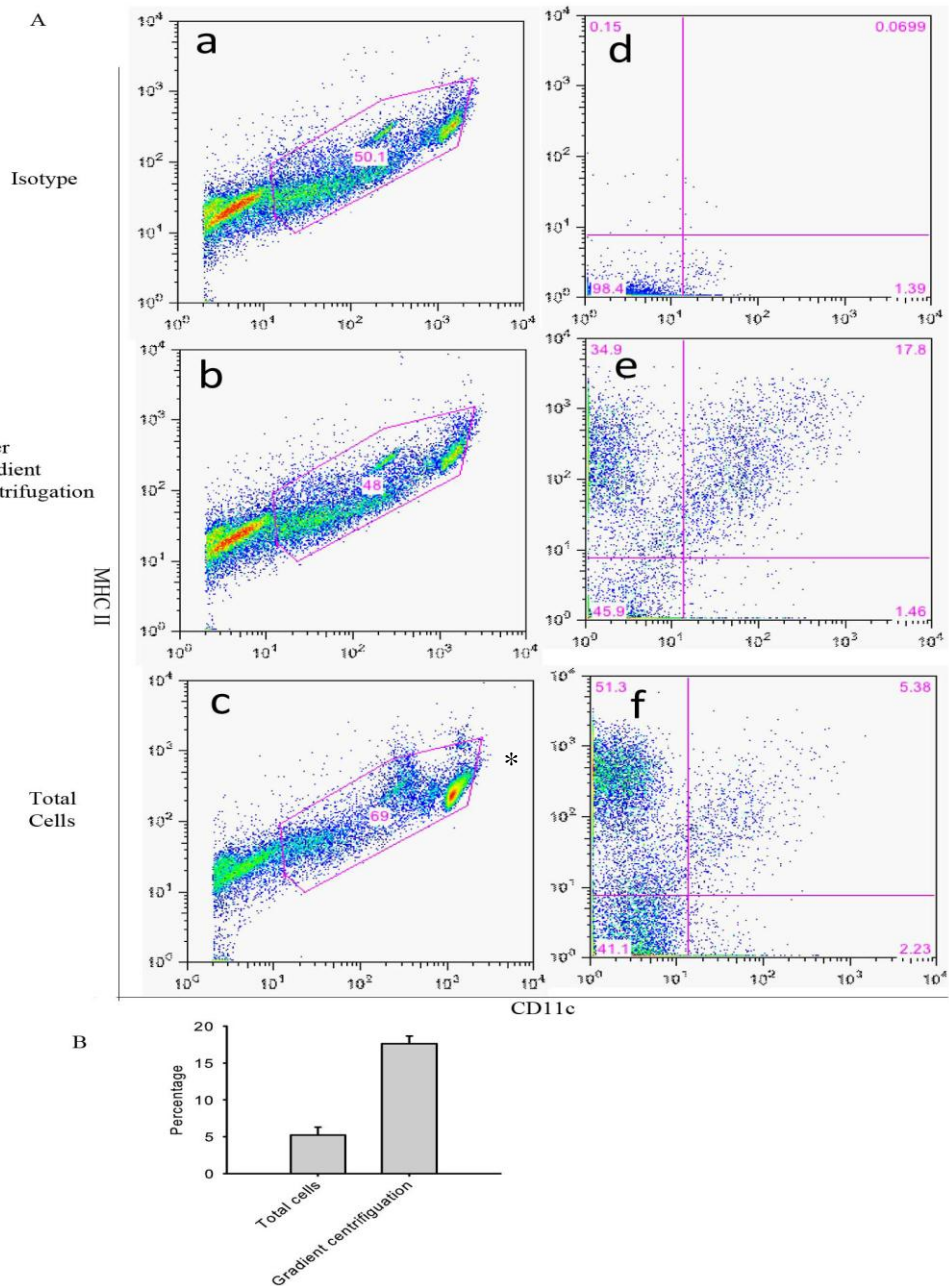


Figure 4-5 Quantification of DCs from lymph node.

Total lymph node-resident cells (A right) and gradient centrifugation enriched cells (A, middle) were characterized by FACS using MHC class II and CD11c as DC markers. The isotype control was shown in A (left). The percentage of DCs in total cells and enriched cells population were analysed, respectively (B). Data are presented as mean \pm SD, *: $P < 0.05$.

4.2.3 Quantification of DCs from lymph node

The inguinal lymph nodes were excised and the total cells were divided into two groups. One part cells were enriched by gradient centrifugation and the other group

kept intact. The cells were then incubated with anti-MHC class II-PE and anti-CD11c-FITC to positively mark dendritic cells.

The FACS profile shows that about 17.8% cells in gradient centrifugation group are MHC class II⁺ and CD11c⁺ compared with only 5.38% double positive cells in total cell group (Figure 4-5, A). The statistics suggests that the DCs are enriched about 3 times by gradient centrifugation (Figure 4-5, B).

4.2.4 LC isolation and identification

The DCs in lymph node consist of several group cells come from different sources, including residing DCs, skin originated LCs and interstitial tissue derived-DCs (Henri et al., 2001). After gradient centrifugation, I stained the enriched DCs with anti-Langerin-PE and anti-IL18R α -APC. The FACS results showed that there are about 8% Langerin positive DCs, and 3% DCs are Langerin and IL18R α double positive (Figure 4-6).

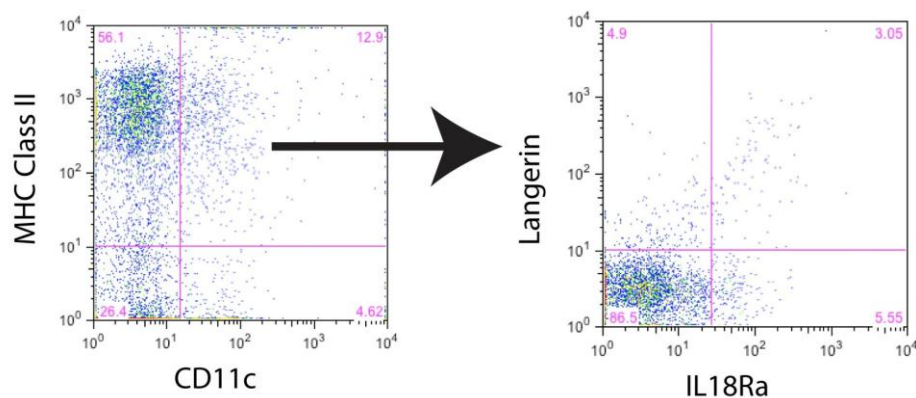


Figure 4-6 Identification LC population in DCs.

The lymph node cells, after gradient centrifugation, were divided into two equal parts. Each part was double stained with MHC class II-PE plus CD11c-FITC (Left) and Langerin-PE plus IL18R α -APC (right), respectively. Arrow: indicates the cells (right) come from which gate area of FACS result (left).

Considering that only 12-17% cells in lymph node are MHC class II⁺ and CD11c⁺, the Langerin positive cells are less than 1% in the whole cell population. Thus the quantification of the proportion of LC in lymph node DCs provides a foundation to explore if exogenous stimuli (EFs and IL-18) could alter the LC migration in epithelia.

4.2.5 Both IL-18 and EFs induce LC migration into lymph node

It has been demonstrated that Fluorescein isothiocyanate (FITC) painting is an effective way to be used as an immunofluorescence marker for cells and a contact sensitizer (Wang et al., 1997). The uptake of FITC by APC can be tracked by fluorescence (Bigby et al., 1987; Szczepanik et al., 1998; Wang et al., 1997). In this study mice were painted on the shaved tail base with 50 μ l ml 0.5% FITC in 1:1 acetone/dibutylphthalate. The experimental mice were divided into four groups: Control (unpainted), FITC painted, FITC painted plus intradermal administration of 50 ng IL-18, FITC painted plus intradermal administration of 50 μ l PGE2 (0.1 mM). The drugs were injected 30 minutes after painting to allow FITC to dry down. The inguinal lymph nodes were collected from the mice 24 hours after treatments. The lymph nodes were gently disrupted and the cells were harvested and enriched as the described in Chapter 2. The enriched cells were consequently analysed with FACS.

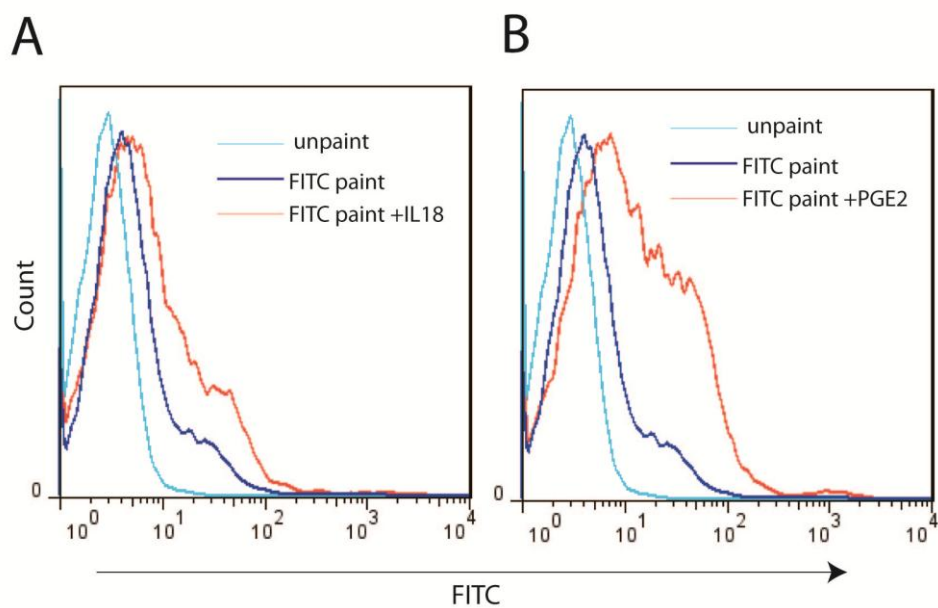


Figure 4-7 Intradermal administration of IL-18 or PGE2 induces LC migration into lymph node

Mouse tail base was painted with FITC (50 $\mu\text{g}/\text{ml}$, 25 μl) and then intradermally injected with IL-18 (50 ng; A) or PGE2 (0.1 mM, 50 μl ; B), respectively. The lymph node resident cells were enriched by gradient centrifugation. The FITC fluorescence intensity of the enriched cells were thus analysed by FACS. The unpainted naïve mice were taken as control.

The histogram in Figure 4- is composed by the number of cells (Y axle) and the strength of fluorescence (x axis). With FITC painting, the histogram (blue) shifts to right, suggesting there are more cells carrying fluorescence in the lymph node than unpainted mice. Intradermal injection of IL-18 enhances the histogram (red) shift to right further (Figure 4-A). This implies that IL-18 induces more APC cells migration compared to FITC painting alone.

PGE2 is a strong chloride channel activator and can stimulate an increase in net chloride efflux in epithelia layer (Shimizu et al., 1993). It has been demonstrated that administration of PGE2 could effectively increase trans-epithelium potential difference, which potentially play essential roles in many physiological and pathological situations including wound healing (Nuccitelli et al., 2008; Song et al., 2002). In this study, I intradermally injected PGE2 to increase the TEPD across the epidermis. Figure 4- (B) illustrates that PGE2-generated TEPD dramatically shifts the histogram to the right, indicating TEPD has induced more FITC positive cells migration to lymph nodes compared to FITC painting and control group.

4.2.6 Quantification of IL-18 induced DC and LC migration

The FITC bearing cells are supposed to be motile APC cells migrated from skin to lymph node. However, except APC the FITC still can be carried into lymph node, such as diffusion. Secondly, the APCs in skin are composite of several subsets, including LC and dermal DC. Thus, to quantitatively analyse the proportion of LC migrated into lymph node, I sorted FITC positive cells out and stained them with either DC specific markers MHC Class II and CD11C or LC specific marker Langerin.

With FITC painting, about 5% FITC bearing DCs showed MHC Class II and CD11c double positive; about 1% Langerin positive LCs migrated into lymph node (Figure 4-, A, B). With IL-18 stimulation, an increased proportion of DCs (~ 11% of total FITC positive cells) appeared in the lymph node, and the proportion of LCs also rose to about 5.6% (Figure 4-, A, B). In comparison with FITC painting alone, IL-18 administration increased the migration of DCs and LCs into lymph node, around 2 folds and 5 folds, respectively (Figure 4-, C and D).

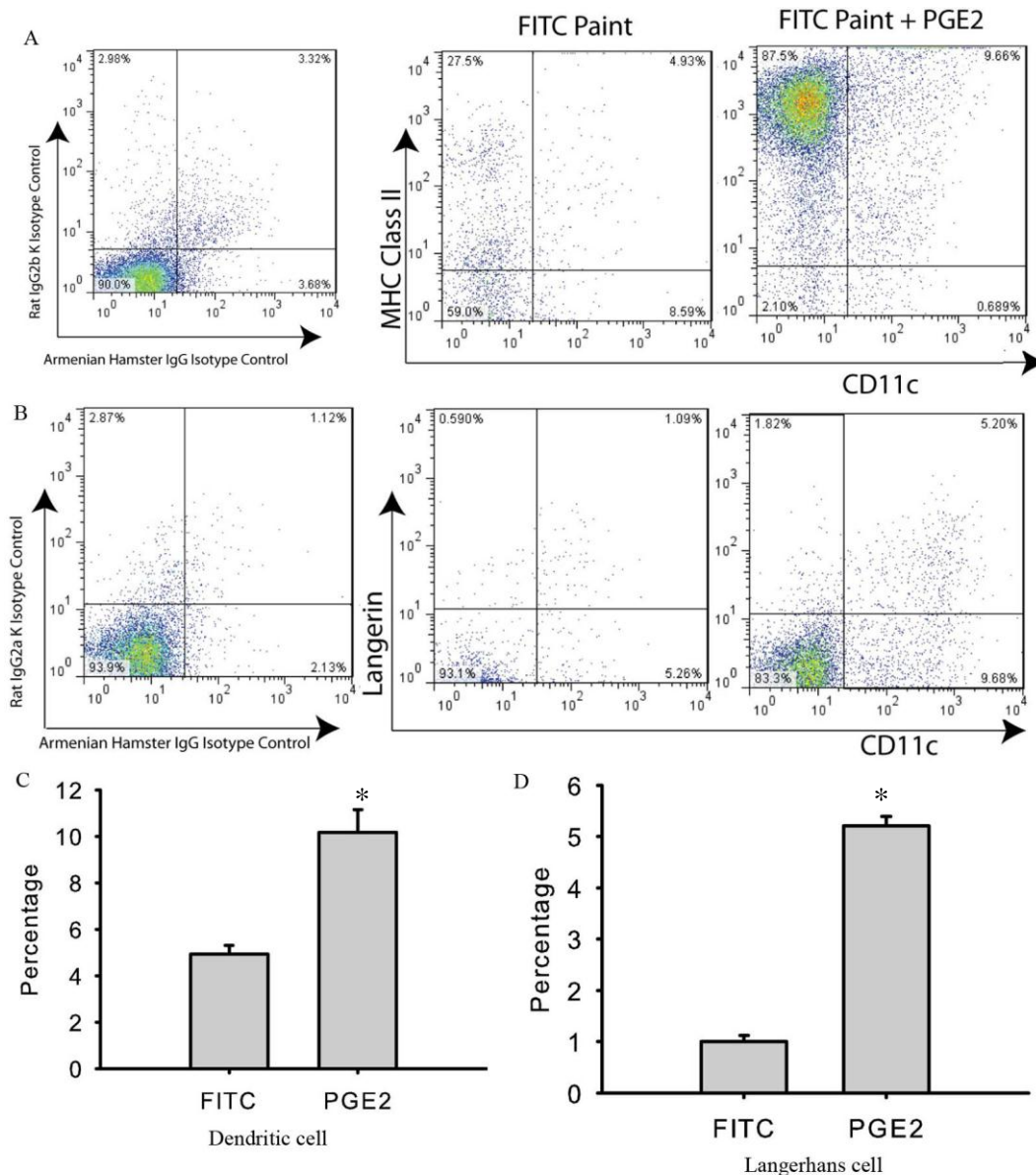


Figure 4-8 IL-18 induces DC/LC migration into lymph node

Mouse tail base was painted with FITC (50 $\mu\text{g/ml}$, 25 μl) and injected with IL-18 (50 ng) and equal amount PBS as control. The next day, dendritic cells from inguinal lymph nodes were enriched and double stained with MHC class II-PE plus CD11c-APC (A) and Langerin-PE plus CD11c-APC (B), respectively, for FACS characterisation.

The portions of emigrated epidermal DCs (C) and LCs (D) in inguinal lymph nodes with FITC painting and IL-18 injection were analyzed, respectively. Data are presented as mean \pm SD, *: $P < 0.05$.

4.2.7 Quantification of DC/LC migration induced by EFs into lymph node

Figure 4- A and B illustrate that the proportion of DC and LC induced by PGE2 generated TEPD are 11% and 5.6%, respectively. The statistical analysis suggests that there are more DCs and LCs migrate into lymph node when treated with PGE2, with about 2 fold and 5 fold more respectively, compared to FITC painting alone (Figure 4-, C and D).

Both IL-18 and PGE2 have been shown to induce more proportion of LC to migrate into lymph node. However, there is no difference between IL-18 and PGE2 group. Considering the possibility that PGE2 may also has pharmacological effects on LC migration (Preliasco et al., 2008), the actual capacity of PGF2 induced EFs in LC migration need to be further investigated with more well-controlled experiments.

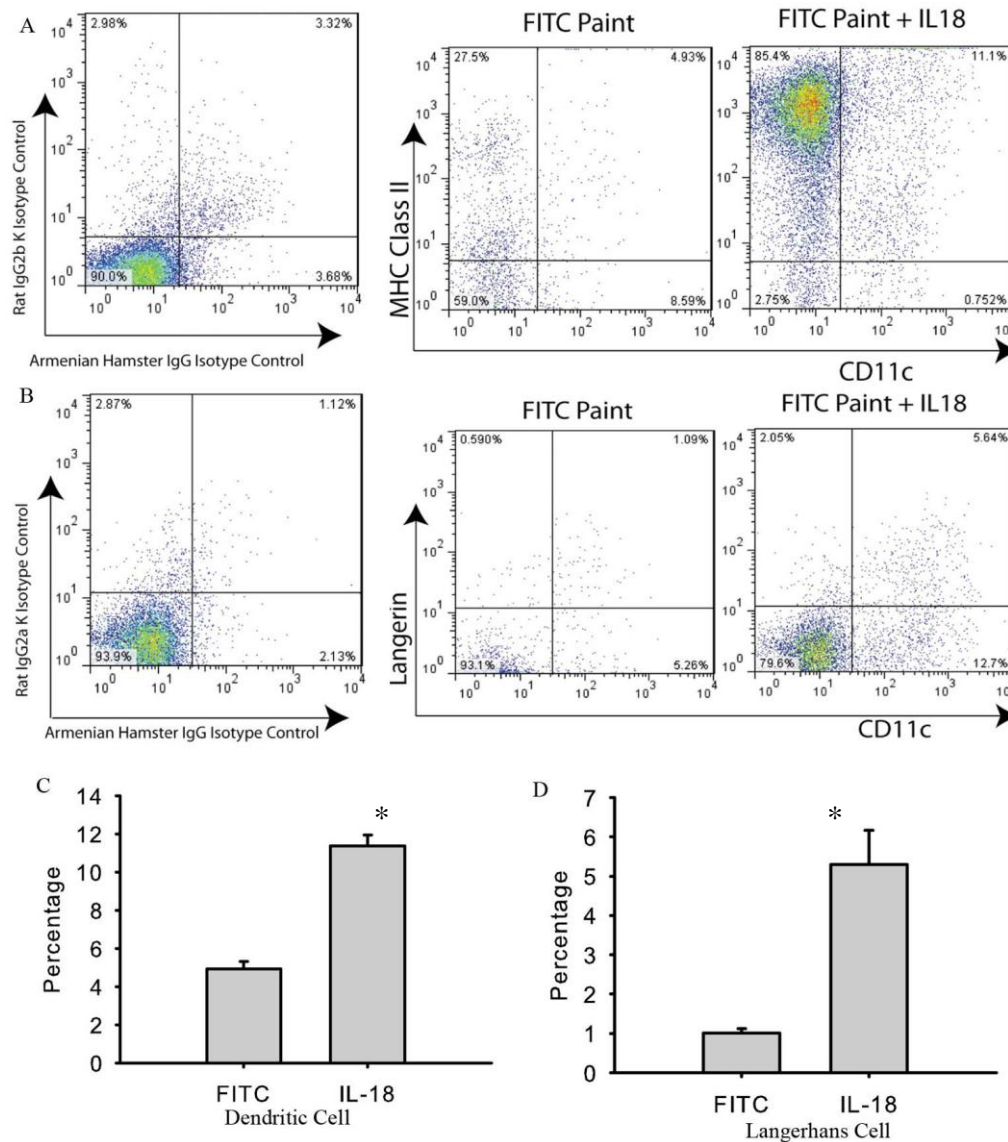


Figure 4-9 EFs induce DC/LC migration into lymph node

Mouse tail base was painted with FITC (50 $\mu\text{g}/\text{ml}$, 25 μl) and injected with PGE2 (0.1 mM, 50 μl) and equal amount PBS as control. The next day, dendritic cells from inguinal lymph nodes were enriched and double stained with MHC class II-PE plus CD11c-APC and Langerin-PE plus CD11c-APC, respectively, for FACS analysis. DCs (A) and LCs (B) were characterised in each experimental condition. The portions of emigrated epidermal DCs (C) and LCs (D) in inguinal lymph nodes with FITC painting and IL-18 injection were analyzed, respectively. Data are presented as mean \pm SD, *: $P < 0.05$.

4.3 Discussion

In this chapter I discussed the migration of LCs induced by electric signals and IL-18, and investigated the hypothesis that EFs promote the sensitivity of LC in response to IL-18 thus the migration of LC is increased.

4.3.1 EFs induce LCs migration towards IL-18 gradient *in vitro*

In this study I investigated the migration of LC under the exposure of either EFs or IL-18 or both in 3D culture. I demonstrated that neither EFs nor IL-18 can alter the migration speed of LCs. However, EFs plus IL-18 synergistically increased LC migration speed. Additionally, I observed that EFs also increased the sensitivity of LCs in response to IL-18, so that LCs directionally migrated towards the source of IL-18 in the presence of EFs.

I first established the experimental approach for studying the effects of EFs on LCs in response to IL-18 in 3D culture. LC is a type of cell which is fragile to artificial environment. Thus I maintained freshly isolated LCs with the full RPMI medium containing cytokines GM-CSF and IL-4, which were reported to be essential for maintaining the viability of LC *in vitro* (Rupec et al., 1996; Yokota et al., 1996). Moreover, Matrigel was used to build up a 3D environment which is suitable to build up cytokines gradient and apply EFs. Using this system, the LC behaviours under stimuli in 3D culture were recorded and analysed thereafter. I demonstrated that EFs synergistically induced LCs directional migration towards IL-18 gradient; however neither IL-18 nor EFs alone could change the directional migration of LCs. Moreover, Wang reported that migratory LCs expressed higher IL-18 than static resident LCs during contact hypersensitivity (Wang et al., 2002). These data indicate that the threshold of LC to sense IL-18 could be potentially reduced by EF and that these mechanisms seemed to work via feed-back loop.

It has been demonstrated that freshly isolated LCs are immature and lack motility (Picut et al., 1988; Schuler and Steinman, 1985b). Cytokines such as IL-1 β and TNF- α are supposed to play an important role to promote their maturation *in vitro* (Noirey et al., 2002; Saitoh et al., 1999; Tang and Udey, 1992; Yokota et al., 1996). IL-18 belongs to IL-1 family and is also suggested to induce LC migration *in vivo* (Wang et

al., 2002). However, in our 3D culture system, IL-18 (50 ng) did not show chemotaxis effects on LCs. This could be due to the low IL-18 concentration or the Matrigel fibres confine the movement of LCs in a certain level.

EFs have been found to either play an important role in controlling a variety of cells directional migration alone (McCaig et al., 2005; Zhao et al., 2002b) or with cytokines together (Li et al., 2011). Moreover, EFs genesis and immune response would overlap temporospatially during the wound healing. It has been demonstrated that EFs can induce lymphocytes directional migration via activation of MAPK signalling cascade (Lin et al., 2008). On the contrary, this study showed that 200 mV/mm EFs stimulation did not evidently convince LC directional migration in 3D culture. That might be because either the EFs was too weak to activate LCs or LCs failed to transduce EF signal into motility.

Interestingly, when both IL-18 gradient and EFs were present, the migration speed of LCs increased significantly. Wu and Lin proposed that redistribution of membrane receptors induced by EFs mediates electrochemical sensing and migration of the cells (Wu and Lin, 2011). Zhao et al proposed that redistribution of EGFRs induced by EFs underlie directional migration of CECs (Zhao et al., 1999). This observation has been confirmed in a variety of cells types such as breast cancer cells (Pu et al., 2007), keratinocytes (Zhao et al., 2006), fibroblasts and *Dictyostelium* (Zhao et al., 2002a). Thus it is tempting to assume that IL-18R would be polarized by EFs and the ligand - receptor binding reaction would then be amplified. IL-18 also has been demonstrated as a chemoattractant, since it can induce actin assembly of dendritic cells thus activate cells motility (Kaser et al., 2004). Thus one of the reasonable explanations is that polarization of IL-18Rs induced by EFs leads to the directional actin assembling.

LCs, like most migratory cells, exist and migrate in a 3D physiological environment. However, the majority of understanding of LC migration is limited to 2D (Haessler et al., 2011). Furthermore, it is suggested that cell migration requires different mechanisms in 3D vs. 2D (Lammermann et al., 2008). Matrigel is a commercially available reconstituted basement membrane mixture, that has been widely used to mimic 3D environment for cell migration (Benhamron et al., 2006; Chabot et al., 2006). However, the limitation of this 3D culture model is that it is difficult to precisely control cytokine (IL-18) release which might potentially lead to the uneven distribution of the gradient. Another shortcoming for EFs study is that Matrigel fails to provide ion flux like skin cells which use ion channels to maintain

TEP, therefore the conductivity is not the same as real physiological situation. This might reduce the sensitivity or the response of cells in Matrigel to the EFs stimulation.

4.3.2 EFs and IL-18 synergistically promote LC emigrant from epidermis explant culture *ex vivo*

In this section I used epidermis explant as a model to investigate LCs migration under the stimulation of IL-18, EF or both. I found that EFs plus IL-18 exerted a synergistic effect on inducing LC emigrant from epidermis. On the contrary, neither IL-18 nor EFs alone showed evident effect on LCs migration in explant culture.

The skin explant culture is a reliable method to study skin cell migration and immune reaction, and it has been recognized as a bridge transferring recognitions from *in vitro* to *in vivo*. Particularly, for the understanding physiological and pathological functions of LC, skin explant culture is a very useful model (Caras et al., 2007; Hofer et al., 2003; Kurz et al., 2002; Lateef et al., 2003; Ratzinger et al., 2002). That is because maturation and migration of skin LCs are tightly linked processes. Apparent migration of LCs can not occur without concomitant maturation (Hofer et al., 2003). LCs in skin explant cultures have presumably received their initial maturation and migration stimulus by the cytokines secreted by nearby keratinocytes or fibroblasts. Therefore, with the right stimulation or signals they are ready to migrate.

In this study, I found that EFs (200 mV/mm) plus IL-18 (100 ng/ml) treatments initiated LC emigrate after 40 minutes exposure. It has been proposed that redistribution of chemoattractant receptors induced by EFs enhances the chemotaxis, when EFs and chemoattractant both are present (Wu and Lin, 2011). Guo reported that it took about 1 hour for dermal fibroblasts to manifest detectable directional migration when exposed to EFs (150 mV/mm) (Guo et al., 2010). It is also suggested that the sensitivity of response to EFs stimulation varies in different cell types or culture systems (Li et al., 2011; Wu and Lin, 2011). Therefore, in our explant culture system, the cells might potentially need ~ 40 minutes to build up an enough potential difference to drive membrane receptor redistribution, and consequently sense the IL-18 gradient.

The advantage of skin explant culture is that the living environment of LCs is relatively intact. However, during enzymatic dissociation and separation, the

surrounding cells such as keratinocytes, fibroblasts will secrete cytokines, apoptosis, or modify gene expression in response to the stress. These changes inevitably would affect LCs behaviour in a certain level. Another concern is that IL-18 and EFs may have effects on other cells as well, it is thus difficult to prove the directional migration of LCs is the direct effects of the stimuli rather than the indirect influence by other cell types.

4.3.3 IL-18 induce LCs migration into draining lymph node *in vivo*

In this section, I semi-quantitatively investigated LCs migration with intradermal administration of IL-18. The results suggested that IL-18 could induce DCs and LCs migration from skin to draining lymph nodes, and the efficiency rose 2 fold and 5 fold compared to control group, respectively.

Within the epidermal environment, keratinocytes and LCs express IL-18, IL-1 β and Caspase-1; when inflammation occurs, the expression of those molecules is upregulated (Antonopoulos et al., 2008; Cumberbatch et al., 2001). It has been reported that IL-18 plays a key role in mediating LC migration, acting upstream of IL-1 β and TNF- α . IL-18 belongs to IL-1 family and shares similar structure to IL-1 β (Dinarello, 2007). Both IL-18 and IL-1 β require proteolytic cleavage by caspase-1 generate mature function form from inactive precursor form. Caspase-1 also has been found to induce LC migration that presumably due to its modulation on IL-1 β and IL-18 expression (Antonopoulos et al., 2001).

However, the recent study by Cumberbatch found that IL-18 and IL-1 β were not able to initiate LC migration in Langerin-Cre-MyD88 mice, indicating there may be an alternative way of IL-18 to induce LC migration. It is also reported that preformed IL-1 α release is important in initiating LC migration without the activation of IL-18 signalling (Cumberbatch et al., 2002).

4.3.4 Electric signals induce LCs migration into draining lymph node *in vivo*

I found that intradermal injection of PGE2 induced LCs migration into draining lymph node. PGE2 is a Cl⁻ channel activator and has prominent effects on increasing physiological electric signals on epithelium (Song et al., 2002).

It has been proposed that physiological EFs control directional cell migration *in vivo* (Song et al., 2002). EFs do establish chemical gradients. Injection of fluorescence

charged protein into the pre-limb-bud region of amphibian embryos – where an EF has been detected, resulted in a comet-tail-like distribution, driven by extracellular electrophoresis, rather than symmetric distribution of the fluorescence probe (McCaig et al., 2005; Zhao et al., 2006). Application of PGE2 into the wound has been proved to increase wound induced EFs and enhance wound healing *in vivo* (Nuccitelli et al., 2008).

The conventional theory for LC migration is based on chemotaxis. It has been demonstrated that CCR7 is crucial for LC migration from epidermis to draining lymph node, because CCR7-deficient mice (which lack the CCR7 ligand CCL19 and CCL-ser) have a severe defect in LC migration to the skin draining lymph node (Villablanca and Mora, 2008). Moreover, immune cells such as T cells and neutrophils could be activated by EFs (Wu and Lin, 2011; Zhao et al., 2006). Recently, Li quantitatively investigated how T cells response to CCL19 with EFs present. They demonstrated that optimal electrotactic migration of activated T cells (in a 7 V/cm EFs) exhibited comparable motility and even higher orientation responses comparing to the optimal T cells chemotaxis to 100nM CCL19 gradient (Li et al., 2011).

Although endogenous electric signals are generated by ions leakage and PGE2 indeed has function in increase endogenous electric signals via consistently opening Cl⁻ channel, it is still difficult to eliminate its pharmacological effects on LCs or surrounding cells. The best way to prove the concept that EFs could induce LCs migration might be physically apply electric current across the skin.

4.4 Summary

The data from this chapter are consistent with my hypothesis: the physiological EFs act on LCs and redistribute membrane receptors, thus initiate cell migration in response to cytokine (IL-18). This finding may permit novel approaches for manipulating the positioning or migration of dendritic cells or other immune cells to enhance immune response or antitumor responses. Additionally, it provides a potential application to optimise vaccine production as a joint effort to combine EFs together.

**CHAPTER 5 ELECTRIC FIELDS CONTROL CHRONIC WOUND
FIBROBLAST MIGRATION**

5.1 Introduction

5.1.1 Chronic wounds

Age-associated diseases such as Alzheimer's disease, diabetes and cancers not only have an enormous economic impact but also are of great concern to patients themselves. Degenerative potential and decline in repair in the skin of aged population may lead to non-healing wounds, such as chronic venous leg ulcers, pressure ulcers, and diabetic ulcers. More than 4 million people in the United States are affected by these wounds each year (Rayment and Upton, 2009), which are becoming a major cause of pain and anxiety and also significantly lessen their mobility and diminish their quality of lives. Large variety of causes and many comorbidities associated with chronic wound not only make clinical treatment into complexity of different management strategies (Campitiello et al., 2005; Falanga, 2005; Sporn and Roberts, 1993) but also dive research into specific branches without a uniform conclusion (Ching et al., 2005; Stojadinovic et al., 2005).

Chronic wound pathology

The tissue damage is primary to some external noxious stimulus, such as edema, mechanical injury, persistent eschar, local pathogens, debris, or infection. Eliminating the primary noxious factors by conservative wound care effectively heals the majority of the wounds (Goldman, 2004). However, there still are some wounds cannot be healed by the conservative methods and persistently reoccur. Generally, those chronic wound can be divided into three categories: Venous chronic wound, pressure ulcer, and diabetic ulcer.

Venous ulcer

The main reason leading to venous ulcer is edema resulting from venous hypertension associated with pericapillary cuffing of fibrin, macroglobins, and other macromolecules (Falanga and Eaglstein, 1993). It has been postulated that fibrin cuffs sequester growth substances, making them unavailable to trigger healing (Braund et al., 2007). Recently it has been reported that venous ulcer is associated with a pro-inflammatory environment with high expression level of IL-1 α , IL-1 β , IFN- γ (Beidler et al., 2009). Additionally,

upregulated protease was thought to overwhelm the antiproteinase shield, perpetuating tissue injury and deactivating growth factors and keeping the healing process delay (Goldman, 2004).

Pressure ulcer

Although the etiology of pressure ulcer is multifactorial, tissue ischemia has long been thought to be the main reason (Saito et al., 2008). Recent studies also indicate that free radical oxidative injury can cause inflammation and severe derangement of the cell recruitment process to the site of injury (Robson et al., 2000). Furthermore impair of endothelial cell function can limit cytokine production and delay wound healing (Saito et al., 2008).

Diabetic ulcer

Diabetic ulcer is a common complication of diabetic mellitus. The factors contributed to the ulcer involve mechanical, vascular, inflammatory, oxidative, endothelial, and nutritive causes (Johansen et al., 2009). There is currently no single treatment with measurable clinical impact available. The main strategy is modulating inflammatory and endothelial function (Torres et al., 2010).

5.1.2 PDGF and wound healing

A wide variety of different growth factors orchestrate cell behaviour and interaction during all stages of wound healing. Platelet derived growth factor (PDGF) is both potent mitogen and chemoattractant in wound healing process (Martin, 1997). PDGF has been shown to improve wound healing in both laboratory (Tyrone et al., 2000; Vasquez et al., 2004) and clinical trials (Koveker, 2000; Margolis et al., 2000; Robson et al., 2000).

PDGF comprises a family of homo or heterodimeric growth factors and the common types are PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD. A few types of cell can produce PDGF including platelets, macrophages, vascular endothelium, fibroblasts and keratinocytes (Barrientos et al., 2008). The conventional signalling

pathway of PDGF is that the ligands bind to PDGF receptor α and PDGF receptor β , which causes dimerization of the receptors resulting in the receptors autophosphorylation. This creates a docking site for SH2 domain whereby several downstream signal pathways are the activated (Werner and Grose, 2003).

PDGF is involved in each stage of wound healing. At early stage, PDGF is released from platelets upon the wound start. The secretion of PDGF in wound bed attracts neutrophils, macrophages and fibroblasts migration to the wound side by chemotaxis (Schneider et al., 2010). It can also induce macrophages to produce other growth factors such as TGF- β . Together with TGF- β , PDGF has been found to enhance granulation tissue formation (Konya et al., 2008). In addition PDGF also has been demonstrated to play an important role in angiogenesis (Xue et al., 2012). It works synergistically with hypoxia to increase the expression of VEGF in ischemia injury (Cao et al., 2003). PDGF is particularly important in blood vessel maturation. *In vivo* experiments suggest that PDGF is essential for recruiting pericytes to the capillaris and increases the structure integrity of vessels (Hellberg et al., 2010).

Moreover, PDGF is also important in reepithelialisation by upregulating the production of IGF-1 and thrombospondin-1 which increases keratinocyte motility and promotes proliferation, respectively (Loot et al., 2002). PDGF has also been proposed to enhance ECM production via increasing proliferation of fibroblasts (Xu and Clark, 1996). Additionally, it stimulates fibroblasts to contract collagen matrices and induces them into myofibroblast phenotype (Leask, 2010).

The decreased expression of PDGF is thought to be one of the reasons for chronic wound (Pierce et al., 1995). It has been shown that PDGF is susceptible to the proteolytic environment found in the chronic wound and its degradation can be reversed by addition of MMP inhibitors (Mast and Schultz, 1996).

Clinically, recombinant human variants of PDGF-BB have been the only successful cytokine treatment in diabetic chronic wound. The gene delivery of PDGF also has been reported that it is effective and safe for chronic wound treatment (Margolis et al., 2000).

5.1.3 Fibroblasts in wound healing

Fibroblast is one of the main driving forces to accomplish wound healing by providing contraction and expressing extracellular matrix and growth factors (Martin, 1997). Upon wound, fibroblasts are activated to differentiate into myofibroblasts, which contract and reduce the wound size (Li and Wang, 2011). A major function of fibroblast is to synthesize ECM proteins, such as collagen type I-VI and XVIII, glycoproteins and proteoglycans for normal growth or during wound healing (Powell et al., 1999).

The contraction is mainly accomplished by the intracellular tension generated by fibroblasts and myofibroblates. The cellular actin-myosin filaments transmit the tension via focal adhesion and as a result cell traction forces are generated (Han et al., 2012). This contraction is regulated by a number of molecules such as myosin light chain kinase (Nobes, 2000). In order to heal the wound, the fibroblasts normally are attracted by chemoattractant and migrate into wound bed.

The migration of fibroblasts therefore is indispensable for wound healing. It has been demonstrated that the small GTPases are critical for fibroblast migration (Nobes, 2000). The major components of the small GTPase involved in cell migration are Rac1, Cdc42 and RhoA. Rac1 induces the formation of lamellipodia protrusions via activating WAVE complex, which provide the driving force for cell migration (Goc et al., 2012). Cdc42 has been proved to establish polarity, and inhibition of Cdc42 results in disrupting the directional migration (Rajnicek et al., 2007b). On the other hand, RhoA induces the contraction of actin filaments to generate contractile force. The RhoA is activated not only at the rear of the migrating cells but also at the front, indicating that it may contact with Rac1 and Cdc42 to generate membrane ruffles (Tsai et al., 2012).

Several growth factors have been found to modulate fibroblast migration and proliferation during wound healing. Schreier studied the role of bFGF in migration and proliferation of fibroblasts in an *in vitro* model of wound healing (Schreier et al., 1993). Chiang reported that PDGF-BB induced human skin fibroblast migration on gelatin and collagen through activation of ERK1/2, p38 and JNK signalling pathways, which could be inhibited by lycopene (Chiang et al., 2007).

Fibroblast dysfunctions, such as loss of response to growth factor (Agren et al., 1999), impaired reorganization of extracellular matrix (Cook et al., 2000), decreased expression of growth factors (Cha et al., 2008b) and gene expression variation (Wall et al., 2008), all play a pivot role in the initiation and development of chronic wounds. Further investigation in fibroblast biology, including activity recovery or rectifying impaired ability of sensing mechanisms to signals may be a potential solution for chronic wound cure.

5.1.4 Electric field and cell migration

McCaig and colleagues systematically documented the interactions between Trans-epithelium potential difference (TEPD), electric signals and cell migration in both physiological and pathological conditions. In physiological condition, TEPD is established across epithelium barrier, by positively generating the efflux of chloride and influx of sodium / potassium, and maintained by the tight junctions between the cells. In pathological condition where the epithelium barrier is disrupted, TEPD is short circuited due to the breach of cell-cell tight junctions, thereby instantly generating endogenous electric fields (EFs) (McCaig et al., 2002, 2005; Song et al., 2004). This consequently increases the epithelial cell motility and guides them to migrate directionally toward wound centre, thus improve wound healing efficiency (Reid et al., 2005; Wang et al., 2000; Zhao et al., 1999). Wound healing process could potentially be obstructed by either blockage of endogenous EFs generation or impaired sensing mechanism of cells to EFs (McCaig et al., 2005; Song et al., 2002; Zhao et al., 2006).

The cytokine receptors have been considered to play a role in EF-induced cell migration during the wound healing. It has been demonstrated that EFs can increase EGFRs expression, and inhibition of MAPK – one of the downstream messenger engaged by EGFR, significantly impairs the response of CECs to EFs (Zhao et al., 1999). In addition, Zhao proposed that EF-polarized EGF receptors caused asymmetric signalling through MAP kinase, which drove directional cell migration in CECs (Zhao et al., 2002b). This however is a cell type dependent event, it has been reported that the sensing and transduction of the electric signals are largely independent of G protein-coupled receptor signalling in *dictyostelium* (Zhao et al., 2002a).

Except growth factor receptors, MAPK signalling pathway has been proved is essential for EF-induced cell migration; PI3K-PTEN is another signalling pathway which has been demonstrated to play an important role in electrotaxis. Zhao reported that EFs triggered activation of Src and inositol-phospholipid signalling, which were polarized to assist the directional cell migration. The genetic disruption of PI3K γ decreased the EF-induced signalling and abolished the directed movements of healing epithelium in response to electric signals. In addition, deletion of the PTEN enhanced signalling and electrotactic responses. This observation has been proved in several different cell types such as: neutrophils, fibroblasts, keratinocytes, indicating it is conservative for cells to response to EF stimulation.

However the interactions between PDGF and EFs during wound process are barely studied. In this study I comparatively investigate the sensitivity and response of three fibroblast cell lines: chronic wound fibroblast (CWF), chronic matched fibroblast (CMF) from the intact skin of the same patient, age-matched healthy fibroblast (HF) from healthy donor, to applied EFs. I evaluated the PDGF treatment to CWF and CMF in response to EFs by directly monitoring the migratory behaviours from both single cell and scratched wound. Moreover, by pharmacological inhibition experiments, I analyzed the molecular pathway of the high concentration PDGF-induced defect of fibroblast to EFs. Our results show that PDGF in optimized concentration could restore CWF's response to EFs, which may be potentially beneficial for clinic application.

5.2 Results

In this chapter three fibroblast cell lines were used: chronic wound fibroblast (CWF) from the patient, chronic matched fibroblast (CMF) from the intact skin of the same patient, age-matched healthy fibroblast (HF) from healthy donor. Cell migration behaviours were assessed in the presence of different guidance cues, including EFs, PDGF and PI3K inhibitors, and small GTPases inhibitors. The assessments were mainly carried out in the following aspects:

1. Comparative studies of the responses of three cell lines to EF stimulation.
2. Comparative studies of the responses of three cell lines exposed to EF and PDGF.
3. The effects of PDGFR inhibitor, PI3K inhibitor and small GTPase inhibitors on EF-induced cell migration.

This allows us to compare and contrast the signalling cascades activated by each guidance cue, and to optimise current PDGF treatment on chronic wound healing potentially in conjunction with EF stimulation.

5.2.1 CWF, CMF and HF behavior in EFs

EF plays an overriding guidance role in directing epithelial cells migration and accelerating wound healing *in vitro* (Wang et al., 2000; Zhao et al., 1999; Zhao et al., 2006) or *in vivo* (Song et al., 2002). Here I utilized a custom-designed chamber (Song et al., 2007b) under a time-lapse microscope to investigate the response of different fibroblast cells in the EFs gradient (Figure 5-1).

On application of EFs from 70 mV/mm to 300 mV/mm to the culture of CWF, CMF and HF, the motility of all cells was raised prominently in a dose-dependent pattern (Figure 5-1A). In the physiological EFs of 150 mV/mm, the motility of CWF, CMF and HF cells was lifted up 11.46%, 12.57% and 13.44% respectively. Additionally, in an even higher EFs strength as 300 mV/mm, CWF motility reached 0.20 $\mu\text{m}/\text{min}$, which was 13.50 % faster than the migration in no-EF group; HF and CMF were up to 0.23 and 0.25 $\mu\text{m}/\text{min}$, respectively. Moreover, CWF cells showed a significant defect in responding to

EFs from 150 mV/mm ($p < 0.05$) compare with CMF and HF cells. It is noticeable that at 70 mV/mm EF stimulation, three cell lines did not show any significant difference regarding migration speed (Figure 5-1 D).

The migration directedness also can be analysed using the software. Figure 5-1(B) shows that EFs induced cells migration to anode (toward EF vector). Similar to motility response, EFs-induced directedness presented a dose-dependent manner. However, CWF cells showed a significant electrotactic defect from 150 mV/mm in directedness ($P < 0.05$) compare with CMF and HF cells. Nevertheless, there was no obvious difference between CMF and HF cells in either EF stimulation or no EF situation. I took the velocity component on EF vector orientation as efficient migration intent to focusing on the effects by EFs. In this evaluation system (Figure 5-1 C), CWF cells showed significantly reduced migration efficiency from 150 mV/mm onwards comparing with both CMF and HF cells, which implicated fibroblast's defect of sensitivity / response to EF might be one of the important factors for the sustention of chronic wound. Considering CMF did not show any difference in phenotype, migration speed and directedness to EF compared to HF, I took CMF cells as the control in the following experiments.

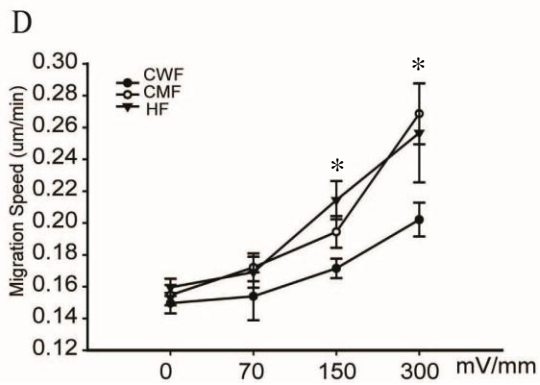
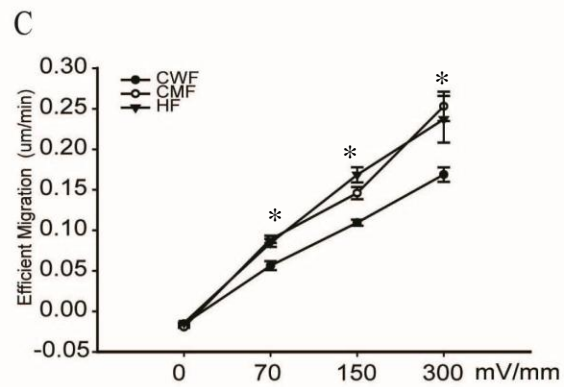
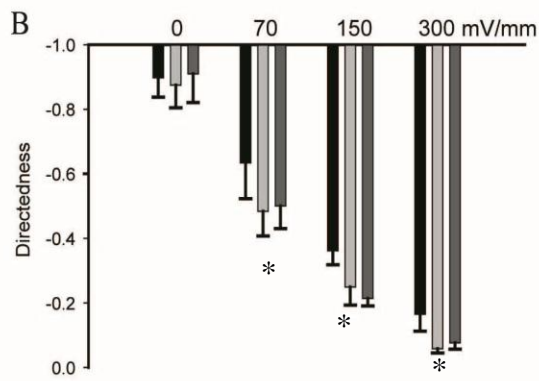
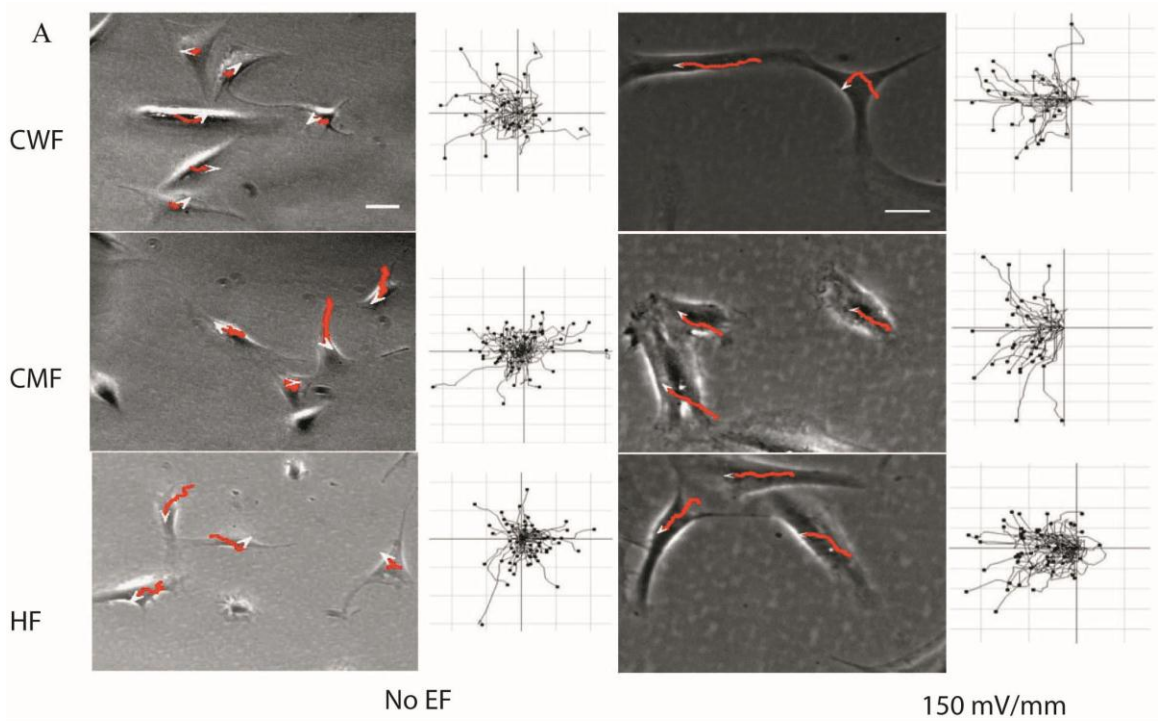


Figure 5-1 Human Chronic Wound Fibroblasts (CWF) lost sensitivity to EFs,

(A) CWF, CMF and HF were plated in the home-made EF chamber in the presence or absence of 150 mV/mm, and cell migration were real-timely recorded for 2 hours; see Materials and Methods. Representative images of CWF, CMF and HF with or without EFs are shown, bar =20 μ m. The cells were tracked by Image J software; the arrows indicate the direction of cell migration. Trajectories of CWF, CMF and HF cells with or without EF stimulation over 2 hours with the starting points positioned at the origin. x and y-axes give distance in μ m. With the EF-treated cells, the EF vector is horizontal with anode to the left and cathode to the right. (B-C) CWF, CMF and HF were treated with EFs from 0 mV/mm to 300 mV/mm and the migration were analyzed by Image J software. The Directedness (B) and migration speed (D) are shown. (C) Efficient migration of CWF, CMF and HF were calculated to indicate how fast the cells migrate along the EFs direction according to the formula see Materials and Methods. Results are presented as Mean \pm SD (*: $P < 0.05$. CWF vs. CMF).

5.2.2 PI3K is involved in EF-induced directional migration

Zhao and colleagues found PI3K and PTEN are essential in controlling EF-induced cell migration and wound healing, and that PI3K and Src signals are asymmetrically activated at the leading edge of cells (Zhao et al., 2006). The CWF and CMF cells were cultured following standard protocol, treated with PI3K inhibitor LY294002 (50 μ M) and exposed to EFs for 2 hours. I fixed the cells at the end of EF stimulation, and then stained with rhodaminephalloidin and an anti-PIP3 antibody to visualize the distribution of actin and PIP3, respectively (Figure 5-2).

Pharmacological inhibition of PI3K with LY204002, triggered a significant reduction of both migration speed and directedness of CWF. The CMF also showed similar trend of reduction when treated with LY294002 (Figure 5-2A,B). In each condition, there was no significant difference between CMF and CWF indicating the reduction was at the same magnitude. This also implied that the PI3K is equally important for both type of cells in the regulation of their responses to EFs.

Immunostaining (Figure 5-2 C) depicts that PIP3 was asymmetrically redistributed at leading edge of electrotaxis cells. In comparison, in the absence of EFs most cells did not show asymmetry of PIP3 distribution. The fluorescence intensity profile showed

consistent result that PIP3 distribution accumulated at anode-facing side of the cells and the fluorescence intensity was about 3 times higher than opposite site of the cells (Figure 5-2 E). On the contrary, fluorescence density of PIP3 was evenly distributed across the cells with no EF treatment (Figure 5-2 D)

In summary, the reduced electrotactic response when blocking PI3K, and the asymmetric distribution of PIP3 by EFs, coherently suggest that EF-induced fibroblast directional migration could be dependent on the PI3K signalling cascade, and this may be modulated by the polarization of PIP3, an immediately downstream effectors of PI3K.

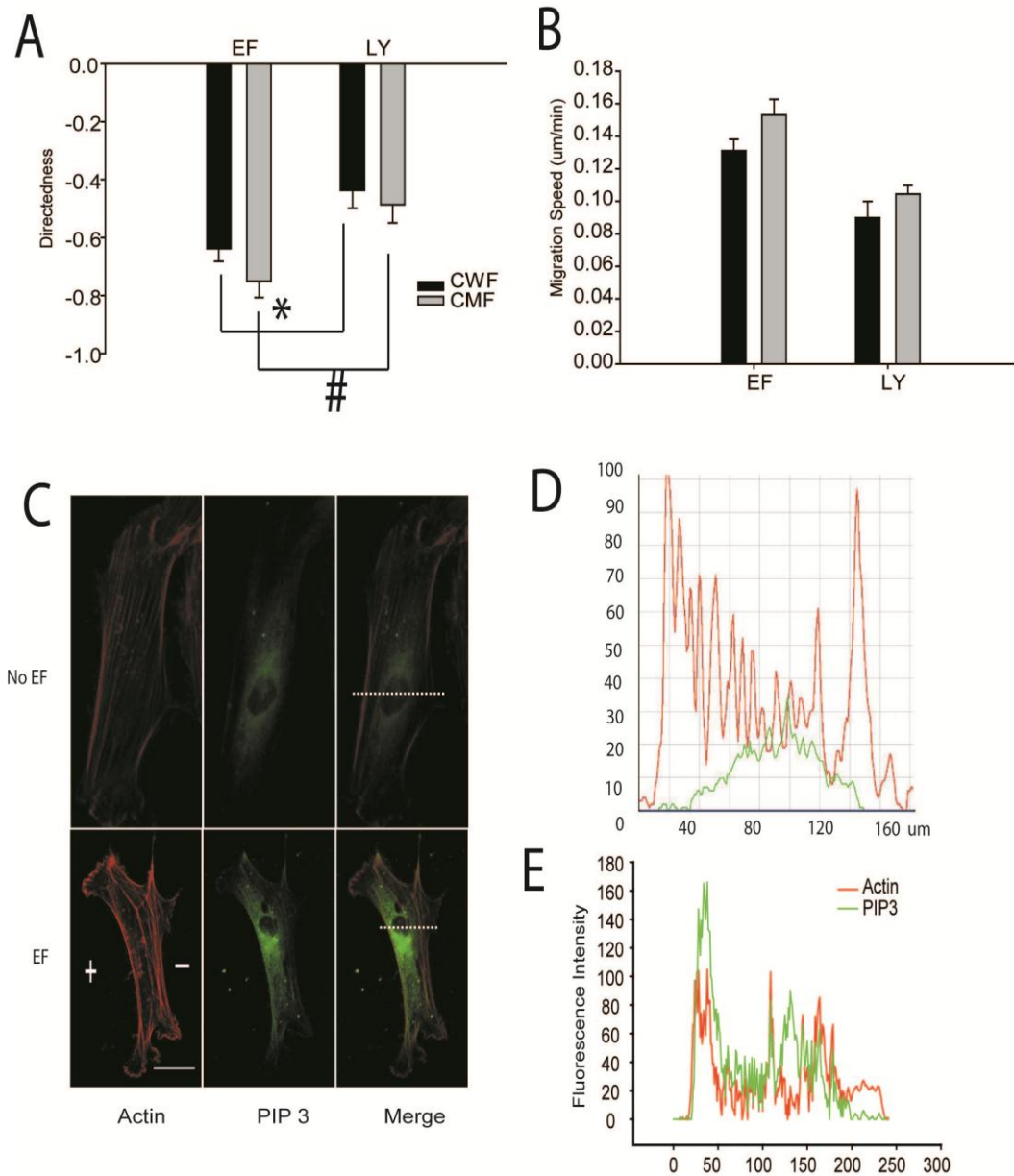


Figure 5-2PI3K is involved in EF-induced directional migration of human dermal fibroblasts

(A-B) CWF and CMF were EFs (150 mV/mm) stimulated with or without the presence of 20 μM LY, and cells migration were recorded. Directedness (A) and migration speed (B) were depicted. (C) After EFs stimulation, CWF and CMF were fixed and stained with Actin and PIP3 antibodies. The representative immunostaining of CWF is shown, Bar = 20 μm, + and - indicate anode and cathode respectively. (D-E) Line profile (white dot line) of fluorescence intensity of CWF without and with EFs (150 mV/mm) treatment are depicted in (D) and (E) respectively. #, *: $P < 0.05$. Results are presented as Mean \pm SD.

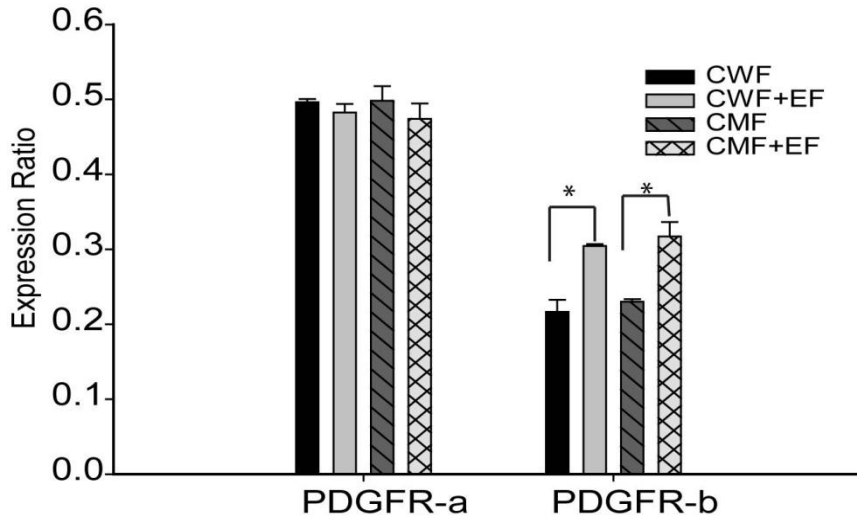


Figure 5-3 PDGFRs expression with/without EFs

CWF and CMF were EFs (150 mV/mm) treated for 4 hours and mRNA level of PDGFR α and β were quantified. Results are presented as Mean \pm SD. (* P <0.05 compared, n=4).

5.2.3 PDGFR- β is upregulated by EFs

The deficit of PDGF was found in chronic wound. Thus the expression levels of PDGFR- α and PDGFR- β in non-EF treated/ EF-treated CWF and CMF were investigated using TaqMan qPCR. The cells were exposed with 150 mV/mm for 4 hours and then total mRNA was extracted for PCR reaction (Figure 5-3).

PDGFR- α was found to mediate 3T3 fibroblast migration stimulated via the Na⁺/H⁺ exchanger (Schneider et al., 2009). Our results indicated that there was no change of PDGFR- α expression between CWF and CMF in the absence of EFs; and EF stimulation did not change these neither on both cell types. Gao suggested that deletion of the PDGFR- β gene affects key fibroblast functions in wound healing (Gao et al., 2005). I found no difference between CMF and CWF regarding PDGFR- β expression in original situation. However, it was upregulated significantly by EFs stimulation in both cell types (Figure 5-3).

5.2.4 EF induced anodal migration is via polarization of p-PDGFR

To investigate if PDGF receptor is involved in signal transduction during electrotaxis, CMF and CWF were pre-treated with PDGF receptor tyrosine kinase blocker Tyrphostin AG1296 30 minutes before EF stimulation (Figure 5-4). The cell migration speed and directedness were analysed by time-lapse tracking cell movement. The cells then were fixed at the end of EF stimulation, sequentially stained with rhodamin-phalloidin and an anti-p-PDGFR antibody, and finally the distribution of actin and p-PDGFR were visualized under the fluorescence microscope.

With the treatment of tyrphostin (10 μ M), the migration directedness of CMF and CWF declined significantly, with the directedness reduced from -0.76 and -0.63 down to -0.51 and -0.44, respectively (Figure 5-4, A). However, the migration speed did not changed with the treatment in both cell types (Figure 5-4, B).

Representative microscopic photos of CWF with PIP3 and p-PDGFR- β staining are presented, respectively in Figure 5-4C. The p-PDGFR- β was activated and polarized to the anterior lamellipodia of the electrotaxis cell toward anode (arrow). Similarly, the PIP3 was also accumulated at the anodal facing lamellipodia (arrow in C). On the contrary, both p-PDGFR- β and PIP3 were evenly distributed across the cell membrane in the absence of EFs. The colocalization of PIP3 and p-PDGFR were then calculated as the intensity correlation similarly as described in the previous study. The intensity correlation indicates the degree of colocalization of signalling molecules. CWF showed significantly increased intensity correlation of p-PDGFR- β and PIP3 when exposed to EF in comparison with no EF treated cells (Figure 5-4, D).

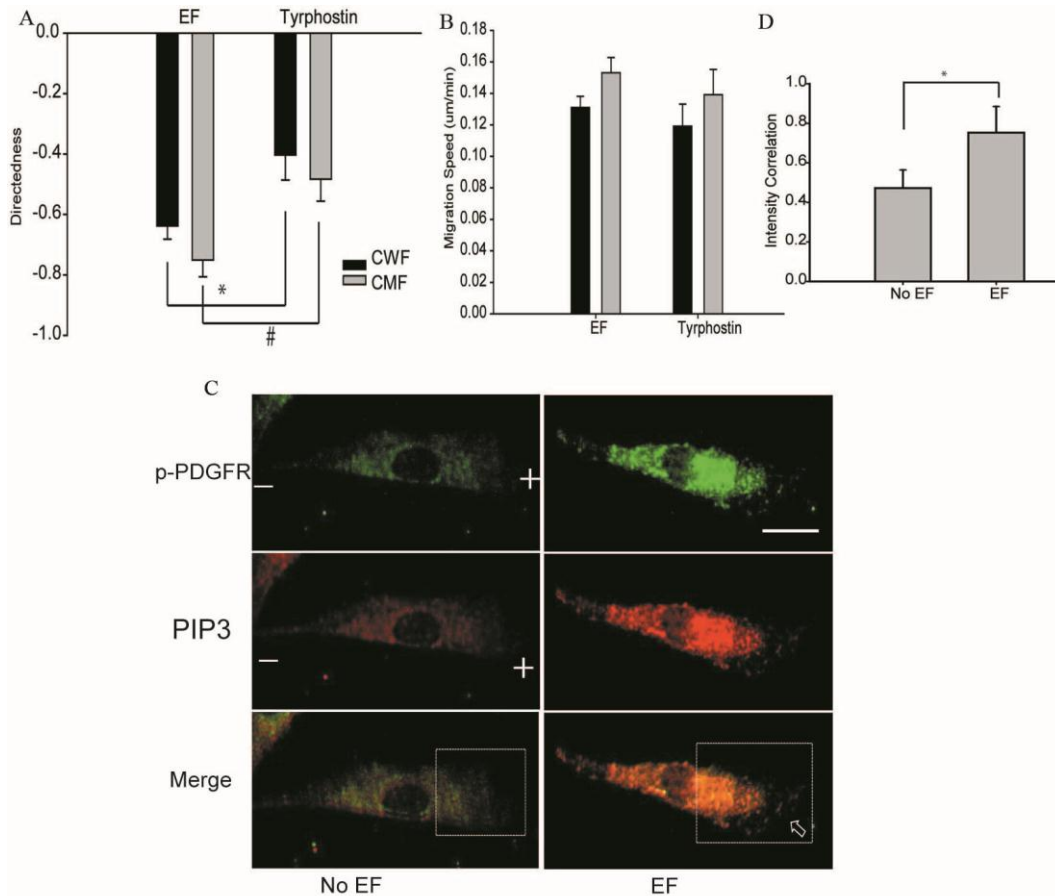


Figure 5-4 PDGFR is required for EF-induced directional migration of human dermal fibroblasts.

(A-B) CWF and CMF were EFs (150 mV/mm) treated with or without the presence of 10 μ M tyrphostin; Directedness (A) and migration speed (B) were depicted. (C) After EFs stimulation, CWF and CMF were fixed and stained with p-PDGFR β and PIP3 antibodies. The representative immunostaining of CWF is shown, Bar = 20 μ m, + and - indicate anode and cathode; #, *: $P < 0.05$. Results are presented as Mean \pm SD; n=80-100. (D) The colocalization of p-PDGFR and PIP3 were analyzed by using Image J. Rectangle area indicates ROI used in software, arrow indicates p-PDGFR and PIP3 colocalized at leading edge of the cells treated with EFs. *: $P < 0.05$. Results are presented as Mean \pm SD; n=35 (no EF), n= 41 (EF).

5.2.5 PDGF raises motility but attenuates directedness of fibroblast in EFs

Previous studies show that PDGF-BB has prominent ability to promote wound repair (Pierce et al., 1994; Robson, 1997; Robson et al., 1992) and recapitulate the activity of human serum in human dermal fibroblast migration assay (Li et al., 2004b). In light of these studies, I hypothesize that PDGF-BB may restore the sensitivity of CWF to EFs thus improve chronic wound healing. Both CWF and CMF were titrated with PDGF from 0 to 40 ng/ml in the presence of 150 mV/mm EFs. The effects of these two guidance cues on CWF and CMF were assessed. The curves of migration speed and directedness vs. PDGF concentration were drawn, respectively.

Since the expression of PDGFR- β was increased by EFs (Figure 5-3), I anticipated that PDGF-BB might potentially restore CWF's sensitivity to EFs. Surprisingly, PDGF-BB treatment significantly decreased the migration directedness of both CWF and CMF when treated with 150 mV/mm EF, in a dose-dependent manner (Figure 5-5, A). Coincidentally, the directedness of both cell types dropped to about -0.3 indicating random movements when the concentration of PDGF-BB was greater than 10 ng/ml.

On the contrary, both CWF and CMF were very sensitive to PDGF-BB regarding cells migration speed (Figure 5-5, B). Even at the lowest concentration of 0.1 ng/ml (\sim >10 times endogenous concentration (Rollman et al., 2003)), the migration speed of CWF and CMF were raised from 0.155 to 0.21 μ m/min and 0.19 to 0.27 μ m/min, respectively. Furthermore, the migration speed of both cell types was increased to the maximum value (\sim 4 times higher than control) with 10 ng/ml PDGF-BB, and started to declined when PDGF was more than 20 ng/ml. The efficient migration (Figure 5-5, B) of cells were calculated according to formula in Chapter 2 to indicate the cell migration ability in EF vector. Figure 5-5C illustrates that both CWF and CMF display a bell-shaped curve against PDGF titration. The CMF reached maximum efficient migration to 0.21 μ m/min when treated with 0.5 ng/ml PDGF, whereas CWF reached maximum efficient migration of 0.17 μ m/min with 5 ng/ml PDGF. This implies that 5 ng/ml PDGF treatment could restore the defect of CWF electrotaxis back to the same level as CMF without PDGF treatment. If electrotactic deficit of CWF in response to endogenous

wound current is one of the factors causing wound healing defect, PDGF treatment can potentially help CWF with the restoration of electrotactic response during wound healing.

Representative migration trajectories of CWF cells are superimposed to illustrate the migration pattern of 3 groups: CWF with EFs, CWF+EFs+PDGF (1.0 ng/ml), CWF+EFs+PDGF (10 ng/ml), respectively (Figure 5-5, D-F). With EFs, the migration pattern is clearly biased toward left (anode), whereas PDGF treatments gradually change the pattern to radial distribution indicating a random migration.

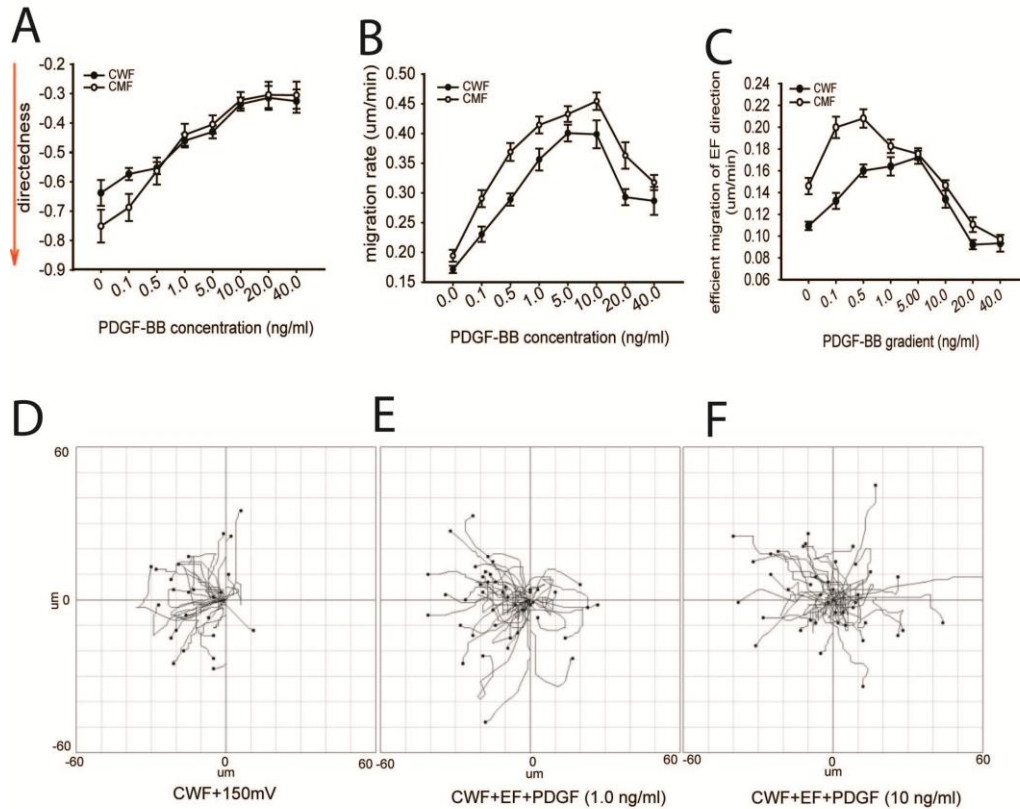


Figure 5-5 PDGF negatively regulated sensitivity of human dermal fibroblast in response to EFs.

(A-C) CWF and CMF were treated with both EF (150 mV/mm) and PDGF (X axis: 0-40 ng/ml), and cell migration was recorded and analyzed. Directedness (A), migration speed (B) and efficient migration (C) of CWF and CMF were drawn against PDGF-BB concentration; details see Materials and Methods. * $p < 0.05$, Results are Mean \pm SD; n=80-100. (D-F) Representative migration trajectories of 3 experimental groups over the 2 hours recording period: CWF+EF, CWF+EF+PDGF (1.0 ng/ml), CWF+EF+PDGF (10 ng/ml), respectively; see Materials and Methods. X and Y axes give distance in μm . The EF vector is horizontal with anode to the left and cathode to the right.

5.2.6 PDGF reduces EF-induced directedness through ROCK

To investigate the mechanism why PDGF compromises EF-induced directedness, inhibitors were used to screen the potential signalling pathway involved (Figure 5-6). The CMF and CWF were exposed with 150 mV/mm EFs plus 1.0 ng/ml PDGF and inhibitors

then were added to test if the migration directedness would be restored. The inhibitors used were tyrphostin, Y-27632 and LY294002 to block PDGFR, ROCK and PI3K, respectively.

Figure 5-6 (A) illustrates that both tyrphostin and Y-27632 restore the directedness of CWF and CMF. The directedness of CMF increased from -0.43 up to -0.77, whilst CWF from -0.41 to -0.62 by tyrphostin. Similarly, Y-27632 lifted the directedness of CMF and CWF up to 0.63 and 0.56 respectively. However, LY294002 the PI3K inhibitor did not alter the directedness of either CWF or CMF. These findings imply that PDGF changes persistence of migration of fibroblasts by PDGFR-ROCK rather than PI3K signalling pathway.

Furthermore, all three inhibitors uniformly reduced PDGF+EF induced migration speed of both CWF and CMF cells (Figure 5-6, B). This suggests that PDGFR, PI3K and ROCK may all be involved in controlling fibroblasts motility induced by EF plus PDGF.

The cells migration trajectories were tracked after various treatments (Figure 5-6, C and supplemental movies).The representative photos of CWF depict the cell behaviours under PDGF, EF and three inhibitors, respectively.

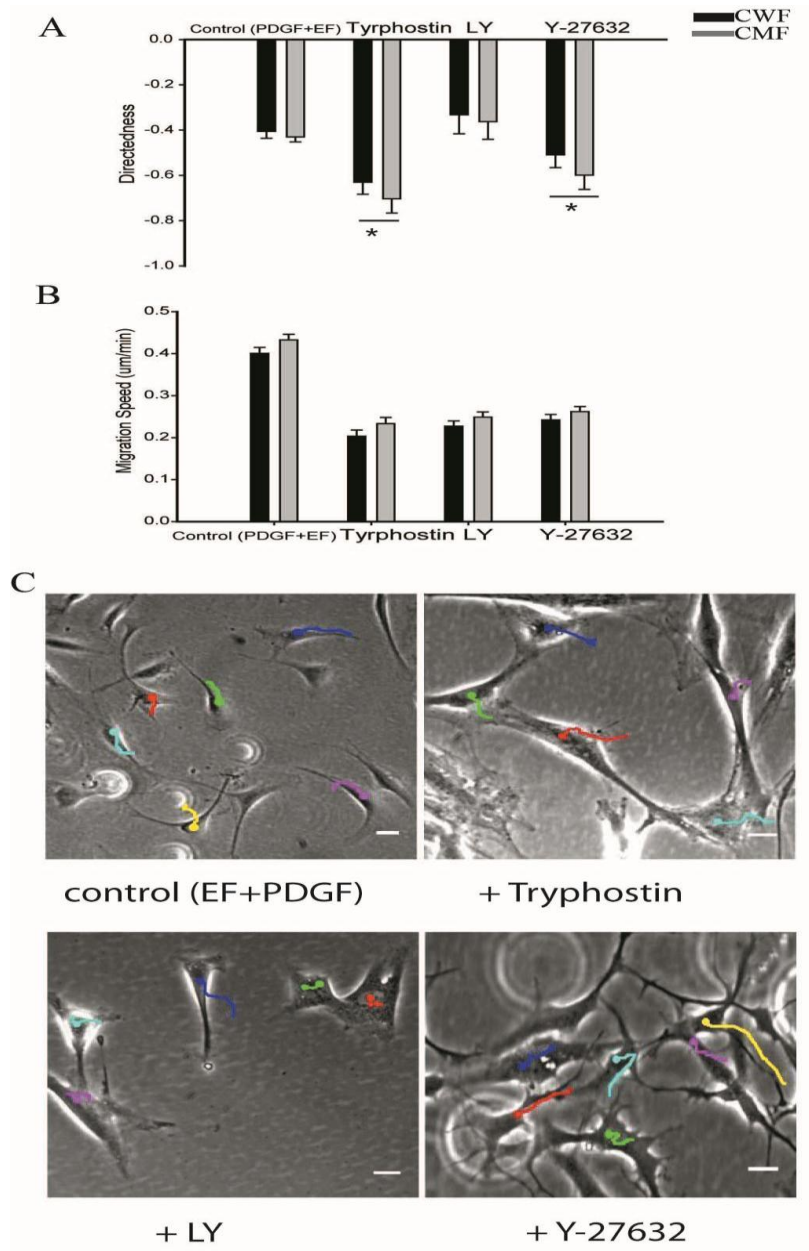


Figure 5-6 PDGF moderates human dermal fibroblasts sensitivity to EFs via PDGFR-ROCK pathway

(A-B) Both CWF and CMF were pretreated with PDGF (1.0 ng/ml) for 30 minutes before co-stimulated with EF (150 mV/mm) and typhostin (10 µM), or LY294002 (20 µM), or Y-27632 (10 µM), respectively. The directedness (A) and migration speed (B) of CWF and CMF were summarized. Values plotted are the Mean \pm SD, *: $P < 0.05$; n=120-150. (C) Representative images of CWF with EF and PDGF as the control and plus treatment of Tryphostin, or LY or Y-27632, respectively are shown, bar=20 µm, arrows indicate the direction of cell migration.

5.2.7 Optimized treatment of PDGF and EFs enhanced chronic wound healing

To investigate whether combination of PDGF and EF could enhance chronic wound healing, the scratch wound assay of CWF and CMF under EFs, PDGF or EFs plus PDGF were carried out, respectively. The CWF and CMF were seeded into the chamber, until the cells became monolayer; the yellow tip was used to scratch across the monolayer to create artificial wounds. The wounds then rinsed and kept into fresh medium for 30 minutes before the treatments. Fibroblasts migrate into scraped space may be affected by two factors: migration and proliferation. PDGF was demonstrated as a strong mitogen for inducing dermal fibroblast proliferation after longer treatment, usually more than 24 hours (Agren et al., 1999). Therefore the whole treatments lasted 6 hours and the cell migration and wound closure process were recorded (Figure 5-7).

When treated with either PDGF-BB (5 ng/ml to CWF; 0.5 ng/ml to CMF), EFs (150 mV/mm) or both, the marked migratory responses of CWF and CMF were observed. Additionally, PDGF plus EFs showed a synergistic effect on promoting wound closure (Figure 5-7, A and supplemental movies). The column graph (Figure 5-7 B) illustrates the wound closure distances in different treatments. All treatments show significant increase in the closure distance comparing to control. To quantitatively evaluate the treatments on chronic wound healing, the ratio of closure distance between CWF and CMF was calculated (Figure 5-7B, line). The PDGF plus EF treatment raised ratio to about 90% from less than 70% in control group, which demonstrated the combined treatment could effectively recovered chronic wound healing by restoring the migration ability of CWF.

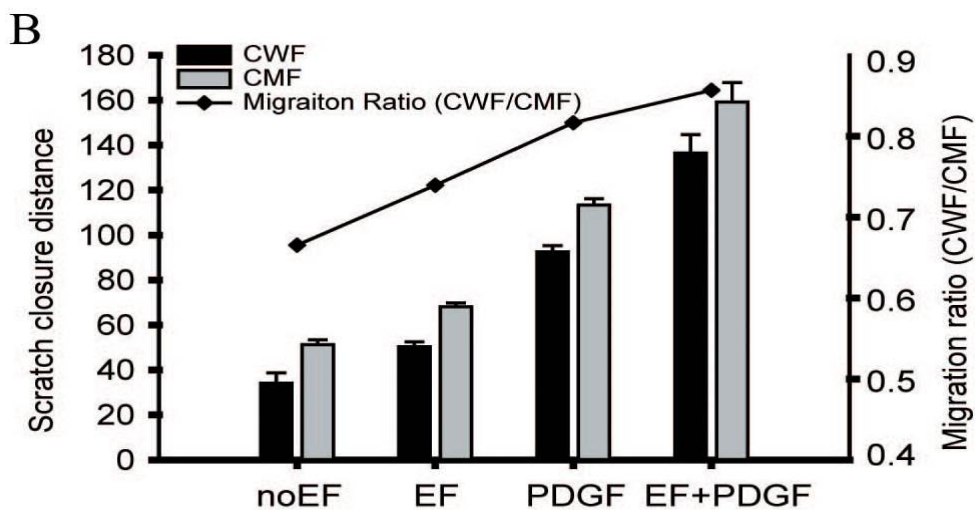
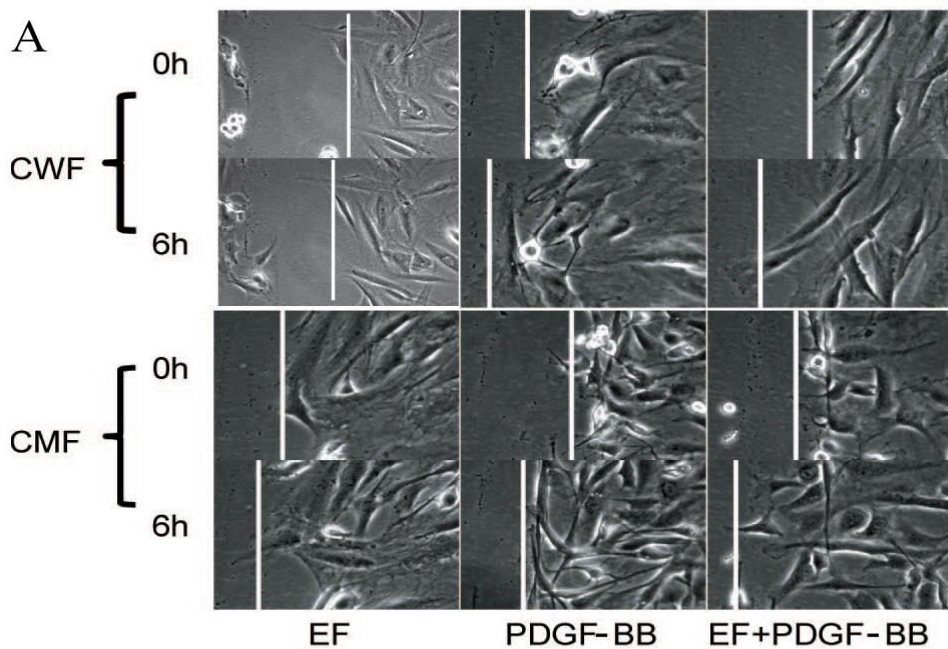


Figure 5-7EFs and optimized concentration of PDGF synergistically enhanced chronic wound healing

(A) Monolayer CWF and CMF were gently scratched by yellow tip and treated with EFs (150 mV/mm), or PDGF-BB (5 ng/ml to CWF; 0.5ng/ml to CMF), or EFs (150 mV/mm) + PDGF-BB (5 ng/ml to CWF; 0.5 ng/ml to CMF), for 6 hours. (B) The scratch closure distance were measured by image J software and summarized referring to left Y axis; the migration ratio, were refer to the right Y axis, calculation formula see Material and Methods. Data are presented as the Mean \pm SD.

5.3 Discussion

In this chapter the chronic wound fibroblasts migration in EFs, PDGF, and PDGF plus EFs is investigated. It is shown that: 1). CWF lost sensitivity to EFs in comparison with CMF; 2). the migration speed of CWF can be enhanced significantly with EFs greater than 150 mV/mm; 3). PDGF treatment attenuates EF-induced directedness of CWF and CMF via PDGFR-ROCK signalling pathway; 4). the combination of PDGF with optimised concentration and EFs can enhance chronic wound healing.

5.3.1 The comparative study of CWF, CMF and HF in EFs

The dermal fibroblasts are the major force to draw the wound bed closure and also contribute to the synthesis, bundling, and alignment of the collagen fibres (Shaw and Martin, 2009). The impaired ability of repopulation and cytokines secretion of fibroblast was the key factors to chronic venous ulcers (Wall et al., 2008). I found that although CWF still show directional migration under EFs exposure in a dose-dependent manner; it showed impaired responses including migration speed and directedness compared to CMF and HF under the same EFs treatment. Endogenous EFs induced by wounds have been proved to play an overriding role in promoting directional migration of various cells (Zhao, 2009). Zhao and colleagues identified the first genes – PI3K/PTEN which modulates directional cell migration in response to electric currents (Zhao et al., 2006). Guo found that the human dermal Fibroblasts also showed responses to EFs stimulation and migrated to anode. Additionally, the dermal fibroblast of p110 γ gene knock-out mouse, with inhibited PI3K activity, exhibit significantly reduced electrotaxis (Guo et al., 2010).

5.3.2 The PDGFR-PI3K pathway mediates the EF-induced directional migration of CWF and CMF

PI3K signalling pathway was investigated to check if it was the reason of defective response of CWF to EFs. In agreement with previous discoveries, our finding revealed that PI3K inhibition reduced the response of both fibroblast cell types to EFs. Moreover I

also identified that PIP3 – the immediate downstream effector of PI3K was polarized at anode-facing side of the fibroblast. In neutrophil, PIP3 induced development of polarity and accumulation of F-actin in the leading lamellae thus forge directional migration (Niggli, 2000).

On the other hand these also imply that PI3K may not be the main driving force leads to the phenotypic difference between CWF and CMF. Jiang reported that PI3K inhibition similarly blocked the chronic wound keratinocytes and healthy keratinocytes migration, suggesting this pathway is central for the migration but not specific to the chronic wound (Jiang et al., 2010).

Most growth factors have been characterized by their capacity to play a role in wound healing due to their mitosis function (Oliva et al., 2012). Antoniades found that the controlled, reversible expression of PDGF and its receptor induced by injury may function in an autocrine/paracrine manner on both epithelial cells and fibroblasts to bring about their sustained proliferation during the normal healing process (Antoniades et al., 1991). It also has been suggested the possibility that a defect in PDGFR may be associated with chronic wound healing.

However, I found there was no significant difference of PDGFR- α and PDGFR- β expression between CWF and CMF. Cook proposed that impaired matrix metalloproteinase-1 and matrix metalloproteinase-2 led to the defective function of CWF (Cook et al., 2000). Seah found that the inhibition of Ras activity partially resulted in the phenotype of CWF (Seah et al., 2005). Interestingly, I observed that PDGFR- β expression were upregulated by EF stimulation. Contrarily, EF stimulation did not significant change PDGFR- α expression on both CWF and CMF. Similarly, EGFR was demonstrated to be upregulated by EF stimulation by CECs (Zhao et al., 1999). On the other hand, Wu and Lin also suggested that the response to EF stimulation is cell type specific, EF stimulation would lead to specific gene expression (Wu and Lin, 2011).

It has been demonstrated that on application of EFs, the MAPK and/or PI3K pathways are activated to produce directional cell migration (Wang et al., 2003; Zhao et al., 1999). The PDGFR is suggested to be upstream of MAPK and PI3K during the cell migration (Choudhury et al., 1997). In our study, inhibition of PDGFR phosphorylation depressed EF-induced directional migration on both CWF and CMF, indicating the activation of

PDGFR was required for EF-induced cell migration. However, the phosphorylation inhibition did not significantly alter the migration speed of both cell types. The inhibitor AG1296 is a very specific tyrosine kinase inhibitor and it can selectively and potently inhibited autophosphorylation of PDGFR- β (Kovalenko et al., 1997; Tse et al., 2001). Moreover, it has been demonstrated that inhibition of PDGFR tyrosine kinase inhibits vascular smooth muscle cell migration (Myllarniemi et al., 1997). Therefore, the inhibition of PDGFR phosphorylation did not attenuate EF-induced cell migration, may be due to the multifunction of EF-induced effects. The blockage of PDGFR pathway may be compensated by other EF-activated pathways, such as ERK1/2. Furthermore, it has been implicated that the directedness is mainly controlled by persistence of cell polarity and the motility is mainly associated with the rate of assembly and disassembly of actin (Glasgow and Daniele, 1994). The other possibility is that two hours EF stimulation experiment may not able to discriminate the migration speed with/without inhibitor in certain concentration.

The result phosphorylation of PDGFR is redistributed facing anodal side by EF stimulation, which is in agreement with previous discoveries. Zhao reported that EGFR were polarized and accumulated to cathodal side of the cell by EF stimulation (Zhao et al., 1999). Moreover I also identified that PIP3 the immediate downstream effector of PI3K was polarized at anode-facing side of the fibroblast. In neutrophil, PIP3 induced development of polarity and accumulation of F-actin in the leading lamellipodia therefore triggers directional migration (Niggli, 2000).

In addition, for the first time our results reported that the colocalization of p-PDGFR and PIP3 was increased at anodal facing protrusion induced by EF stimulation, which suggests the regulation of fibroblasts directional migration. This is also consistent with the previous report that EGFR accumulation to the cathodal side of bovine corneal epithelial cells was induced by EF exposure (Zhao et al., 1999). Therefore the accumulation and redistribution of PIP3 may potentially control the persistence of migration in a coordinated manner in association with PDGFR.

5.3.3 PDGF attenuates EF-induced directedness via ROCK

The functions of PDGF through PDGFR-PI3K pathway to mediate cytoskeleton dynamic and cell migration have been observed in several different cell types (Jimenez et al., 2000; Pukac et al., 1998). PDGF also has been widely used for treatment of chronic wound (Bennett and Schultz, 1993; Pierce et al., 1995). Unexpectedly I found PDGF negatively regulated fibroblasts migration directedness induced by EF stimulation.

The electric signals controlled migration directedness is suggested to rely on the redistribution of membrane growth receptors which leads to polarized actin polymerization (Zhao, 2009; Zhao et al., 2006). PDGF has been demonstrated to induce actin stress fibre disassembly, and reduce focal adhesion complex, thus influence on protrusion and lamellipodium formation (Guan et al., 2009). In addition, it was previously reported that PI3K was essential for PDGF receptor-induced cytoskeletal changes and cell migration (Jimenez et al., 2000). Therefore, EFs and PDGF may competitively exert force on fibroblast to induce cell directional migration, because they potentially share the same signalling pathway. The competition induced by PDGF thus attenuates the influence by EFs. Even more, the EF-induced directional migration is build up by formation of leading edge and tail contraction, the addition of PDGF probably blocks the consistent head-tail establishment therefore results in random migration.

To investigate which pathway PDGF uses to antagonise EFs, the inhibitors of PDGFR, PI3K and ROCK were screened. I showed that blockage of PDGFR and ROCK rescued EF-induced directedness, on the contrary LY294002, the inhibitor of PI3K did not. These results indicate that PDGF attenuate EF-induced directional migration through activation of PDGFR-ROCK pathway. It was previously reported that PDGF stimulated oral fibroblast contraction through Rho kinase and P38 signalling pathway in vitro (Watts and Rose, 2010). Additionally, Jackson found that ROCK mediated phosphorylation of myosin light chain which led to cell contraction (Jackson et al., 2011). This demonstrates that PDGF not only can activate actin dynamic through PI3K but also function on myosin contraction via ROCK. However, the EF-induced directedness is based on actin dynamic, thus the inhibition of PI3K cannot rescue the antagonist effect of PDGF.

5.3.4 Defective response of CWF to EFs

The reason leading to chronic wound is still not yet very well understood. Phillips et al suggested that the microenvironment was the culprit. They reported that chronic wound fluid arrested new-born fibroblast growth by preventing the cell cycle (Phillips et al., 1998). It has been demonstrated that reduced activity of TIMP-1, TIMP-2, and MMP-2 is associated with dysfunction of CWF (Cook et al., 2000).

I explored for the first time the response of CWF to EF stimulation, and found CWF showed impaired responses including migration speed and directedness to EFs in comparison to CMF and HF. Sequentially, the EF-induced responses were titrated with PDGF from 0 to 40 ng/ml. The efficient migration was created by accumulating of the cell migration on EF vector to figure the best ratio of two cues: EF and PDGF in promoting chronic wound healing. I found that CWF required 5 ng/ml PDGF whereas CMF needed 0.5 ng/ml to achieve their best efficient migration in the presence of 150 mV/mm EF.

The result that CWF needs as much as 10 times PDGF to migrate similarly as CMF is in agreement with previous studies. It has been demonstrated that PDGF is defective in CWF and PDGF-BB treatment increases chronic wound healing (Pierce et al., 1995; Shukla et al., 1998). Moreover, PDGF-BB becomes the only growth factor approved by the FDA for daily application in treating wound healing (Goldman, 2004).

5.3.5 Combined treatment of PDGF and EFs enhances chronic wound healing

In scratch wound assay, I revealed that combined treatment of EFs plus PDGF significantly increased fibroblast cell migration. Additionally, the combined treatment recovered the migration capacity of CWF back to CMF during the wound healing. However, due to the effect that PDGF impairs EF-induced directedness in single cell level *in vitro*, there are two feasible solutions: 1). optimising PDGF concentration to achieve the best efficient migration; and 2). using oscillating EFs, which has been previously tested in spinal cord injury treatment (Borgens et al., 1981).

In addition to the cytokine treatment, exogenously applied EFs have been shown as a promising treatment of chronic wounds (Guo et al., 2010). Considering that several other

growth factors (such as IGF, EGF and FGF) have been demonstrated to enhance wound healing, and the ligand and receptor reaction can be amplified by EF stimulation, it would be very useful to comprehensively test combination treatment with other growth factor and EFs. This new treatment would be potentially a practicable approach to enhance chronic wound healing in the future.

5.4 Summary

The data from this chapter can be summarized as following:

- EFs induce human dermal fibroblast directional migration in a voltage dependent manner
- CWF shows impaired sensitivity in response to EFs compared to CMF and HF
- Activation of PDGFR and PI3K are both required for EF-induced directional migration
- PDGF attenuates EF-induced migration directedness through PDGFR-ROCK other than PI3K pathway
- Optimised concentration of PDGF plus physiological EFs enhance chronic wound healing

CHAPTER 6 CONCLUSIONS AND FUTURE EXPERIMENTS

6.1 Conclusions

6.1.1 EFs promote dendritic cells (DCs) / Langerhans cells (LCs) migration in response to IL-18

1. EFs promoted the responses of KG-1 cells to IL-18, leading to an increase of IFN- γ expression in a dose-dependent manner.
2. 3D chemotaxis of KG-1 cell, KG-1-derived DCs and primary cultured Langerhans cells in response to IL-18 was also enhanced by EF stimulation.
3. The EF-induced activation of KG-1 cells was modulated through phosphorylation of P38 MAPK rather than up-regulation of IL18R α expression.
4. The EF-induced redistribution of IL18R α colocalized with asymmetric assembly of actin, which led to directional migration of DCs.
5. EFs and IL-18 synergistically induced LCs emigrate from epidermis *ex vivo*
6. Both IL-18 and EFs induced LCs migration from epithelia to draining lymph node *in vivo*

6.1.2 EFs and PDGF modulate cell migration in chronic wound

1. CWF showed defective migration speed and directedness in EFs stimulation in comparison to CMF and HF.
2. Fibroblasts directional migration was guided through PI3K signalling pathway.
3. Excessive PDGF treatment impaired directedness of fibroblast migration induced by EFs via ROCK pathway.
4. Optimised PDGF dosage and EFs synergistically promote chronic wound healing.

6.2 General discussion and clinical applications

6.2.1 Electric signals and immunomodulation

DCs are the most potent antigen presenting cells and play a critical role in mediating immune response. Activation and maturation of DCs is induced by infectious agents and inflammatory products leading to increased expression of co-stimulatory MHC class II

molecules and cytokine products (Frick et al., 2010). Toll-like receptors (TLR) ligands and cytokines have been demonstrated to affect DCs maturation and migration. Particularly, the proinflammatory cytokines such as TNF- α , IL-6, IL-12, IL-1 β and IL-18 are essential for DC induced immune responses.

IL-18 has been proved to be a multifunctional cytokine. It can induce several different cells including spleen cells and T cells to express IFN- γ (Okamura et al., 1998). In addition, it has been demonstrated that except for IFN- γ induction, IL-18 upregulates Fas ligand expression on NK cells and T cells thus resulting in apoptosis in activated lymphocytes during hepatic injury (Tsutsui et al., 1997). Moreover, IL-18 is required for initiating LCs migration in contact hypersensitivity (Nakano et al., 2003; Stoll et al., 1998; Wang et al., 2002). Those finding suggest that IL-18 play essential roles in various immunological events in physiological and pathological situation.

In recent years, McCaig and colleagues systematically demonstrated and reviewed the important roles of EFs exerted on directional cell migration, cell division, gene expression, embryogenesis, nerve growth and wound healing both *in vitro* and *in vivo* (McCaig et al., 2002, 2005). In addition, there are emerging evidences supporting that EFs may also play an important role in immunomodulation .

Firstly, it has been demonstrated that EFs regulate immune cells metabolism. Anticevich found that EFs treatment increased responsiveness of sensitized human bronchial tissue to neutrophil supernatant (Anticevich et al., 1996). A further study showed that low frequency EFs promoted neutrophil extension, metabolic resonance and DNA damage when phase-matched with metabolic oscillators *in vitro* (Kindzelskii and Petty, 2000). Furthermore, Yoshikawa, et al. reported low frequency electromagnetic field enhanced nitric oxide generation by neutrophils *in vivo*. Moreover, an EF was applied to macrophage cells led to enhance NAD(P)H amplitude, implying that EFs might influence macrophage metabolic signals (Adachi et al., 1999).

Secondly, EFs are found to induce directional migration of immune cells. It has been demonstrated that neutrophils can be induced to migrate directionally towards cathode in an applied EF (McCaig et al., 2005; Zhao et al., 2006). Lin comparatively investigated electrotaxis and chemotaxis on human PBLs and found that, electrical stimulation

activates intracellular kinase signalling pathways in association with chemoattractant. Additionally, they also found motile cutaneous T cells actively migrate toward the cathode of an applied EF *in vivo* (Lin et al., 2008). Our results also suggest that EFs can promote the sensitization of several types of DCs in response to IL-18. These data indicate that EFs may play an essential role in regulating immune cell recruitment within tissues during inflammation and infection, which could mediate the process of immune response.

Although, the molecular mechanism of how EFs regulate cell behaviours is still not clear, several models / theories have been reported. Ion channel clustering has been demonstrated to regulate EF-induced neutrophil polarization (Kindzelskii and Petty, 2005). Moreover, Shanley reported that influx of extracellular Ca^{2+} is necessary for electrotaxis in *Dictyostelium*, which is different from those induced by cAMP and folic acid (Shanley et al., 2006). Another theory is that asymmetrical redistribution of cell surface receptors induced by EFs controls cell behaviours such as sensing chemoattractant gradient, directional migration, division (McCaig et al., 2002, 2005; McCaig and Zhao, 1997; Song et al., 2002). In addition, a recent study suggested EF-induced cell polarization was modulated via intracellular pH and cell polarity inducing factors in multiple directions (Minc and Chang, 2010).

6.2.2 Therapeutic applications of EFs

One major clinical application of EFs is cancer treatment. Nishi reported that using pulsed electric field in combination with chemotherapeutic agents or genes achieved an efficient result as a clinical treatment of cutaneous tumours and head / neck cancers (Nishi et al., 1997). In addition Plotnikov found that low EFs effectively enhanced chemotherapy for mouse metastatic prostate cancer (Plotnikov et al., 2006). Moreover, it has been demonstrated that EFs can induce human breast cancer cell directional migration, indicating electric signals are involved cancer metastasis (Pu et al., 2007).

A recent study found application of short pulses of electric current immediately after an injection of a genetic vaccine significantly enhanced the transfection efficacy and the subsequent vaccine-specific immune responses (Brave et al., 2010). In agreement with

this finding, our study for the first time established a molecular basis of how EFs increase DCs activity in response to cytokines. These data shall shed light on novel adjuvant technology essential in the design / development of high efficiency vaccination. Moreover, EF-induced directional migration of DCs also can be used for target-specific and precisely controlled cancer therapy.

6.2.3 EFs and wound healing

The wound repair is one of the most complex biological process involving not only biological guidance cues but also non-biological factors to accomplish the wound closure. In general, the wound healing process occurs in almost all types of tissue damage / disruption. A huge clinical burden in treating wound healing resulted from inefficient handling and treatment due to long and comprehensive process and multiple factors, especially when dealing with non-healing chronic wound.

It has been demonstrated that EFs almost take part in all stages of wound repair. Song et al reported that increasing trans corneal potential difference (TCPD) enhanced cornea wound healing and decreasing TCPD prolong the wound healing *in vivo* (Song et al., 2002). In addition, wound-generated EFs regulated epithelial cell proliferation during wound healing (Song et al., 2002). Moreover, it has been demonstrated that EFs can induce fibroblast directional migration via PI3K signalling pathway (Guo et al., 2010). The membrane growth factor receptors redistribution and Golgi apparatus polarization have been thought to be the molecular basis of EF-induced directional migration (Pu et al., 2007; Pu and Zhao, 2005; Zhao et al., 2002b).

6.2.4 Application of EFs in wound healing treatment

Clinically, application of EFs is a new and challenging avenue to pursue better wound healing and regeneration. Adams et al. reported that using pharmacological manipulation to activate ion transportation thus speed up wound healing (Adams et al., 2007). Because of the intrinsic signalling system of cells to EFs, application of “therapeutic” current for wound healing is expected to have positive effects as well (Zhao, 2009). While numerous clinical trial have been done and some with very promising results, one device (the

Stadyn Dermapulse) has undergone controlled animal and human test (Gentzkow and Miller, 1991), and an application requesting approval for treating dermal ulcers has been submitted to FDA.

Our results, apart from elaborating the molecular mechanism of EF-induced fibroblasts directional migration, also raise the possibility that the deficient response to physiological EF may contribute at least in part to the slow healing / non-healing of chronic wound. Given the fact that cytokine treatment is prevailing in clinical treatment of chronic wound, and that endogenous EFs regulate directional cell migration during wound healing, our rather surprising finding that over dose of PDGF would attenuate electrotactic response of fibroblasts, indicates a clear warning that we must be cautious with the strategy of using cytokines in the clinical treatment of chronic wound. Moreover, the optimised concentration of PDGF combined with EFs also achieved better wound healing efficiency, which may shed light on a comprehensive treatment of chronic wound combining cytokines, electrical signals and other factors.

6.3 Future experiments

6.3.1 EF's function in immunomodulation

Although, I have found that EF can modulate DCs sensitization in response to IL-18, there still are some gaps to be filled in order to achieve definitive and convincing conclusion.

Firstly, a loss-of-function analysis with either IL18R inhibition or IL18R receptor deficiency would be a valuable evidence to verify the conclusion retrospectively.

Secondly, the study is lack of firm and deliberate proof for the EF-induced signalling cascade downstream of IL18Rs. A screening of potential downstream effectors should be conducted to measure the alteration of other signalling pathways parallel to P38 MAPK, such as Erk1/2, JNK.

Thirdly, an *in vivo* experiment to investigate if pharmacologically enhanced TEPD plus IL-18 treatment could change LCs migration from skin to draining lymph node will be a strong evidence for the regulating effects of EFs on LCs.

Fourthly, application of electrical stimulation locally on the mouse skin would eliminate the pharmacological effects brought by chemicals and provide a definitive answer to the EF stimulation effects.

Fifthly, transgenic mice will always be useful to support and verify the hypothesis; therefore further confirmation on IL18R knockout mice would significantly facilitate the reliability of the study.

6.3.2 EFs Application on chronic wound

To further prove the discovery and conclusions that CWF is lack of response to EFs and PDGF impairs fibroblast response to EF stimulation, the following experiments would be beneficial.

Firstly, direct measurement of wound-induced EFs on chronic wound can further confirm whether the wound healing defect in chronic wound was due to the defect of the CWFs to sense the wound EFs, or the lack of wound EFs.

Secondly, because Rho GTPases interact with each other closely, it would be necessary to test if Cdc42, Rac are involved in the PDGF-induced random migration when EF is present.

Thirdly, RhoA downstream effector myosin controls cell contraction, thus the study of the relation between EF-induced motility and myosin controlled contraction should provide a distinctive explanation of PDGF's function in electrotaxis.

Reference

- Ackerman, A.L., and Cresswell, P. (2003). Regulation of MHC class I transport in human dendritic cells and the dendritic-like cell line KG-1. *Journal of Immunology* *170*, 4178-4188.
- Adachi, Y., Kindzelskii, A.L., Ohno, N., Yadomae, T., and Petty, H.R. (1999). Amplitude and frequency modulation of metabolic signals in leukocytes: synergistic role of IFN-gamma in IL-6- and IL-2-mediated cell activation. *J Immunol* *163*, 4367-4374.
- Adams, D.S., Masi, A., and Levin, M. (2007). H⁺ pump-dependent changes in membrane voltage are an early mechanism necessary and sufficient to induce *Xenopus* tail regeneration. *Development* *134*, 1323-1335.
- Agren, M.S., Steenfoss, H.H., Dabelsteen, S., Hansen, J.B., and Dabelsteen, E. (1999). Proliferation and mitogenic response to PDGF-BB of fibroblasts isolated from chronic venous leg ulcers is ulcer-age dependent. *J Invest Dermatol* *112*, 463-469.
- Alblas, J., Ulfman, L., Hordijk, P., and Koenderman, L. (2001). Activation of RhoA and ROCK are essential for detachment of migrating leukocytes. *Mol Biol Cell* *12*, 2137-2145.
- Alboni, S., Cervia, D., Sugama, S., and Conti, B. (2010). Interleukin 18 in the CNS. *Journal of Neuroinflammation* *7*, 9.
- Aller, M.A., Arias, J.L., and Arias, J. (2007). The mast cell integrates the splanchnic and systemic inflammatory response in portal hypertension. *J Transl Med* *5*, 44.
- Alvarez, D., Vollmann, E.H., and von Andrian, U.H. (2008). Mechanisms and consequences of dendritic cell migration. *Immunity* *29*, 325-342.
- Amann, K.J., and Pollard, T.D. (2001). The Arp2/3 complex nucleates actin filament branches from the sides of pre-existing filaments. *Nat Cell Biol* *3*, 306-310.
- Amendt, C., Mann, A., Schirmacher, P., and Blessing, M. (2002). Resistance of keratinocytes to TGFbeta-mediated growth restriction and apoptosis induction accelerates re-epithelialization in skin wounds. *J Cell Sci* *115*, 2189-2198.
- Andrae, J., Gallini, R., and Betsholtz, C. (2008). Role of platelet-derived growth factors in physiology and medicine. *Genes Dev* *22*, 1276-1312.
- Anjuere, F., Martinez del Hoyo, G., Martin, P., and Ardavin, C. (2000). Langerhans cells acquire a CD8⁺ dendritic cell phenotype on maturation by CD40 ligation. *J Leukoc Biol* *67*, 206-209.

Anthis, N.J., Wegener, K.L., Ye, F., Kim, C., Goult, B.T., Lowe, E.D., Vakonakis, I., Bate, N., Critchley, D.R., Ginsberg, M.H., *et al.* (2009). The structure of an integrin/talin complex reveals the basis of inside-out signal transduction. *EMBO J* 28, 3623-3632.

Anticevich, S.Z., Hughes, J.M., Black, J.L., and Armour, C.L. (1996). Induction of hyperresponsiveness in human airway tissue by neutrophils--mechanism of action. *Clinical and experimental allergy : journal of the British Society for Allergy and Clinical Immunology* 26, 549-556.

Antoniades, H.N., Galanopoulos, T., Neville-Golden, J., Kiritsy, C.P., and Lynch, S.E. (1991). Injury induces in vivo expression of platelet-derived growth factor (PDGF) and PDGF receptor mRNAs in skin epithelial cells and PDGF mRNA in connective tissue fibroblasts. *Proc Natl Acad Sci U S A* 88, 565-569.

Antonopoulos, C., Cumberbatch, M., Dearman, R.J., Daniel, R.J., Kimber, I., and Groves, R.W. (2001). Functional caspase-1 is required for Langerhans cell migration and optimal contact sensitization in mice. *J Immunol* 166, 3672-3677.

Antonopoulos, C., Cumberbatch, M., Mee, J.B., Dearman, R.J., Wei, X.Q., Liew, F.Y., Kimber, I., and Groves, R.W. (2008). IL-18 is a key proximal mediator of contact hypersensitivity and allergen-induced Langerhans cell migration in murine epidermis. *J Leukoc Biol* 83, 361-367.

Arber, S., Barbayannis, F.A., Hanser, H., Schneider, C., Stanyon, C.A., Bernard, O., and Caroni, P. (1998). Regulation of actin dynamics through phosphorylation of cofilin by LIM-kinase. *Nature* 393, 805-809.

Arend, W.P., Palmer, G., and Gabay, C. (2008). IL - 1, IL - 18, and IL - 33 families of cytokines. *Immunological reviews* 223, 20-38.

Ashcroft, G.S., Yang, X., Glick, A.B., Weinstein, M., Letterio, J.L., Mizel, D.E., Anzano, M., Greenwell-Wild, T., Wahl, S.M., Deng, C., *et al.* (1999). Mice lacking Smad3 show accelerated wound healing and an impaired local inflammatory response. *Nat Cell Biol* 1, 260-266.

Askari, J.A., Buckley, P.A., Mould, A.P., and Humphries, M.J. (2009). Linking integrin conformation to function. *J Cell Sci* 122, 165-170.

Bachmann, M., Dragoi, C., Poleganov, M.A., Pfeilschifter, J., and Muhl, H. (2007a). Interleukin-18 directly activates T-bet expression and function via p38 mitogen-activated protein kinase and nuclear factor-kappaB in acute myeloid leukemia-derived predendritic KG-1 cells. *Mol Cancer Ther* 6, 723-731.

Bachmann, M., Dragoi, C., Poleganov, M.A., Pfeilschifter, J., and Muhl, H. (2007b). Interleukin-18 directly activates T-bet expression and function via p38 mitogen-activated protein kinase and nuclear factor-kappaB in acute myeloid leukemia-derived predendritic KG-1 cells. *Mol Cancer Ther* 6, 723-731.

Bamboot, Z.M., Stableford, J.A., Plitas, G., Burt, B.M., Nguyen, H.M., Welles, A.P., Gonen, M., Young, J.W., and DeMatteo, R.P. (2009). Human liver dendritic cells promote T cell hyporesponsiveness. *J Immunol* 182, 1901-1911.

Banchereau, J., Briere, F., Caux, C., Davoust, J., Lebecque, S., Liu, Y.J., Pulendran, B., and Palucka, K. (2000). Immunobiology of dendritic cells. *Annu Rev Immunol* 18, 767-811.

Barbet, G., Demion, M., Moura, I.C., Serafini, N., Leger, T., Vrtovsnik, F., Monteiro, R.C., Guinamard, R., Kinet, J.P., and Launay, P. (2008). The calcium-activated nonselective cation channel TRPM4 is essential for the migration but not the maturation of dendritic cells. *Nature immunology* 9, 1148-1156.

Barrientos, S., Stojadinovic, O., Golinko, M.S., Brem, H., and Tomic-Canic, M. (2008). Growth factors and cytokines in wound healing. *Wound Repair Regen* 16, 585-601.

Beidler, S.K., Douillet, C.D., Berndt, D.F., Keagy, B.A., Rich, P.B., and Marston, W.A. (2009). Inflammatory cytokine levels in chronic venous insufficiency ulcer tissue before and after compression therapy. *J Vasc Surg* 49, 1013-1020.

Benhamron, S., Nechushtan, H., Verbovetski, I., Krispin, A., Abboud-Jarrous, G., Zcharia, E., Edovitsky, E., Nahari, E., Peretz, T., Vlodaysky, I., *et al.* (2006). Translocation of active heparanase to cell surface regulates degradation of extracellular matrix heparan sulfate upon transmigration of mature monocyte-derived dendritic cells. *J Immunol* 176, 6417-6424.

Bennett, N.T., and Schultz, G.S. (1993). Growth factors and wound healing: Part II. Role in normal and chronic wound healing. *Am J Surg* 166, 74-81.

Benson, H.L., Suzuki, H., Lott, J., Fisher, A.J., Walline, C., Heidler, K.M., Brutkiewicz, R., Blum, J.S., and Wilkes, D.S. (2012). Donor lung derived myeloid and plasmacytoid dendritic cells differentially regulate T cell proliferation and cytokine production. *Respiratory research* 13, 25.

Betz, W.J., Caldwell, J.H., Ribchester, R.R., Robinson, K.R., and Stump, R.F. (1980). Endogenous electric field around muscle fibres depends on the Na⁺-K⁺ pump. *Nature* 287, 235-237.

Bharadwaj, U., Zhang, R.X., Yang, H., Li, M., Doan, L.X., Chen, C.Y., and Yao, Q.Z. (2005). Effects of cyclophilin A on myeloblastic cell line KG-1 derived dendritic like cells (DLC) through p38 MAP kinase activation. *Journal of Surgical Research* 127, 29-38.

Bienenstock, J., Tomioka, M., Stead, R., Ernst, P., Jordana, M., Gauldie, J., Dolovich, J., and Denburg, J. (1987). Mast cell involvement in various inflammatory processes. *Am Rev Respir Dis* 135, S5-8.

Bigby, M., Kwan, T., and Sy, M.S. (1987). Ratio of Langerhans cells to Thy-1+ dendritic epidermal cells in murine epidermis influences the intensity of contact hypersensitivity. *J Invest Dermatol* 89, 495-499.

Blanchoin, L., Amann, K.J., Higgs, H.N., Marchand, J.B., Kaiser, D.A., and Pollard, T.D. (2000). Direct observation of dendritic actin filament networks nucleated by Arp2/3 complex and WASP/Scar proteins. *Nature* 404, 1007-1011.

Bolte, S., and Cordelieres, F.P. (2006). A guided tour into subcellular colocalization analysis in light microscopy. *J Microsc* 224, 213-232.

Borgens, R.B., Roederer, E., and Cohen, M.J. (1981). Enhanced spinal cord regeneration in lamprey by applied electric fields. *Science* 213, 611-617.

Braun, S., auf dem Keller, U., Steiling, H., and Werner, S. (2004). Fibroblast growth factors in epithelial repair and cytoprotection. *Philos Trans R Soc Lond B Biol Sci* 359, 753-757.

Braund, R., Hook, S., and Medlicott, N.J. (2007). The role of topical growth factors in chronic wounds. *Current drug delivery* 4, 195-204.

Brave, A., Gudmundsdotter, L., Sandstrom, E., Haller, B.K., Hallengard, D., Maltais, A.K., King, A.D., Stout, R.R., Blomberg, P., Hoglund, U., *et al.* (2010). Biodistribution, persistence and lack of integration of a multigene HIV vaccine delivered by needle-free intradermal injection and electroporation. *Vaccine* 28, 8203-8209.

Bufler, P., Azam, T., Gamboni-Robertson, F., Reznikov, L.L., Kumar, S., Dinarello, C.A., and Kim, S.H. (2002). A complex of the IL-1 homologue IL-1F7b and IL-18-binding protein reduces IL-18 activity. *Proc Natl Acad Sci U S A* 99, 13723-13728.

Calderwood, D.A., Fujioka, Y., de Pereda, J.M., Garcia-Alvarez, B., Nakamoto, T., Margolis, B., McGlade, C.J., Liddington, R.C., and Ginsberg, M.H. (2003). Integrin beta cytoplasmic domain interactions with phosphotyrosine-binding domains: a structural prototype for diversity in integrin signaling. *Proc Natl Acad Sci U S A* 100, 2272-2277.

Campitiello, F., Della Corte, A., Fattopace, A., D'Acunzi, D., and Canonico, S. (2005). The use of artificial dermis in the treatment of chronic and acute wounds: Regeneration of dermis and wound healing. *Acta Biomedica de l'Ateneo Parmense* 76, 69-71.

Cantarelli, V.V., Kodama, T., Nijstad, N., Abolghait, S.K., Iida, T., and Honda, T. (2006). Cortactin is essential for F-actin assembly in enteropathogenic *Escherichia coli* (EPEC)- and enterohaemorrhagic *E. coli* (EHEC)-induced pedestals and the alpha-helical region is involved in the localization of cortactin to bacterial attachment sites. *Cell Microbiol* 8, 769-780.

Cao, R., Brakenhielm, E., Pawliuk, R., Wariaro, D., Post, M.J., Wahlberg, E., Leboulch, P., and Cao, Y. (2003). Angiogenic synergism, vascular stability and improvement of hind-limb ischemia by a combination of PDGF-BB and FGF-2. *Nat Med* 9, 604-613.

Caras, I., Tucureanu, C., Lerescu, L., and Salageanu, A. (2007). Bacterial product cantastim derived from *Pseudomonas aeruginosa* induces migration and maturation of dendritic cells. *Roumanian archives of microbiology and immunology* 66, 5-9.

Carlier, M.F., Laurent, V., Santolini, J., Melki, R., Didry, D., Xia, G.X., Hong, Y., Chua, N.H., and Pantaloni, D. (1997). Actin depolymerizing factor (ADF/cofilin) enhances the rate of filament turnover: implication in actin-based motility. *J Cell Biol* 136, 1307-1322.

Castronuovo, J.J., Jr., Ghobrial, I., Giusti, A.M., Rudolph, S., and Smiell, J.M. (1998). Effects of chronic wound fluid on the structure and biological activity of becaplermin (rhPDGF-BB) and becaplermin gel. *Am J Surg* 176, 61S-67S.

Caux, C., Vanbervliet, B., Massacrier, C., Dezutter-Dambuyant, C., de Saint-Vis, B., Jacquet, C., Yoneda, K., Imamura, S., Schmitt, D., and Banchereau, J. (1996). CD34+ hematopoietic progenitors from human cord blood differentiate along two independent dendritic cell pathways in response to GM-CSF+TNF alpha. *J Exp Med* 184, 695-706.

Cha, J., Kwak, T., Butmarc, J., Kim, T.A., Yufit, T., Carson, P., Kim, S.J., and Falanga, V. (2008a). Fibroblasts from non-healing human chronic wounds show decreased expression of beta ig-h3, a TGF-beta inducible protein. *J Dermatol Sci* 50, 15-23.

Cha, J., Kwak, T., Butmarc, J., Kim, T.A., Yufit, T., Carson, P., Kim, S.J., and Falanga, V. (2008b). Fibroblasts from non-healing human chronic wounds show decreased expression of Îg-h3, a TGF- Î-inducible protein. *Journal of Dermatological Science* 50, 15-23.

Chabot, V., Reverdiau, P., Iochmann, S., Rico, A., Senecal, D., Goupille, C., Sizaret, P.Y., and Sensebe, L. (2006). CCL5-enhanced human immature dendritic cell migration through the basement membrane in vitro depends on matrix metalloproteinase-9. *J Leukoc Biol* 79, 767-778.

Chen, L., Zhang, J.J., and Huang, X.Y. (2008). cAMP inhibits cell migration by interfering with Rac-induced lamellipodium formation. *J Biol Chem* 283, 13799-13805.

Chiang, H.S., Wu, W.B., Fang, J.Y., Chen, D.F., Chen, B.H., Huang, C.C., Chen, Y.T., and Hung, C.F. (2007). Lycopene inhibits PDGF-BB-induced signaling and migration in human dermal fibroblasts through interaction with PDGF-BB. *Life Sci* 81, 1509-1517.

Ching, C.S., Phillips, T.J., Howard, C.E., Panova, I.P., Hayes, C.M., Asandra, A.S., and Park, H.Y. (2005). Chronic wound fluid suppresses proliferation of dermal fibroblasts through a Ras-mediated signaling pathway. *Journal of Investigative Dermatology* 124, 466-474.

Chmielowiec, J., Borowiak, M., Morkel, M., Stradal, T., Munz, B., Werner, S., Wehland, J., Birchmeier, C., and Birchmeier, W. (2007). c-Met is essential for wound healing in the skin. *J Cell Biol* 177, 151-162.

Choi, C.K., Vicente-Manzanares, M., Zareno, J., Whitmore, L.A., Mogilner, A., and Horwitz, A.R. (2008). Actin and alpha-actinin orchestrate the assembly and maturation of nascent adhesions in a myosin II motor-independent manner. *Nat Cell Biol* 10, 1039-1050.

Choudhury, G.G., Karamitsos, C., Hernandez, J., Gentilini, A., Bardgette, J., and Abboud, H.E. (1997). PI-3-kinase and MAPK regulate mesangial cell proliferation and migration in response to PDGF. *Am J Physiol* 273, F931-938.

Co, C., Wong, D.T., Gierke, S., Chang, V., and Taunton, J. (2007). Mechanism of actin network attachment to moving membranes: barbed end capture by N-WASP WH2 domains. *Cell* 128, 901-913.

Codner, M.A., Shuster, B.A., Steinman, R.M., Harper, A.D., LaTrenta, G.S., and Hoffman, L.A. (1990). Migration of donor leukocytes from limb allografts into host lymphoid tissues. *Ann Plast Surg* 25, 353-359.

Conti, B., Jahng, J.W., Tinti, C., Son, J.H., and Joh, T.H. (1997). Induction of interferon-gamma inducing factor in the adrenal cortex. *J Biol Chem* 272, 2035-2037.

Cook, H., Davies, K.J., Harding, K.G., and Thomas, D.W. (2000). Defective extracellular matrix reorganization by chronic wound fibroblasts is associated with alterations in TIMP-1, TIMP-2, and MMP-2 activity. *J Invest Dermatol* 115, 225-233.

Cooper, D.M., Yu, E.Z., Hennessey, P., Ko, F., and Robson, M.C. (1994). Determination of endogenous cytokines in chronic wounds. *Ann Surg* 219, 688-691; discussion 691-682.

Cooper, M.S., and Keller, R.E. (1984). Perpendicular orientation and directional migration of amphibian neural crest cells in dc electrical fields. *Proc Natl Acad Sci U S A* 81, 160-164.

Cumberbatch, M., Dearman, R.J., Antonopoulos, C., Groves, R.W., and Kimber, I. (2001). Interleukin (IL)-18 induces Langerhans cell migration by a tumour necrosis factor-alpha- and IL-1beta-dependent mechanism. *Immunology* 102, 323-330.

Cumberbatch, M., Dearman, R.J., Groves, R.W., Antonopoulos, C., and Kimber, I. (2002). Differential regulation of epidermal langerhans cell migration by interleukins (IL)-1alpha and IL-1beta during irritant- and allergen-induced cutaneous immune responses. *Toxicol Appl Pharmacol* 182, 126-135.

Cumberbatch, M., Dearman, R.J., and Kimber, I. (1997a). Interleukin 1 beta and the stimulation of Langerhans cell migration: comparisons with tumour necrosis factor alpha. *Arch Dermatol Res* 289, 277-284.

Cumberbatch, M., Dearman, R.J., and Kimber, I. (1997b). Stimulation of Langerhans cell migration in mice by tumour necrosis factor alpha and interleukin 1 beta. *Adv Exp Med Biol* 417, 121-124.

Dao, T., Ohashi, K., Kayano, T., Kurimoto, M., and Okamura, H. (1996). Interferon-gamma-inducing factor, a novel cytokine, enhances Fas ligand-mediated cytotoxicity of murine T helper 1 cells. *Cell Immunol* 173, 230-235.

Daub, H., Gevaert, K., Vandekerckhove, J., Sobel, A., and Hall, A. (2001). Rac/Cdc42 and p65PAK regulate the microtubule-destabilizing protein stathmin through phosphorylation at serine 16. *J Biol Chem* 276, 1677-1680.

De Ugarte, D.A., Roberts, R.L., Lerdluedeeporn, P., Stiehm, E.R., and Atkinson, J.B. (2002). Treatment of chronic wounds by local delivery of granulocyte-macrophage colony-stimulating factor in patients with neutrophil dysfunction. *Pediatric surgery international* 18, 517-520.

Deodhar, A.K., and Rana, R.E. (1997). Surgical physiology of wound healing: a review. *J Postgrad Med* 43, 52-56.

Didry, D., Carlier, M.F., and Pantaloni, D. (1998). Synergy between actin depolymerizing factor/cofilin and profilin in increasing actin filament turnover. *J Biol Chem* 273, 25602-25611.

Dieu, M.C., Vanbervliet, B., Vicari, A., Bridon, J.M., Oldham, E., Ait-Yahia, S., Briere, F., Zlotnik, A., Lebecque, S., and Caux, C. (1998). Selective recruitment of immature and mature dendritic cells by distinct chemokines expressed in different anatomic sites. *J Exp Med* 188, 373-386.

Dinarello, C.A. (1999). IL-18: A TH1-inducing, proinflammatory cytokine and new member of the IL-1 family. *J Allergy Clin Immunol* 103, 11-24.

Dinarello, C.A. (2007). Interleukin-18 and the pathogenesis of inflammatory diseases. *Seminars in nephrology* 27, 98-114.

Dovi, J.V., Szpaderska, A.M., and DiPietro, L.A. (2004). Neutrophil function in the healing wound: adding insult to injury? *Thromb Haemost* 92, 275-280.

Eden, S., Rohatgi, R., Podtelejnikov, A.V., Mann, M., and Kirschner, M.W. (2002). Mechanism of regulation of WAVE1-induced actin nucleation by Rac1 and Nck. *Nature* 418, 790-793.

Egozi, E.I., Ferreira, A.M., Burns, A.L., Gamelli, R.L., and DiPietro, L.A. (2003). Mast cells modulate the inflammatory but not the proliferative response in healing wounds. *Wound Repair Regen* 11, 46-54.

Elbe, A., Tschachler, E., Steiner, G., Binder, A., Wolff, K., and Stingl, G. (1989). Maturation steps of bone marrow-derived dendritic murine epidermal cells. Phenotypic and functional studies on Langerhans cells and Thy-1+ dendritic epidermal cells in the perinatal period. *J Immunol* 143, 2431-2438.

Eming, S.A., Krieg, T., and Davidson, J.M. (2007). Inflammation in wound repair: Molecular and cellular mechanisms. *Journal of Investigative Dermatology* 127, 514-525.

Etienne-Manneville, S., and Hall, A. (2001). Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCzeta. *Cell* 106, 489-498.

Falanga, V. (2005). Advanced treatments for non-healing chronic wounds. *World Wide Wounds* 2005.

Falanga, V., and Eaglstein, W.H. (1993). The "trap" hypothesis of venous ulceration. *Lancet* 341, 1006-1008.

Flint, M.S., Dearman, R.J., Kimber, I., and Hotchkiss, S.A. (1998). Production and in situ localization of cutaneous tumour necrosis factor alpha (TNF-alpha) and interleukin 6 (IL-6) following skin sensitization. *Cytokine* 10, 213-219.

Forster, R., Schubel, A., Breitfeld, D., Kremmer, E., Renner-Muller, I., Wolf, E., and Lipp, M. (1999). CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell* 99, 23-33.

Frelinger, J.G., Hood, L., Hill, S., and Frelinger, J.A. (1979). Mouse epidermal Ia molecules have a bone marrow origin. *Nature* 282, 321-323.

Frick, J.S., Grunebach, F., and Autenrieth, I.B. (2010). Immunomodulation by semi-mature dendritic cells: a novel role of Toll-like receptors and interleukin-6. *Int J Med Microbiol* 300, 19-24.

Fu, F., Li, Y., Qian, S., Lu, L., Chambers, F.D., Starzl, T.E., Fung, J.J., and Thomson, A.W. (1997). Costimulatory molecule-deficient dendritic cell progenitors induce T cell hyporesponsiveness in vitro and prolong the survival of vascularized cardiac allografts. *Transplant Proc* 29, 1310.

Fu, X., Shen, Z., Guo, Z., Zhang, M., and Sheng, Z. (2002). Healing of chronic cutaneous wounds by topical treatment with basic fibroblast growth factor. *Chin Med J (Engl)* 115, 331-335.

Fukata, M., Nakagawa, M., and Kaibuchi, K. (2003). Roles of Rho-family GTPases in cell polarisation and directional migration. *Curr Opin Cell Biol* 15, 590-597.

Funamoto, S., Meili, R., Lee, S., Parry, L., and Firtel, R.A. (2002). Spatial and temporal regulation of 3-phosphoinositides by PI 3-kinase and PTEN mediates chemotaxis. *Cell* 109, 611-623.

Gao, Z., Sasaoka, T., Fujimori, T., Oya, T., Ishii, Y., Sabit, H., Kawaguchi, M., Kurotaki, Y., Naito, M., Wada, T., *et al.* (2005). Deletion of the PDGFR-beta gene affects key fibroblast functions important for wound healing. *J Biol Chem* 280, 9375-9389.

Geissmann, F., Prost, C., Monnet, J.P., Dy, M., Brousse, N., and Hermine, O. (1998). Transforming growth factor beta1, in the presence of granulocyte/macrophage colony-stimulating factor and interleukin 4, induces differentiation of human peripheral blood monocytes into dendritic Langerhans cells. *J Exp Med* 187, 961-966.

Gentzkow, G.D., and Miller, K.H. (1991). Electrical stimulation for dermal wound healing. *Clinics in podiatric medicine and surgery* 8, 827-841.

Gerberick, G.F., Ryan, C.A., Von Bargen, E.C., Stuard, S.B., and Ridder, G.M. (1991). Examination of tetrachlorosalicylanilide (TCSA) photoallergy using in vitro photohapten-modified Langerhans cell-enriched epidermal cells. *J Invest Dermatol* 97, 210-218.

Glasgow, J.E., and Daniele, R.P. (1994). Role of microtubules in random cell migration: stabilization of cell polarity. *Cell Motil Cytoskeleton* 27, 88-96.

Goc, A., Abdalla, M., Al-Azayzih, A., and Somanath, P.R. (2012). Rac1 activation driven by 14-3-3zeta dimerization promotes prostate cancer cell-matrix interactions, motility and transendothelial migration. *PLoS One* 7, e40594.

Goldman, R. (2004). Growth factors and chronic wound healing: past, present, and future. *Adv Skin Wound Care* 17, 24-35.

Gournier, H., Goley, E.D., Niederstrasser, H., Trinh, T., and Welch, M.D. (2001). Reconstitution of human Arp2/3 complex reveals critical roles of individual subunits in complex structure and activity. *Mol Cell* 8, 1041-1052.

Gracie, J.A., Robertson, S.E., and McInnes, I.B. (2003). Interleukin-18. *J Leukoc Biol* 73, 213-224.

Granucci, F., Ferrero, E., Foti, M., Aggujaro, D., Vettoreto, K., and Ricciardi-Castagnoli, P. (1999). Early events in dendritic cell maturation induced by LPS. *Microbes Infect* 1, 1079-1084.

Guan, S., Fan, J., Han, A., Chen, M., Woodley, D.T., and Li, W. (2009). Non-compensating roles between Nckalpha and Nckbeta in PDGF-BB signaling to promote human dermal fibroblast migration. *J Invest Dermatol* 129, 1909-1920.

Guo, A., Song, B., Reid, B., Gu, Y., Forrester, J.V., Jahoda, C.A., and Zhao, M. (2010). Effects of physiological electric fields on migration of human dermal fibroblasts. *J Invest Dermatol* 130, 2320-2327.

Gupton, S.L., Anderson, K.L., Kole, T.P., Fischer, R.S., Ponti, A., Hitchcock-DeGregori, S.E., Danuser, G., Fowler, V.M., Wirtz, D., Hanein, D., *et al.* (2005). Cell migration without a lamellipodium: translation of actin dynamics into cell movement mediated by tropomyosin. *J Cell Biol* 168, 619-631.

Gurtner, G.C., Werner, S., Barrandon, Y., and Longaker, M.T. (2008). Wound repair and regeneration. *Nature* 453, 314-321.

Gutzmer, R., Langer, K., Mommert, S., Wittmann, M., Kapp, A., and Werfel, T. (2003). Human dendritic cells express the IL-18R and are chemoattracted to IL-18. *J Immunol* *171*, 6363-6371.

Haessler, U., Pisano, M., Wu, M., and Swartz, M.A. (2011). Dendritic cell chemotaxis in 3D under defined chemokine gradients reveals differential response to ligands CCL21 and CCL19. *Proc Natl Acad Sci U S A* *108*, 5614-5619.

Han, G., Nguyen, L.N., Macherla, C., Chi, Y., Friedman, J.M., Nosanchuk, J.D., and Martinez, L.R. (2012). Nitric oxide-releasing nanoparticles accelerate wound healing by promoting fibroblast migration and collagen deposition. *Am J Pathol* *180*, 1465-1473.

Harding, K.G., Morris, H.L., and Patel, G.K. (2002). Science, medicine and the future: healing chronic wounds. *BMJ* *324*, 160-163.

Hart, D.N., and Fabre, J.W. (1981a). Demonstration and characterization of Ia-positive dendritic cells in the interstitial connective tissues of rat heart and other tissues, but not brain. *J Exp Med* *154*, 347-361.

Hart, D.N., and Fabre, J.W. (1981b). Localization of MHC antigens in long surviving rat renal allografts: probable implication of passenger leukocytes in graft adaptation. *Transplant Proc* *13*, 95-99.

Hart, D.N., and Fabre, J.W. (1981c). Major histocompatibility complex antigens in rat kidney, ureter, and bladder. Localization with monoclonal antibodies and demonstration of Ia-positive dendritic cells. *Transplantation* *31*, 318-325.

Hart, D.N., and Fabre, J.W. (1982). Mechanism of induction of passive enhancement. Evidence for an interaction of enhancing antibody with donor interstitial dendritic cells. *Transplantation* *33*, 319-322.

Hart, D.N., Fuggle, S.V., Williams, K.A., Fabre, J.W., Ting, A., and Morris, P.J. (1981). Localization of HLA-ABC and DR antigens in human kidney. *Transplantation* *31*, 428-433.

Haugh, J.M., Codazzi, F., Teruel, M., and Meyer, T. (2000). Spatial sensing in fibroblasts mediated by 3' phosphoinositides. *J Cell Biol* *151*, 1269-1280.

Havran, W.L., and Jameson, J.M. (2010). Epidermal T cells and wound healing. *J Immunol* *184*, 5423-5428.

He, Q., You, H., Li, X.M., Liu, T.H., Wang, P., and Wang, B.E. (2012). HMGB1 Promotes the Synthesis of Pro-IL-1beta and Pro-IL-18 by Activation of p38 MAPK and NF-kappaB Through Receptors for Advanced Glycation End-products in Macrophages. *Asian Pacific journal of cancer prevention : APJCP* *13*, 1365-1370.

Hellberg, C., Ostman, A., and Heldin, C.H. (2010). PDGF and vessel maturation. Recent results in cancer research Fortschritte der Krebsforschung Progres dans les recherches sur le cancer *180*, 103-114.

Henri, S., Vremec, D., Kamath, A., Waithman, J., Williams, S., Benoist, C., Burnham, K., Saeland, S., Handman, E., and Shortman, K. (2001). The dendritic cell populations of mouse lymph nodes. *J Immunol* *167*, 741-748.

Hertzog, M., Yarmola, E.G., Didry, D., Bubb, M.R., and Carlier, M.F. (2002). Control of actin dynamics by proteins made of beta-thymosin repeats: the actobindin family. *J Biol Chem* *277*, 14786-14792.

Heufler, C., Koch, F., and Schuler, G. (1988). Granulocyte/macrophage colony-stimulating factor and interleukin 1 mediate the maturation of murine epidermal Langerhans cells into potent immunostimulatory dendritic cells. *J Exp Med* *167*, 700-705.

Hikawa, H., Lotze, M.T., and Rosenblum, W.D. (2007). Induction of peripheral tolerance by local delivery of dendritic cell progenitors to cardiac allografts in a murine heterotopic heart transplantation model. *General thoracic and cardiovascular surgery* *55*, 307-314.

Himmel, M., Ritter, A., Rothmund, S., Pauling, B.V., Rottner, K., Gingras, A.R., and Ziegler, W.H. (2009). Control of high affinity interactions in the talin C terminus: how talin domains coordinate protein dynamics in cell adhesions. *J Biol Chem* *284*, 13832-13842.

Hofer, S., Ivarsson, L., Stoitzner, P., Auffinger, M., Rainer, C., Romani, N., and Heufler, C. (2003). Adenosine slows migration of dendritic cells but does not affect other aspects of dendritic cell maturation. *J Invest Dermatol* *121*, 300-307.

Howe, R., Dillon, S., Rogers, L., McCarter, M., Kelly, C., Gonzalez, R., Madinger, N., and Wilson, C.C. (2009). Evidence for dendritic cell-dependent CD4(+) T helper-1 type responses to commensal bacteria in normal human intestinal lamina propria. *Clin Immunol* *131*, 317-332.

Inaba, K., Inaba, M., Romani, N., Aya, H., Deguchi, M., Ikehara, S., Muramatsu, S., and Steinman, R.M. (1992). Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* *176*, 1693-1702.

Ishizaki, T., Morishima, Y., Okamoto, M., Furuyashiki, T., Kato, T., and Narumiya, S. (2001). Coordination of microtubules and the actin cytoskeleton by the Rho effector mDia1. *Nat Cell Biol* *3*, 8-14.

Ito, T., Inaba, M., Inaba, K., Toki, J., Sogo, S., Iguchi, T., Adachi, Y., Yamaguchi, K., Amakawa, R., Valladeau, J., *et al.* (1999). A CD1a+/CD11c+ subset of human blood dendritic cells is a direct precursor of Langerhans cells. *J Immunol* *163*, 1409-1419.

Itoh, R.E., Kurokawa, K., Ohba, Y., Yoshizaki, H., Mochizuki, N., and Matsuda, M. (2002). Activation of rac and cdc42 video imaged by fluorescent resonance energy transfer-based single-molecule probes in the membrane of living cells. *Mol Cell Biol* 22, 6582-6591.

Iwasa, J.H., and Mullins, R.D. (2007). Spatial and temporal relationships between actin-filament nucleation, capping, and disassembly. *Curr Biol* 17, 395-406.

Jackson, B., Peyrollier, K., Pedersen, E., Basse, A., Karlsson, R., Wang, Z., Lefever, T., Ochsenein, A.M., Schmidt, G., Aktories, K., *et al.* (2011). RhoA is dispensable for skin development, but crucial for contraction and directed migration of keratinocytes. *Mol Biol Cell* 22, 593-605.

Jaffe, L.F., and Poo, M.M. (1979). Neurites grow faster towards the cathode than the anode in a steady field. *J Exp Zool* 209, 115-128.

Jameson, J., Ugarte, K., Chen, N., Yachi, P., Fuchs, E., Boismenu, R., and Havran, W.L. (2002). A role for skin gammadelta T cells in wound repair. *Science* 296, 747-749.

Jiang, W.G., Ye, L., Patel, G., and Harding, K.G. (2010). Expression of WAVEs, the WASP (Wiskott-Aldrich syndrome protein) family of verprolin homologous proteins in human wound tissues and the biological influence on human keratinocytes. *Wound Repair Regen* 18, 594-604.

Jimenez, C., Portela, R.A., Mellado, M., Rodriguez-Frade, J.M., Collard, J., Serrano, A., Martinez, A.C., Avila, J., and Carrera, A.C. (2000). Role of the PI3K regulatory subunit in the control of actin organization and cell migration. *J Cell Biol* 151, 249-262.

Jin, T., Xu, X., and Hereld, D. (2008). Chemotaxis, chemokine receptors and human disease. *Cytokine* 44, 1-8.

Johansen, O.E., Birkeland, K.I., Jorgensen, A.P., Orvik, E., Sorgard, B., Torjussen, B.R., Ueland, T., Aukrust, P., and Gullestad, L. (2009). Diabetic foot ulcer burden may be modified by high-dose atorvastatin: A 6-month randomized controlled pilot trial. *Journal of diabetes* 1, 182-187.

Johnson, S.L., and Woodbury, J.W. (1964). Membrane Resistance of Human Red Cells. *The Journal of general physiology* 47, 827-837.

Kabashima, K., Shiraishi, N., Sugita, K., Mori, T., Onoue, A., Kobayashi, M., Sakabe, J., Yoshiki, R., Tamamura, H., Fujii, N., *et al.* (2007). CXCL12-CXCR4 engagement is required for migration of cutaneous dendritic cells. *Am J Pathol* 171, 1249-1257.

Kalina, U., Kauschat, D., Koyama, N., Nuernberger, H., Ballas, K., Koschmieder, S., Bug, G., Hofmann, W.K., Hoelzer, D., and Ottmann, O.G. (2000). IL-18 activates STAT3 in the natural killer cell line 92, augments cytotoxic activity, and mediates IFN-gamma production by the stress kinase p38 and by the extracellular regulated kinases p44erk-1 and p42erk-21. *J Immunol* 165, 1307-1313.

Kaser, A., Kaser, S., Kaneider, N.C., Enrich, B., Wiedermann, C.J., and Tilg, H. (2004). Interleukin-18 attracts plasmacytoid dendritic cells (DC2s) and promotes Th1 induction by DC2s through IL-18 receptor expression. *Blood* 103, 648-655.

Kim, M.H., Liu, W., Borjesson, D.L., Curry, F.R., Miller, L.S., Cheung, A.L., Liu, F.T., Isseroff, R.R., and Simon, S.I. (2008). Dynamics of neutrophil infiltration during cutaneous wound healing and infection using fluorescence imaging. *J Invest Dermatol* 128, 1812-1820.

Kindzelskii, A.L., and Petty, H.R. (2000). Extremely low frequency pulsed DC electric fields promote neutrophil extension, metabolic resonance and DNA damage when phase-matched with metabolic oscillators. *Biochim Biophys Acta* 1495, 90-111.

Kindzelskii, A.L., and Petty, H.R. (2005). Ion channel clustering enhances weak electric field detection by neutrophils: apparent roles of SKF96365-sensitive cation channels and myeloperoxidase trafficking in cellular responses. *European biophysics journal : EBJ* 35, 1-26.

Kiss, Z., Deli, E., Shoji, M., Koeffler, H.P., Pettit, G.R., Vogler, W.R., and Kuo, J.F. (1987). Differential-Effects of Various Protein-Kinase-C Activators on Protein-Phosphorylation in Human Acute Myeloblastic-Leukemia Cell-Line Kg-1 and Its Phorbol Ester-Resistant Subline Kg-1a. *Cancer Research* 47, 1302-1307.

Kobayashi, K., Kuroda, S., Fukata, M., Nakamura, T., Nagase, T., Nomura, N., Matsuura, Y., Yoshida-Kubomura, N., Iwamatsu, A., and Kaibuchi, K. (1998). p140Sra-1 (specifically Rac1-associated protein) is a novel specific target for Rac1 small GTPase. *J Biol Chem* 273, 291-295.

Kobayashi, Y., Staquet, M.J., Dezutter-Dambuyant, C., and Schmitt, D. (1994). Development of motility of Langerhans cell through extracellular matrix by in vitro haptent contact. *Eur J Immunol* 24, 2254-2257.

Kobayashi, Y., Staquet, M.J., Dezutter-Dambuyant, C., and Schmitt, D. (1995). In vitro migration capacity of epidermal Langerhans cells. *Adv Exp Med Biol* 378, 169-171.

Koeffler, H.P., Bar-Eli, M., and Territo, M.C. (1981). Phorbol ester effect on differentiation of human myeloid leukemia cell lines blocked at different stages of maturation. *Cancer Res* 41, 919-926.

Koeffler, H.P., and Golde, D.W. (1978). Acute myelogenous leukemia: a human cell line responsive to colony-stimulating activity. *Science* 200, 1153-1154.

Kohka, H., Yoshino, T., Iwagaki, H., Sakuma, I., Tanimoto, T., Matsuo, Y., Kurimoto, M., Orita, K., Akagi, T., and Tanaka, N. (1998). Interleukin-18/interferon-gamma-inducing factor, a novel cytokine, up-regulates ICAM-1 (CD54) expression in KG-1 cells. *Journal of Leukocyte Biology* 64, 519-527.

Kojima, H., Aizawa, Y., Yanai, Y., Nagaoka, K., Takeuchi, M., Ohta, T., Ikegami, H., Ikeda, M., and Kurimoto, M. (1999). An essential role for NF-kappa B in IL-18-induced IFN-gamma expression in KG-1 cells. *J Immunol* *162*, 5063-5069.

Komai-Koma, M., Gracie, J.A., Wei, X.Q., Xu, D., Thomson, N., McInnes, I.B., and Liew, F.Y. (2003). Chemoattraction of human T cells by IL-18. *J Immunol* *170*, 1084-1090.

Konishi, K., Tanabe, F., Taniguchi, M., Yamauchi, H., Tanimoto, T., Ikeda, M., Orita, K., and Kurimoto, M. (1997). A simple and sensitive bioassay for the detection of human interleukin-18/interferon-gamma-inducing factor using human myelomonocytic KG-1 cells. *J Immunol Methods* *209*, 187-191.

Konya, D., Gercek, A., Akakin, A., Akakin, D., Tural, S., Cetinel, S., Ozgen, S., and Pamir, M.N. (2008). The effects of inflammatory response associated with traumatic spinal cord injury in cutaneous wound healing and on expression of transforming growth factor-beta1 (TGF-beta1) and platelet-derived growth factor (PDGF)-A at the wound site in rats. *Growth Factors* *26*, 74-79.

Koutoulaki, A., Langley, M., Sloan, A.J., Aeschlimann, D., and Wei, X.Q. (2010a). TNF alpha and TGF-beta 1 influence IL-18-induced IFN gamma production through regulation of IL-18 receptor and T-bet expression. *Cytokine* *49*, 177-184.

Koutoulaki, A., Langley, M., Sloan, A.J., Aeschlimann, D., and Wei, X.Q. (2010b). TNFalpha and TGF-beta1 influence IL-18-induced IFNgamma production through regulation of IL-18 receptor and T-bet expression. *Cytokine* *49*, 177-184.

Kovalenko, M., Ronnstrand, L., Heldin, C.H., Loubtchenkov, M., Gazit, A., Levitzki, A., and Bohmer, F.D. (1997). Phosphorylation site-specific inhibition of platelet-derived growth factor beta-receptor autophosphorylation by the receptor blocking tyrophostin AG1296. *Biochemistry* *36*, 6260-6269.

Koveker, G.B. (2000). Growth factors in clinical practice. *Int J Clin Pract* *54*, 590-593.

Kraynov, V.S., Chamberlain, C., Bokoch, G.M., Schwartz, M.A., Slabaugh, S., and Hahn, K.M. (2000). Localized Rac activation dynamics visualized in living cells. *Science* *290*, 333-337.

Kroschewski, R., Hall, A., and Mellman, I. (1999). Cdc42 controls secretory and endocytic transport to the basolateral plasma membrane of MDCK cells. *Nat Cell Biol* *1*, 8-13.

Kucerova, R., Walczysko, P., Reid, B., Ou, J., Leiper, L.J., Rajnicek, A.M., McCaig, C.D., Zhao, M., and Collinson, J.M. (2011). The role of electrical signals in murine corneal wound re-epithelialization. *Journal of cellular physiology* *226*, 1544-1553.

Kurz, S.M., Diebold, S.S., Hieronymus, T., Gust, T.C., Bartunek, P., Sachs, M., Birchmeier, W., and Zenke, M. (2002). The impact of c-met/scatter factor receptor on dendritic cell migration. *Eur J Immunol* 32, 1832-1838.

Lai, J.J., Lai, K.P., Chuang, K.H., Chang, P., Yu, I.C., Lin, W.J., and Chang, C. (2009). Monocyte/macrophage androgen receptor suppresses cutaneous wound healing in mice by enhancing local TNF-alpha expression. *J Clin Invest* 119, 3739-3751.

Lai, P.S., Fresco, J.M., Pinilla, M.A., Macias, A.A., Brown, R.D., Englert, J.A., Hofmann, O., Lederer, J.A., Hide, W., Christiani, D.C., *et al.* (2012). Chronic endotoxin exposure produces airflow obstruction and lung dendritic cell expansion. *Am J Respir Cell Mol Biol* 47, 209-217.

Lammermann, T., Bader, B.L., Monkley, S.J., Worbs, T., Wedlich-Soldner, R., Hirsch, K., Keller, M., Forster, R., Critchley, D.R., Fassler, R., *et al.* (2008). Rapid leukocyte migration by integrin-independent flowing and squeezing. *Nature* 453, 51-55.

Lateef, Z., Fleming, S., Halliday, G., Faulkner, L., Mercer, A., and Baird, M. (2003). Orf virus-encoded interleukin-10 inhibits maturation, antigen presentation and migration of murine dendritic cells. *J Gen Virol* 84, 1101-1109.

Lauffenburger, D.A., and Horwitz, A.F. (1996). Cell migration: a physically integrated molecular process. *Cell* 84, 359-369.

Le Clainche, C., and Carlier, M.F. (2008). Regulation of actin assembly associated with protrusion and adhesion in cell migration. *Physiol Rev* 88, 489-513.

Le Clainche, C., Didry, D., Carlier, M.F., and Pantaloni, D. (2001). Activation of Arp2/3 complex by Wiskott-Aldrich Syndrome protein is linked to enhanced binding of ATP to Arp2. *J Biol Chem* 276, 46689-46692.

Leask, A. (2010). Potential therapeutic targets for cardiac fibrosis: TGFbeta, angiotensin, endothelin, CCN2, and PDGF, partners in fibroblast activation. *Circ Res* 106, 1675-1680.

Leung, B.P., Culshaw, S., Gracie, J.A., Hunter, D., Canetti, C.A., Campbell, C., Cunha, F., Liew, F.Y., and McInnes, I.B. (2001). A role for IL-18 in neutrophil activation. *J Immunol* 167, 2879-2886.

Levin, M. (2009). Bioelectric mechanisms in regeneration: Unique aspects and future perspectives. *Semin Cell Dev Biol* 20, 543-556.

Li, B., and Wang, J.H. (2011). Fibroblasts and myofibroblasts in wound healing: force generation and measurement. *Journal of tissue viability* 20, 108-120.

Li, G., Gustafson-Brown, C., Hanks, S.K., Nason, K., Arbeit, J.M., Pogliano, K., Wisdom, R.M., and Johnson, R.S. (2003a). c-Jun is essential for organization of the epidermal leading edge. *Dev Cell* 4, 865-877.

- Li, J., Mbow, M.L., Sun, L., Li, L., Yang, G., Griswold, D.E., Schantz, A., Shealy, D.J., Goletz, T.J., Wan, J., *et al.* (2004a). Induction of dendritic cell maturation by IL-18. *Cellular immunology* *227*, 103-108.
- Li, J., Nandagopal, S., Wu, D., Romanuik, S.F., Paul, K., Thomson, D.J., and Lin, F. (2011). Activated T lymphocytes migrate toward the cathode of DC electric fields in microfluidic devices. *Lab Chip* *11*, 1298-1304.
- Li, J., Zhang, Y.P., and Kirsner, R.S. (2003b). Angiogenesis in wound repair: Angiogenic growth factors and the extracellular matrix. *Microsc Res Tech* *60*, 107-114.
- Li, W., Fan, J., Chen, M., Guan, S., Sawcer, D., Bokoch, G.M., and Woodley, D.T. (2004b). Mechanism of human dermal fibroblast migration driven by type I collagen and platelet-derived growth factor-BB. *Mol Biol Cell* *15*, 294-309.
- Li, Z., Jiang, H., Xie, W., Zhang, Z., Smrcka, A.V., and Wu, D. (2000). Roles of PLC-beta2 and -beta3 and PI3Kgamma in chemoattractant-mediated signal transduction. *Science* *287*, 1046-1049.
- Lin, F., Baldessari, F., Gyenge, C.C., Sato, T., Chambers, R.D., Santiago, J.G., and Butcher, E.C. (2008). Lymphocyte electrotaxis in vitro and in vivo. *J Immunol* *181*, 2465-2471.
- Liu, K., Waskow, C., Liu, X., Yao, K., Hoh, J., and Nussenzweig, M. (2007). Origin of dendritic cells in peripheral lymphoid organs of mice. *Nature immunology* *8*, 578-583.
- Loot, M.A., Kenter, S.B., Au, F.L., van Galen, W.J., Middelkoop, E., Bos, J.D., and Mekkes, J.R. (2002). Fibroblasts derived from chronic diabetic ulcers differ in their response to stimulation with EGF, IGF-I, bFGF and PDGF-AB compared to controls. *Eur J Cell Biol* *81*, 153-160.
- Loots, M.A., Lamme, E.N., Mekkes, J.R., Bos, J.D., and Middelkoop, E. (1999). Cultured fibroblasts from chronic diabetic wounds on the lower extremity (non-insulin-dependent diabetes mellitus) show disturbed proliferation. *Arch Dermatol Res* *291*, 93-99.
- Lorenz, M., Yamaguchi, H., Wang, Y., Singer, R.H., and Condeelis, J. (2004). Imaging sites of N-wasp activity in lamellipodia and invadopodia of carcinoma cells. *Curr Biol* *14*, 697-703.
- Lovvorn, H.N., 3rd, Cheung, D.T., Nimni, M.E., Perelman, N., Estes, J.M., and Adzick, N.S. (1999). Relative distribution and crosslinking of collagen distinguish fetal from adult sheep wound repair. *J Pediatr Surg* *34*, 218-223.
- Ma, Y.Q., Qin, J., Wu, C., and Plow, E.F. (2008). Kindlin-2 (Mig-2): a co-activator of $\beta 3$ integrins. *The Journal of cell biology* *181*, 439-446.

Margolis, D.J., Crombleholme, T., and Herlyn, M. (2000). Clinical protocol: Phase I trial to evaluate the safety of H5.020CMV.PDGF-B for the treatment of a diabetic insensate foot ulcer. *Wound Repair Regen* 8, 480-493.

Martin, P. (1997). Wound healing--aiming for perfect skin regeneration. *Science* 276, 75-81.

Mast, B.A., and Schultz, G.S. (1996). Interactions of cytokines, growth factors, and proteases in acute and chronic wounds. *Wound Repair Regen* 4, 411-420.

McCaig, C.D. (1986). Dynamic aspects of amphibian neurite growth and the effects of an applied electric field. *J Physiol* 375, 55-69.

McCaig, C.D. (1987). Spinal neurite reabsorption and regrowth in vitro depend on the polarity of an applied electric field. *Development* 100, 31-41.

McCaig, C.D. (1989). Studies on the mechanism of embryonic frog nerve orientation in a small applied electric field. *J Cell Sci* 93 (Pt 4), 723-730.

McCaig, C.D., and Dover, P.J. (1991). Factors influencing perpendicular elongation of embryonic frog muscle cells in a small applied electric field. *J Cell Sci* 98 (Pt 4), 497-506.

McCaig, C.D., and Dover, P.J. (1993). Raised cyclic-AMP and a small applied electric field influence differentiation, shape, and orientation of single myoblasts. *Dev Biol* 158, 172-182.

McCaig, C.D., Rajnicek, A.M., Song, B., and Zhao, M. (2002). Has electrical growth cone guidance found its potential? *Trends Neurosci* 25, 354-359.

McCaig, C.D., Rajnicek, A.M., Song, B., and Zhao, M. (2005). Controlling cell behavior electrically: current views and future potential. *Physiol Rev* 85, 943-978.

McCaig, C.D., Song, B., and Rajnicek, A.M. (2009). Electrical dimensions in cell science. *J Cell Sci* 122, 4267-4276.

McCaig, C.D., and Zhao, M. (1997). Physiological electrical fields modify cell behaviour. *Bioessays* 19, 819-826.

McKenzie, J.L., Beard, M.E., and Hart, D.N. (1984). Depletion of donor kidney dendritic cells prolongs graft survival. *Transplant Proc* 16, 948-951.

McLaughlin, S., and Poo, M.M. (1981). The role of electro-osmosis in the electric-field-induced movement of charged macromolecules on the surfaces of cells. *Biophys J* 34, 85-93.

Mee, J.B., Alam, Y., and Groves, R.W. (2000). Human keratinocytes constitutively produce but do not process interleukin-18. *Br J Dermatol* 143, 330-336.

- Merad, M., Ginhoux, F., and Collin, M. (2008). Origin, homeostasis and function of Langerhans cells and other langerin-expressing dendritic cells. *Nat Rev Immunol* 8, 935-947.
- Mercurio, A.M., Rabinovitz, I., and Shaw, L.M. (2001). The alpha 6 beta 4 integrin and epithelial cell migration. *Curr Opin Cell Biol* 13, 541-545.
- Midwood, K.S., Williams, L.V., and Schwarzbauer, J.E. (2004). Tissue repair and the dynamics of the extracellular matrix. *Int J Biochem Cell Biol* 36, 1031-1037.
- Minc, N., and Chang, F. (2010). Electrical control of cell polarization in the fission yeast *Schizosaccharomyces pombe*. *Curr Biol* 20, 710-716.
- Miyoshi, T., Tsuji, T., Higashida, C., Hertzog, M., Fujita, A., Narumiya, S., Scita, G., and Watanabe, N. (2006). Actin turnover-dependent fast dissociation of capping protein in the dendritic nucleation actin network: evidence of frequent filament severing. *J Cell Biol* 175, 947-955.
- Morotomi-Yano, K., Akiyama, H., and Yano, K. (2011). Nanosecond pulsed electric fields activate MAPK pathways in human cells. *Arch Biochem Biophys* 515, 99-106.
- Munder, M., Mallo, M., Eichmann, K., and Modolell, M. (1998). Murine macrophages secrete interferon gamma upon combined stimulation with interleukin (IL)-12 and IL-18: A novel pathway of autocrine macrophage activation. *J Exp Med* 187, 2103-2108.
- Myllarniemi, M., Calderon, L., Lemstrom, K., Buchdunger, E., and Hayry, P. (1997). Inhibition of platelet-derived growth factor receptor tyrosine kinase inhibits vascular smooth muscle cell migration and proliferation. *FASEB J* 11, 1119-1126.
- Nakano, H., Tsutsui, H., Terada, M., Yasuda, K., Matsui, K., Yumikura-Futatsugi, S., Yamanaka, K., Mizutani, H., Yamamura, T., and Nakanishi, K. (2003). Persistent secretion of IL-18 in the skin contributes to IgE response in mice. *Int Immunol* 15, 611-621.
- Neuss, S., Becher, E., Woltje, M., Tietze, L., and Jahnen-Dechent, W. (2004). Functional expression of HGF and HGF receptor/c-met in adult human mesenchymal stem cells suggests a role in cell mobilization, tissue repair, and wound healing. *Stem Cells* 22, 405-414.
- Ng, L.G., Hsu, A., Mandell, M.A., Roediger, B., Hoeller, C., Mrass, P., Iparraguirre, A., Cavanagh, L.L., Triccas, J.A., Beverley, S.M., *et al.* (2008). Migratory dermal dendritic cells act as rapid sensors of protozoan parasites. *PLoS Pathog* 4, e1000222.
- Niggli, V. (2000). A membrane-permeant ester of phosphatidylinositol 3,4, 5-trisphosphate (PIP(3)) is an activator of human neutrophil migration. *FEBS Lett* 473, 217-221.

- Nishi, T., Dev, S.B., Yoshizato, K., Kuratsu, J., and Ushio, Y. (1997). Treatment of cancer using pulsed electric field in combination with chemotherapeutic agents or genes. *Human cell* 10, 81-86.
- Niyonsaba, F., Ushio, H., Nagaoka, I., Okumura, K., and Ogawa, H. (2005). The human beta-defensins (-1, -2, -3, -4) and cathelicidin LL-37 induce IL-18 secretion through p38 and ERK MAPK activation in primary human keratinocytes. *J Immunol* 175, 1776-1784.
- Nobes, C.D. (2000). Rho GTPases and cell migration-fibroblast wound healing. *Methods Enzymol* 325, 441-449.
- Nobes, C.D., and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell* 81, 53-62.
- Noirey, N., Staquet, M.J., Gariazzo, M.J., Serres, M., Andre, C., Schmitt, D., and Vincent, C. (2002). Relationship between expression of matrix metalloproteinases and migration of epidermal and in vitro generated Langerhans cells. *Eur J Cell Biol* 81, 383-389.
- Novick, D., Kim, S.H., Fantuzzi, G., Reznikov, L.L., Dinarello, C.A., and Rubinstein, M. (1999). Interleukin-18 binding protein: a novel modulator of the Th1 cytokine response. *Immunity* 10, 127-136.
- Nuccitelli, R., Nuccitelli, P., Ramlatchan, S., Sanger, R., and Smith, P.J.S. (2008). Imaging the electric field associated with mouse and human skin wounds. *Wound Repair and Regeneration* 16, 432-441.
- Nwomeh, B.C., Yager, D.R., and Cohen, I.K. (1998). Physiology of the chronic wound. *Clinics in plastic surgery* 25, 341-356.
- Ohtsuki, T., Micallef, M.J., Kohno, K., Tanimoto, T., Ikeda, M., and Kurimoto, M. (1997). Interleukin 18 enhances Fas ligand expression and induces apoptosis in Fas-expressing human myelomonocytic KG-1 cells. *Anticancer Res* 17, 3253-3258.
- Okamura, H., Kashiwamura, S., Tsutsui, H., Yoshimoto, T., and Nakanishi, K. (1998). Regulation of interferon-gamma production by IL-12 and IL-18. *Curr Opin Immunol* 10, 259-264.
- Okamura, H., Tsutsui, H., Komatsu, T., Yutsudo, M., Hakura, A., Tanimoto, T., Torigoe, K., Okura, T., Nukada, Y., Hattori, K., *et al.* (1995). Cloning of a new cytokine that induces IFN-gamma production by T cells. *Nature* 378, 88-91.
- Oliva, F., Gatti, S., Porcellini, G., Forsyth, N.R., and Maffulli, N. (2012). Growth factors and tendon healing. *Medicine and sport science* 57, 53-64.
- Patel, N., and Poo, M.M. (1982). Orientation of neurite growth by extracellular electric fields. *J Neurosci* 2, 483-496.

Patel, N.B., and Poo, M.M. (1984). Perturbation of the direction of neurite growth by pulsed and focal electric fields. *J Neurosci* 4, 2939-2947.

Perejaslov, A., Chooklin, S., and Bihalsky, I. (2008). Implication of interleukin 18 and intercellular adhesion molecule (ICAM)-1 in acute pancreatitis. *Hepato-gastroenterology* 55, 1806-1813.

Perez, S.E., Cano, D.A., Dao-Pick, T., Rougier, J.P., Werb, Z., and Hebrok, M. (2005). Matrix metalloproteinases 2 and 9 are dispensable for pancreatic islet formation and function in vivo. *Diabetes* 54, 694-701.

Phillips, T.J., al-Amoudi, H.O., Leverkus, M., and Park, H.Y. (1998). Effect of chronic wound fluid on fibroblasts. *J Wound Care* 7, 527-532.

Picut, C.A., Lee, C.S., Dougherty, E.P., Anderson, K.L., and Lewis, R.M. (1988). Immunostimulatory capabilities of highly enriched Langerhans cells in vitro. *J Invest Dermatol* 90, 201-206.

Pierce, G.F., Tarpley, J.E., Allman, R.M., Goode, P.S., Serdar, C.M., Morris, B., Mustoe, T.A., and Vande Berg, J. (1994). Tissue repair processes in healing chronic pressure ulcers treated with recombinant platelet-derived growth factor BB. *Am J Pathol* 145, 1399-1410.

Pierce, G.F., Tarpley, J.E., Tseng, J., Bready, J., Chang, D., Kenney, W.C., Rudolph, R., Robson, M.C., Vande Berg, J., Reid, P., *et al.* (1995). Detection of platelet-derived growth factor (PDGF)-AA in actively healing human wounds treated with recombinant PDGF-BB and absence of PDGF in chronic nonhealing wounds. *J Clin Invest* 96, 1336-1350.

Plotnikov, A., Niego, B., Ophir, R., Korenstein, R., and Keisari, Y. (2006). Effective treatment of mouse metastatic prostate cancer by low electric field enhanced chemotherapy. *Prostate* 66, 1620-1630.

Poleganov, M.A., Bachmann, M., Pfeilschifter, J., and Muhl, H. (2008). Genome-wide analysis displays marked induction of EB13/IL-27B in IL-18-activated AML-derived KG1 cells: critical role of two kappaB binding sites in the human EB13 promoter. *Mol Immunol* 45, 2869-2880.

Ponti, A., Machacek, M., Gupton, S.L., Waterman-Storer, C.M., and Danuser, G. (2004). Two distinct actin networks drive the protrusion of migrating cells. *Science* 305, 1782-1786.

Powell, D.W., Mifflin, R.C., Valentich, J.D., Crowe, S.E., Saada, J.I., and West, A.B. (1999). Myofibroblasts. I. Paracrine cells important in health and disease. *Am J Physiol* 277, C1-9.

Preliasco, V.F., Benchuya, C., Pavan, V., de la Cal, C., Ganzinelli, S., and Sterin-Borda, L. (2008). IL-1 beta and PGE2 levels are increased in the saliva of children with

Langerhans cell histiocytosis. *Journal of oral pathology & medicine : official publication of the International Association of Oral Pathologists and the American Academy of Oral Pathology* 37, 522-527.

Price, A.A., Cumberbatch, M., Kimber, I., and Ager, A. (1997). Alpha 6 integrins are required for Langerhans cell migration from the epidermis. *J Exp Med* 186, 1725-1735.

Pu, J., McCaig, C.D., Cao, L., Zhao, Z., Segall, J.E., and Zhao, M. (2007). EGF receptor signalling is essential for electric-field-directed migration of breast cancer cells. *J Cell Sci* 120, 3395-3403.

Pu, J., and Zhao, M. (2005). Golgi polarization in a strong electric field. *J Cell Sci* 118, 1117-1128.

Pukac, L., Huangpu, J., and Karnovsky, M.J. (1998). Platelet-derived growth factor-BB, insulin-like growth factor-I, and phorbol ester activate different signaling pathways for stimulation of vascular smooth muscle cell migration. *Exp Cell Res* 242, 548-560.

Raftopoulou, M., and Hall, A. (2004). Cell migration: Rho GTPases lead the way. *Dev Biol* 265, 23-32.

Rajnicek, A.M., Foubister, L.E., and McCaig, C.D. (2006a). Growth cone steering by a physiological electric field requires dynamic microtubules, microfilaments and Rac-mediated filopodial asymmetry. *J Cell Sci* 119, 1736-1745.

Rajnicek, A.M., Foubister, L.E., and McCaig, C.D. (2006b). Temporally and spatially coordinated roles for Rho, Rac, Cdc42 and their effectors in growth cone guidance by a physiological electric field. *J Cell Sci* 119, 1723-1735.

Rajnicek, A.M., Foubister, L.E., and McCaig, C.D. (2007a). Prioritising guidance cues: directional migration induced by substratum contours and electrical gradients is controlled by a rho/cdc42 switch. *Developmental Biology* 312, 448-460.

Rajnicek, A.M., Foubister, L.E., and McCaig, C.D. (2007b). Prioritising guidance cues: directional migration induced by substratum contours and electrical gradients is controlled by a rho/cdc42 switch. *Dev Biol* 312, 448-460.

Rambukkana, A., Pistor, F.H., Bos, J.D., Kapsenberg, M.L., and Das, P.K. (1996). Effects of contact allergens on human Langerhans cells in skin organ culture: migration, modulation of cell surface molecules, and early expression of interleukin-1 beta protein. *Lab Invest* 74, 422-436.

Ratzinger, G., Stoitzner, P., Ebner, S., Lutz, M.B., Layton, G.T., Rainer, C., Senior, R.M., Shipley, J.M., Fritsch, P., Schuler, G., *et al.* (2002). Matrix metalloproteinases 9 and 2 are necessary for the migration of Langerhans cells and dermal dendritic cells from human and murine skin. *J Immunol* 168, 4361-4371.

Rayment, E.A., and Upton, Z. (2009). Review: Finding the culprit: A review of the influences of proteases on the chronic wound environment. *International Journal of Lower Extremity Wounds* 8, 19-27.

Reid, B., Song, B., McCaig, C.D., and Zhao, M. (2005). Wound healing in rat cornea: the role of electric currents. *FASEB J* 19, 379-386.

Ridley, A.J. (2001). Rho proteins, PI 3-kinases, and monocyte/macrophage motility. *FEBS Lett* 498, 168-171.

Ridley, A.J., Schwartz, M.A., Burridge, K., Firtel, R.A., Ginsberg, M.H., Borisy, G., Parsons, J.T., and Horwitz, A.R. (2003). Cell migration: integrating signals from front to back. *Science* 302, 1704-1709.

Robinson, K.R. (1985). The responses of cells to electrical fields: a review. *J Cell Biol* 101, 2023-2027.

Robson, M.C. (1997). The role of growth factors in the healing of chronic wounds. *Wound Repair Regen* 5, 12-17.

Robson, M.C., Hill, D.P., Smith, P.D., Wang, X., Meyer-Siegler, K., Ko, F., VandeBerg, J.S., Payne, W.G., Ochs, D., and Robson, L.E. (2000). Sequential cytokine therapy for pressure ulcers: clinical and mechanistic response. *Ann Surg* 231, 600-611.

Robson, M.C., Phillips, L.G., Thomason, A., Robson, L.E., and Pierce, G.F. (1992). Platelet-derived growth factor BB for the treatment of chronic pressure ulcers. *Lancet* 339, 23-25.

Roediger, B., Ng, L.G., Smith, A.L., Fazekas de St Groth, B., and Weninger, W. (2008). Visualizing dendritic cell migration within the skin. *Histochem Cell Biol* 130, 1131-1146.

Rohatgi, R., Nollau, P., Ho, H.Y., Kirschner, M.W., and Mayer, B.J. (2001). Nck and phosphatidylinositol 4,5-bisphosphate synergistically activate actin polymerization through the N-WASP-Arp2/3 pathway. *J Biol Chem* 276, 26448-26452.

Rollman, O., Jensen, U.B., Ostman, A., Bolund, L., Gustafsdottir, S.M., and Jensen, T.G. (2003). Platelet derived growth factor (PDGF) responsive epidermis formed from human keratinocytes transduced with the PDGF beta receptor gene. *J Invest Dermatol* 120, 742-749.

Romani, N., Holzmann, S., Tripp, C.H., Koch, F., and Stoitzner, P. (2003). Langerhans cells - dendritic cells of the epidermis. *APMIS : acta pathologica, microbiologica, et immunologica Scandinavica* 111, 725-740.

Rook, A., and Burns, T. (2010). *Rook's textbook of dermatology*, Vol 4 (Wiley-Blackwell).

Ross, R., Ross, X.L., Langin, T., and Reske-Kunz, A.B. (1999). Maturation of epidermal Langerhans cells: increased expression of beta- and gamma-actin isoforms as a basis of specialized cell functions. *Exp Dermatol* 8, 487-494.

Ross, R., Ross, X.L., Schwing, J., Langin, T., and Reske-Kunz, A.B. (1998). The actin-bundling protein fascin is involved in the formation of dendritic processes in maturing epidermal Langerhans cells. *J Immunol* 160, 3776-3782.

Rupec, R., Magerstaedt, R., Schirren, C.G., Sander, E., and Bieber, T. (1996). Granulocyte/macrophage-colony-stimulating factor induces the migration of human epidermal Langerhans cells in vitro. *Exp Dermatol* 5, 115-119.

Ryan, T.J. (1989). Structure and function of lymphatics. *J Invest Dermatol* 93, 18S-24S.

Saito, Y., Hasegawa, M., Fujimoto, M., Matsushita, T., Horikawa, M., Takenaka, M., Ogawa, F., Sugama, J., Steeber, D.A., Sato, S., *et al.* (2008). The loss of MCP-1 attenuates cutaneous ischemia-reperfusion injury in a mouse model of pressure ulcer. *J Invest Dermatol* 128, 1838-1851.

Saitoh, A., Yasaka, N., Osada, A., Nakamura, K., Furue, M., and Tamaki, K. (1999). Migration of Langerhans cells in an in vitro organ culture system: IL-6 and TNF-alpha are partially responsible for migration into the epidermis. *J Dermatol Sci* 19, 166-174.

Sandeman, S.R., Allen, M.C., Liu, C., Faragher, R.G., and Lloyd, A.W. (2000). Human keratocyte migration into collagen gels declines with in vitro ageing. *Mech Ageing Dev* 119, 149-157.

Saunders, D., Lucas, K., Ismaili, J., Wu, L., Maraskovsky, E., Dunn, A., and Shortman, K. (1996). Dendritic cell development in culture from thymic precursor cells in the absence of granulocyte/macrophage colony-stimulating factor. *J Exp Med* 184, 2185-2196.

Scales, T.M., and Parsons, M. (2011). Spatial and temporal regulation of integrin signalling during cell migration. *Curr Opin Cell Biol* 23, 562-568.

Scandella, E., Men, Y., Gillessen, S., Forster, R., and Groettrup, M. (2002). Prostaglandin E2 is a key factor for CCR7 surface expression and migration of monocyte-derived dendritic cells. *Blood* 100, 1354-1361.

Scheicher, C., Mehlig, M., Zecher, R., and Reske, K. (1992). Dendritic cells from mouse bone marrow: in vitro differentiation using low doses of recombinant granulocyte-macrophage colony-stimulating factor. *J Immunol Methods* 154, 253-264.

Schneider, L., Cammer, M., Lehman, J., Nielsen, S.K., Guerra, C.F., Veland, I.R., Stock, C., Hoffmann, E.K., Yoder, B.K., Schwab, A., *et al.* (2010). Directional cell migration and chemotaxis in wound healing response to PDGF-AA are coordinated by the primary cilium in fibroblasts. *Cell Physiol Biochem* 25, 279-292.

Schneider, L., Stock, C.M., Dieterich, P., Jensen, B.H., Pedersen, L.B., Satir, P., Schwab, A., Christensen, S.T., and Pedersen, S.F. (2009). The Na⁺/H⁺ exchanger NHE1 is required for directional migration stimulated via PDGFR-alpha in the primary cilium. *J Cell Biol* 185, 163-176.

Schreier, T., Degen, E., and Baschong, W. (1993). Fibroblast migration and proliferation during in vitro wound healing. A quantitative comparison between various growth factors and a low molecular weight blood dialysate used in the clinic to normalize impaired wound healing. *Res Exp Med (Berl)* 193, 195-205.

Schuler, G., and Steinman, R.M. (1985a). Murine Epidermal Langerhans Cells Mature into Potent Immunostimulatory Dendritic Cells-Invitro. *Journal of Experimental Medicine* 161, 526-546.

Schuler, G., and Steinman, R.M. (1985b). Murine epidermal Langerhans cells mature into potent immunostimulatory dendritic cells in vitro. *J Exp Med* 161, 526-546.

Seah, C.C., Phillips, T.J., Howard, C.E., Panova, I.P., Hayes, C.M., Asandra, A.S., and Park, H.Y. (2005). Chronic wound fluid suppresses proliferation of dermal fibroblasts through a Ras-mediated signaling pathway. *J Invest Dermatol* 124, 466-474.

Sepp, K.J., and Auld, V.J. (2003). RhoA and Rac1 GTPases mediate the dynamic rearrangement of actin in peripheral glia. *Development* 130, 1825-1835.

Servant, G., Weiner, O.D., Herzmark, P., Balla, T., Sedat, J.W., and Bourne, H.R. (2000). Polarization of chemoattractant receptor signaling during neutrophil chemotaxis. *Science* 287, 1037-1040.

Shanley, L.J., Walczysko, P., Bain, M., MacEwan, D.J., and Zhao, M. (2006). Influx of extracellular Ca²⁺ is necessary for electrotaxis in Dictyostelium. *J Cell Sci* 119, 4741-4748.

Shaw, T.J., and Martin, P. (2009). Wound repair at a glance. *J Cell Sci* 122, 3209-3213.

Shimizu, T., Nakamura, M., Yoshitomi, K., and Imai, M. (1993). Effects of prostaglandin E2 on membrane voltage of the connecting tubule and cortical collecting duct from rabbits. *J Physiol* 462, 275-289.

Shirakata, Y., Kimura, R., Nanba, D., Iwamoto, R., Tokumaru, S., Morimoto, C., Yokota, K., Nakamura, M., Sayama, K., Mekada, E., *et al.* (2005). Heparin-binding EGF-like growth factor accelerates keratinocyte migration and skin wound healing. *J Cell Sci* 118, 2363-2370.

Shortman, K., and Naik, S.H. (2007). Steady-state and inflammatory dendritic-cell development. *Nat Rev Immunol* 7, 19-30.

Shukla, A., Dubey, M.P., Srivastava, R., and Srivastava, B.S. (1998). Differential expression of proteins during healing of cutaneous wounds in experimental normal and chronic models. *Biochem Biophys Res Commun* 244, 434-439.

Smith, D.E. (2011). The biological paths of IL-1 family members IL-18 and IL-33. *J Leukoc Biol* 89, 383-392.

Song, B., Gu, Y., Pu, J., Reid, B., Zhao, Z., and Zhao, M. (2007a). Application of direct current electric fields to cells and tissues in vitro and modulation of wound electric field in vivo. *Nat Protoc* 2, 1479-1489.

Song, B., Gu, Y., Pu, J., Reid, B., Zhao, Z., and Zhao, M. (2007b). Application of direct current electric fields to cells and tissues in vitro and modulation of wound electric field in vivo. *Nat Protocols* 2, 1479-1489.

Song, B., Zhao, M., Forrester, J., and McCaig, C. (2004). Nerve regeneration and wound healing are stimulated and directed by an endogenous electrical field in vivo. *J Cell Sci* 117, 4681-4690.

Song, B., Zhao, M., Forrester, J.V., and McCaig, C.D. (2002). Electrical cues regulate the orientation and frequency of cell division and the rate of wound healing in vivo. *Proc Natl Acad Sci U S A* 99, 13577-13582.

Sozzani, S., Luini, W., Borsatti, A., Polentarutti, N., Zhou, D., Piemonti, L., D'Amico, G., Power, C.A., Wells, T.N., Gobbi, M., *et al.* (1997). Receptor expression and responsiveness of human dendritic cells to a defined set of CC and CXC chemokines. *J Immunol* 159, 1993-2000.

Sporn, M.B., and Roberts, A.B. (1993). A major advance in the use of growth factors to enhance wound healing. *J Clin Invest* 92, 2565-2566.

Springer, T.A. (1994). Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell* 76, 301-314.

St Louis, D.C., Woodcock, J.B., Franzoso, G., Blair, P.J., Carlson, L.M., Murillo, M., Wells, M.R., Williams, A.J., Smoot, D.S., Kaushal, S., *et al.* (1999). Evidence for distinct intracellular signaling pathways in CD34+ progenitor to dendritic cell differentiation from a human cell line model. *Journal of immunology* 162, 3237-3248.

Stadelmann, W.K., Digenis, A.G., and Tobin, G.R. (1998). Physiology and healing dynamics of chronic cutaneous wounds. *American Journal of Surgery* 176, 26S-38S.

Steinman, R.M. (1991). The dendritic cell system and its role in immunogenicity. *Annu Rev Immunol* 9, 271-296.

Steinman, R.M., Adams, J.C., and Cohn, Z.A. (1975). Identification of a Novel Cell Type in Peripheral Lymphoid Organs of Mice .4. Identification and Distribution in Mouse Spleen. *Journal of Experimental Medicine* 141, 804-820.

Steinman, R.M., and Banchereau, J. (2007). Taking dendritic cells into medicine. *Nature* 449, 419-426.

Steinman, R.M., and Cohn, Z.A. (1973). Identification of a Novel Cell Type in Peripheral Lymphoid Organs of Mice. *Journal of Experimental Medicine* 137, 1142-1162.

Steinman, R.M., and Cohn, Z.A. (1974). Identification of a Novel Cell Type in Peripheral Lymphoid Organs of Mice .2. Functional Properties in-Vitro. *Journal of Experimental Medicine* 139, 380-397.

Steinman, R.M., Kaplan, G., Witmer, M.D., and Cohn, Z.A. (1979). Identification of a Novel Cell Type in Peripheral Lymphoid Organs of Mice .5. Purification of Spleen Dendritic Cells, New Surface Markets, and Maintenance Invitro. *Journal of Experimental Medicine* 149, 1-16.

Steinman, R.M., Lustig, D.S., and Cohn, Z.A. (1974). Identification of a Novel Cell Type in Peripheral Lymphoid Organs of Mice .3. Functional Properties Invivo. *Journal of Experimental Medicine* 139, 1431-1445.

Stephens, L., Ellson, C., and Hawkins, P. (2002). Roles of PI3Ks in leukocyte chemotaxis and phagocytosis. *Curr Opin Cell Biol* 14, 203-213.

Stephens, P., Cook, H., Hilton, J., Jones, C.J., Haughton, M.F., Wyllie, F.S., Skinner, J.W., Harding, K.G., Kipling, D., and Thomas, D.W. (2003). An analysis of replicative senescence in dermal fibroblasts derived from chronic leg wounds predicts that telomerase therapy would fail to reverse their disease-specific cellular and proteolytic phenotype. *Exp Cell Res* 283, 22-35.

Stojadinovic, O., Brem, H., Vouthounis, C., Lee, B., Fallon, J., Stallcup, M., Merchant, A., Galiano, R.D., and Tomic-Canic, M. (2005). Molecular pathogenesis of chronic wounds: the role of beta-catenin and c-myc in the inhibition of epithelialization and wound healing. *Am J Pathol* 167, 59-69.

Stoll, S., Jonuleit, H., Schmitt, E., Muller, G., Yamauchi, H., Kurimoto, M., Knop, J., and Enk, A.H. (1998). Production of functional IL-18 by different subtypes of murine and human dendritic cells (DC): DC-derived IL-18 enhances IL-12-dependent Th1 development. *Eur J Immunol* 28, 3231-3239.

Stradal, T.E., and Scita, G. (2006). Protein complexes regulating Arp2/3-mediated actin assembly. *Curr Opin Cell Biol* 18, 4-10.

Stuart, H.C., Jia, Z., Messenberg, A., Joshi, B., Underhill, T.M., Moukhles, H., and Nabi, I.R. (2008). Localized Rho GTPase activation regulates RNA dynamics and compartmentalization in tumor cell protrusions. *J Biol Chem* 283, 34785-34795.

Sun, Y., Martin, A.C., and Drubin, D.G. (2006). Endocytic internalization in budding yeast requires coordinated actin nucleation and myosin motor activity. *Dev Cell* 11, 33-46.

Svitkina, T.M., and Borisy, G.G. (1999). Arp2/3 complex and actin depolymerizing factor/cofilin in dendritic organization and treadmilling of actin filament array in lamellipodia. *J Cell Biol* 145, 1009-1026.

Svitkina, T.M., Verkhovsky, A.B., McQuade, K.M., and Borisy, G.G. (1997). Analysis of the actin-myosin II system in fish epidermal keratocytes: mechanism of cell body translocation. *J Cell Biol* 139, 397-415.

Szczepanik, M., Lewis, J., Geba, G.P., Ptak, W., and Askenase, P.W. (1998). Positive regulatory gamma delta T cells in contact sensitivity: augmented responses by in vivo treatment with anti-gamma delta monoclonal antibody, or anti-V gamma 5 or V delta 4. *Immunological investigations* 27, 1-15.

Takashima, A., and Bergstresser, P.R. (1996). Cytokine-mediated communication by keratinocytes and Langerhans cells with dendritic epidermal T cells. *Semin Immunol* 8, 333-339.

Takayama, K., Yokozeki, H., Ghoreishi, M., Satoh, T., Katayama, I., Umeda, T., and Nishioka, K. (1999). IL-4 inhibits the migration of human Langerhans cells through the downregulation of TNF receptor II expression. *J Invest Dermatol* 113, 541-546.

Takeda, K., Tsutsui, H., Yoshimoto, T., Adachi, O., Yoshida, N., Kishimoto, T., Okamura, H., Nakanishi, K., and Akira, S. (1998). Defective NK cell activity and Th1 response in IL-18-deficient mice. *Immunity* 8, 383-390.

Takenawa, T., and Suetsugu, S. (2007). The WASP-WAVE protein network: connecting the membrane to the cytoskeleton. *Nat Rev Mol Cell Biol* 8, 37-48.

Tang, A., and Udey, M.C. (1992). Effects of ultraviolet radiation on murine epidermal Langerhans cells: doses of ultraviolet radiation that modulate ICAM-1 (CD54) expression and inhibit Langerhans cell function cause delayed cytotoxicity in vitro. *J Invest Dermatol* 99, 83-89.

Teobald, I., Dunnion, D.J., Whitbread, M., Curnow, S.J., and Browning, M.J. (2008). Phenotypic and functional differentiation of KG-1 into dendritic-like cells. *Immunobiology* 213, 75-86.

Theilgaard-Monch, K., Knudsen, S., Follin, P., and Borregaard, N. (2004). The transcriptional activation program of human neutrophils in skin lesions supports their important role in wound healing. *J Immunol* 172, 7684-7693.

Thomas, R., and Lipsky, P.E. (1994). Human peripheral blood dendritic cell subsets. Isolation and characterization of precursor and mature antigen-presenting cells. *Journal of immunology* 153, 4016-4028.

Thomassen, E., Bird, T.A., Renshaw, B.R., Kennedy, M.K., and Sims, J.E. (1998). Binding of interleukin-18 to the interleukin-1 receptor homologous receptor IL-1Rrp1 leads to activation of signaling pathways similar to those used by interleukin-1. *Journal of*

interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research *18*, 1077-1088.

Torres, T., Rosmaninho, A., Caetano, M., and Selores, M. (2010). Malignant melanoma misdiagnosed as a diabetic foot ulcer. *Diabetic medicine : a journal of the British Diabetic Association* *27*, 1302-1303.

Tsai, C.H., Lin, B.J., and Chao, P.H. (2012). $\alpha 2\beta 1$ integrin and RhoA mediates electric field-induced ligament fibroblast migration directionality. *J Orthop Res*.

Tse, K.F., Novelli, E., Civin, C.I., Bohmer, F.D., and Small, D. (2001). Inhibition of FLT3-mediated transformation by use of a tyrosine kinase inhibitor. *Leukemia* *15*, 1001-1010.

Tsutsui, H., Matsui, K., Kawada, N., Hyodo, Y., Hayashi, N., Okamura, H., Higashino, K., and Nakanishi, K. (1997). IL-18 accounts for both TNF- α - and Fas ligand-mediated hepatotoxic pathways in endotoxin-induced liver injury in mice. *J Immunol* *159*, 3961-3967.

Tyrone, J.W., Mogford, J.E., Chandler, L.A., Ma, C., Xia, Y., Pierce, G.F., and Mustoe, T.A. (2000). Collagen-embedded platelet-derived growth factor DNA plasmid promotes wound healing in a dermal ulcer model. *J Surg Res* *93*, 230-236.

Usui, M.L., Mansbridge, J.N., Carter, W.G., Fujita, M., and Olerud, J.E. (2008). Keratinocyte migration, proliferation, and differentiation in chronic ulcers from patients with diabetes and normal wounds. *J Histochem Cytochem* *56*, 687-696.

van de Veerdonk, F.L., Netea, M.G., Dinarello, C.A., and Joosten, L.A. (2011). Inflammasome activation and IL-1 β and IL-18 processing during infection. *Trends in immunology* *32*, 110-116.

van Leeuwen, J.E., Paik, P.K., and Samelson, L.E. (1999). Activation of nuclear factor of activated T cells-(NFAT) and activating protein 1 (AP-1) by oncogenic 70Z Cbl requires an intact phosphotyrosine binding domain but not Crk(L) or p85 phosphatidylinositol 3-kinase association. *J Biol Chem* *274*, 5153-5162.

Varol, C., Vallon-Eberhard, A., Elinav, E., Aychek, T., Shapira, Y., Luche, H., Fehling, H.J., Hardt, W.D., Shakhar, G., and Jung, S. (2009). Intestinal lamina propria dendritic cell subsets have different origin and functions. *Immunity* *31*, 502-512.

Vasquez, R., Marien, B.J., Gram, C., Goodwin, D.G., Menzoian, J.O., and Raffetto, J.D. (2004). Proliferative capacity of venous ulcer wound fibroblasts in the presence of platelet-derived growth factor. *Vasc Endovascular Surg* *38*, 355-360.

Vestweber, D., and Blanks, J.E. (1999). Mechanisms that regulate the function of the selectins and their ligands. *Physiol Rev* *79*, 181-213.

Villablanca, E.J., and Mora, J.R. (2008). A two-step model for Langerhans cell migration to skin-draining LN. *Eur J Immunol* 38, 2975-2980.

Volc-Platzer, B., Stingl, G., Wolff, K., Hinterberg, W., and Schnedl, W. (1984). Cytogenetic identification of allogeneic epidermal Langerhans cells in a bone-marrow-graft recipient. *N Engl J Med* 310, 1123-1124.

von Wichert, G., Haimovich, B., Feng, G.S., and Sheetz, M.P. (2003). Force-dependent integrin-cytoskeleton linkage formation requires downregulation of focal complex dynamics by Shp2. *EMBO J* 22, 5023-5035.

Wall, I.B., Moseley, R., Baird, D.M., Kipling, D., Giles, P., Laffafian, I., Price, P.E., Thomas, D.W., and Stephens, P. (2008). Fibroblast dysfunction is a key factor in the non-healing of chronic venous leg ulcers. *J Invest Dermatol* 128, 2526-2540.

Wang, B., Amerio, P., and Sauder, D.N. (1999a). Role of cytokines in epidermal Langerhans cell migration. *J Leukoc Biol* 66, 33-39.

Wang, B., Feliciani, C., Howell, B.G., Freed, I., Cai, Q., Watanabe, H., and Sauder, D.N. (2002). Contribution of Langerhans cell-derived IL-18 to contact hypersensitivity. *J Immunol* 168, 3303-3308.

Wang, B., Fujisawa, H., Zhuang, L., Kondo, S., Shivji, G.M., Kim, C.S., Mak, T.W., and Sauder, D.N. (1997). Depressed Langerhans cell migration and reduced contact hypersensitivity response in mice lacking TNF receptor p75. *J Immunol* 159, 6148-6155.

Wang, B., Zhuang, L., Fujisawa, H., Shinder, G.A., Feliciani, C., Shivji, G.M., Suzuki, H., Amerio, P., Toto, P., and Sauder, D.N. (1999b). Enhanced epidermal Langerhans cell migration in IL-10 knockout mice. *J Immunol* 162, 277-283.

Wang, E., Zhao, M., Forrester, J.V., and CD, M.C. (2000). Re-orientation and faster, directed migration of lens epithelial cells in a physiological electric field. *Exp Eye Res* 71, 91-98.

Wang, E., Zhao, M., Forrester, J.V., and McCaig, C.D. (2003). Electric fields and MAP kinase signaling can regulate early wound healing in lens epithelium. *Invest Ophthalmol Vis Sci* 44, 244-249.

Wang, Y., Liu, J., and Segall, J.E. (1998). MAP kinase function in amoeboid chemotaxis. *J Cell Sci* 111 (Pt 3), 373-383.

Watts, E.J., and Rose, M.T. (2010). Platelet-derived growth factor acts via both the Rho-kinase and p38 signaling enzymes to stimulate contraction in an in vitro model of equine wound healing. *Domest Anim Endocrinol* 38, 253-259.

Weed, S.A., Karginov, A.V., Schafer, D.A., Weaver, A.M., Kinley, A.W., Cooper, J.A., and Parsons, J.T. (2000). Cortactin localization to sites of actin assembly in lamellipodia requires interactions with F-actin and the Arp2/3 complex. *J Cell Biol* 151, 29-40.

Werner, S., and Grose, R. (2003). Regulation of wound healing by growth factors and cytokines. *Physiol Rev* 83, 835-870.

Werner, S., Smola, H., Liao, X., Longaker, M.T., Krieg, T., Hofschneider, P.H., and Williams, L.T. (1994). The function of KGF in morphogenesis of epithelium and reepithelialization of wounds. *Science* 266, 819-822.

Wiesner, S., Helfer, E., Didry, D., Ducouret, G., Lafuma, F., Carlier, M.F., and Pantaloni, D. (2003). A biomimetic motility assay provides insight into the mechanism of actin-based motility. *J Cell Biol* 160, 387-398.

Witmer-Pack, M.D., Olivier, W., Valinsky, J., Schuler, G., and Steinman, R.M. (1987). Granulocyte/macrophage colony-stimulating factor is essential for the viability and function of cultured murine epidermal Langerhans cells. *J Exp Med* 166, 1484-1498.

Witmer-Pack, M.D., Valinsky, J., Olivier, W., and Steinman, R.M. (1988). Quantitation of surface antigens on cultured murine epidermal Langerhans cells: rapid and selective increase in the level of surface MHC products. *J Invest Dermatol* 90, 387-394.

Wittmann, T., and Waterman-Storer, C.M. (2001). Cell motility: can Rho GTPases and microtubules point the way? *J Cell Sci* 114, 3795-3803.

Wu, D., and Lin, F. (2011). A receptor-electromigration-based model for cellular electrotactic sensing and migration. *Biochem Biophys Res Commun* 411, 695-701.

Xu, J., and Clark, R.A. (1996). Extracellular matrix alters PDGF regulation of fibroblast integrins. *J Cell Biol* 132, 239-249.

Xu, K.P., and Yu, F.S. (2007). Cross talk between c-Met and epidermal growth factor receptor during retinal pigment epithelial wound healing. *Invest Ophthalmol Vis Sci* 48, 2242-2248.

Xu, W., Baribault, H., and Adamson, E.D. (1998). Vinculin knockout results in heart and brain defects during embryonic development. *Development* 125, 327-337.

Xu, Y., Bismar, T.A., Su, J., Xu, B., Kristiansen, G., Varga, Z., Teng, L., Ingber, D.E., Mammoto, A., Kumar, R., *et al.* (2010). Filamin A regulates focal adhesion disassembly and suppresses breast cancer cell migration and invasion. *J Exp Med* 207, 2421-2437.

Xue, Y., Lim, S., Yang, Y., Wang, Z., Jensen, L.D., Hedlund, E.M., Andersson, P., Sasahara, M., Larsson, O., Galter, D., *et al.* (2012). PDGF-BB modulates hematopoiesis and tumor angiogenesis by inducing erythropoietin production in stromal cells. *Nat Med* 18, 100-110.

Yokota, K., Ariizumi, K., Kitajima, T., Bergstresser, P.R., Street, N.E., and Takashima, A. (1996). Cytokine-mediated communication between dendritic epidermal T cells and Langerhans cells. In vitro studies using cell lines. *J Immunol* 157, 1529-1537.

Yu, B., Ueta, H., Kitazawa, Y., Tanaka, T., Adachi, K., Kimura, H., Morita, M., Sawanobori, Y., Qian, H.X., Kodama, T., *et al.* (2012). Two immunogenic passenger dendritic-cell subsets in the rat liver have distinct trafficking patterns and radiosensitivities. *Hepatology*.

Zaidel-Bar, R., Itzkovitz, S., Ma'ayan, A., Iyengar, R., and Geiger, B. (2007). Functional atlas of the integrin adhesome. *Nat Cell Biol* 9, 858-867.

Zhao, M. (2007). PTEN: a promising pharmacological target to enhance epithelial wound healing. *Br J Pharmacol* 152, 1141-1144.

Zhao, M. (2009). Electrical fields in wound healing-An overriding signal that directs cell migration. *Seminars in Cell and Developmental Biology* 20, 674-682.

Zhao, M., Bai, H., Wang, E., Forrester, J.V., and McCaig, C.D. (2004). Electrical stimulation directly induces pre-angiogenic responses in vascular endothelial cells by signaling through VEGF receptors. *J Cell Sci* 117, 397-405.

Zhao, M., Dick, A., Forrester, J.V., and McCaig, C.D. (1999). Electric field-directed cell motility involves up-regulated expression and asymmetric redistribution of the epidermal growth factor receptors and is enhanced by fibronectin and laminin. *Mol Biol Cell* 10, 1259-1276.

Zhao, M., Jin, T., McCaig, C.D., Forrester, J.V., and Devreotes, P.N. (2002a). Genetic analysis of the role of G protein-coupled receptor signaling in electrotaxis. *J Cell Biol* 157, 921-927.

Zhao, M., McCaig, C.D., Agius-Fernandez, A., Forrester, J.V., and Araki-Sasaki, K. (1997). Human corneal epithelial cells reorient and migrate cathodally in a small applied electric field. *Curr Eye Res* 16, 973-984.

Zhao, M., Pu, J., Forrester, J.V., and McCaig, C.D. (2002b). Membrane lipids, EGF receptors, and intracellular signals colocalize and are polarized in epithelial cells moving directionally in a physiological electric field. *FASEB J* 16, 857-859.

Zhao, M., Song, B., Pu, J., Wada, T., Reid, B., Tai, G., Wang, F., Guo, A., Walczysko, P., Gu, Y., *et al.* (2006). Electrical signals control wound healing through phosphatidylinositol-3-OH kinase-gamma and PTEN. *Nature* 442, 457-460.

- Alblas, J., Ulfman, L., Hordijk, P., and Koenderman, L. (2001). Activation of RhoA and ROCK are essential for detachment of migrating leukocytes. *Mol Biol Cell* *12*, 2137-2145.
- Alvarez, D., Vollmann, E.H., and von Andrian, U.H. (2008). Mechanisms and consequences of dendritic cell migration. *Immunity* *29*, 325-342.
- Burr, H.S. (1941). Field properties of the developing frog's egg. *Proceedings of the National Academy of Sciences of the United States of America* *27*, 276-281.
- Lauffenburger, D.A., and Horwitz, A.F. (1996). Cell migration: a physically integrated molecular process. *Cell* *84*, 359-369.
- Lund, E.J. (1928). Relation between continuous bio-electric currents and cell respiration. V. The quantitative relation between E-p and cell oxidation as shown by the effects of cyanide and oxygen. *Journal of Experimental Zoology* *51*, 327-337.
- Novick, D., Kim, S.H., Fantuzzi, G., Reznikov, L.L., Dinarello, C.A., and Rubinstein, M. (1999). Interleukin-18 binding protein: a novel modulator of the Th1 cytokine response. *Immunity* *10*, 127-136.
- Robinson, K.R. (1985). The responses of cells to electrical fields: a review. *J Cell Biol* *101*, 2023-2027.
- Ross, R., Ross, X.L., Schwing, J., Langin, T., and Reske-Kunz, A.B. (1998). The actin-bundling protein fascin is involved in the formation of dendritic processes in maturing epidermal Langerhans cells. *J Immunol* *160*, 3776-3782.
- Scales, T.M., and Parsons, M. (2011). Spatial and temporal regulation of integrin signalling during cell migration. *Curr Opin Cell Biol* *23*, 562-568.