

12-2010

PLASMACYTOID DENDRITIC CELLS SENSE SKIN INJURY AND PROMOTE WOUND HEALING THROUGH TYPE I INTERFERONS

Josh D. Gregorio

Follow this and additional works at: https://digitalcommons.library.tmc.edu/utgsbs_dissertations



Part of the [Biology Commons](#), [Immune System Diseases Commons](#), [Immunity Commons](#), [Other Immunology and Infectious Disease Commons](#), and the [Skin and Connective Tissue Diseases Commons](#)

Recommended Citation

Gregorio, Josh D., "PLASMACYTOID DENDRITIC CELLS SENSE SKIN INJURY AND PROMOTE WOUND HEALING THROUGH TYPE I INTERFERONS" (2010). *The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences Dissertations and Theses (Open Access)*. 92.
https://digitalcommons.library.tmc.edu/utgsbs_dissertations/92

This Dissertation (PhD) is brought to you for free and open access by the The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences at DigitalCommons@TMC. It has been accepted for inclusion in The University of Texas MD Anderson Cancer Center UTHealth Graduate School of Biomedical Sciences Dissertations and Theses (Open Access) by an authorized administrator of DigitalCommons@TMC. For more information, please contact digitalcommons@library.tmc.edu.

**PLASMACYTOID DENDRITIC CELLS SENSE SKIN INJURY AND PROMOTE
WOUND HEALING THROUGH TYPE I INTERFERONS**

By

Josh Gregorio, B.S., J.D.

APPROVED:

Michel Gilliet, MD, Supervisory Professor

Gary Gallick, PhD

Yong-Jun Liu, MD, PhD

Willem Overwijk, PhD

Stephen Ullrich, PhD

APPROVED:

George M. Stancel, PhD
Dean, The University of Texas
Graduate School of Biomedical Sciences at Houston

**PLASMACYTOID DENDRITIC CELLS SENSE SKIN INJURY AND PROMOTE
WOUND HEALING THROUGH TYPE I INTERFERONS**

A

DISSERTATION

Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M.D. Anderson Cancer Center
Graduate School of Biomedical Sciences

In Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

By

Josh Gregorio, B.S., J.D.

Houston, Texas

December, 2010

Dedicated to Guin and Jacob for all the sacrifices you have made.

ACKNOWLEDGEMENTS

I would first like to express my gratitude and appreciation to my wonderful compassionate wife. Guin, your love and support through these past five years have enabled me to concentrate on following my passion. I would like to also express my gratitude to my infant son Jacob, who came into life during my graduate training and whose birth gave me a new perspective on life and on family. I couldn't have done it without both of you. I would also like to thank Dante and Merlyn Sansano who encouraged me to go back to school. Your support and generosity will always be remembered.

Additionally, I would like to thank my mentor, Dr Gilliet. Although we sometimes never saw eye to eye on things I will always appreciate your candor, patience, scientific acuity and your enthusiasm to discover new things. You taught me how to think scientifically which will serve me well in my future endeavors. I would also like to thank all past and current members of my advisory, candidacy, and supervisory committees. Your thoughtful and meaningful communications helped me become a better scientist.

Lastly, I would like to thank all past and current members of the Gilliet laboratory for your kindness and your willingness to help me whenever I needed it.

**Plasmacytoid dendritic cells sense skin injury and promote wound
healing through type I interferons**

Publication No. _____

Josh Gregorio, B.S., J.D.

Supervisory Professor: Michel Gilliet, M.D.

Plasmacytoid dendritic cells (pDCs) are a rare population of circulating cells, which selectively express intracellular Toll-like receptors (TLR)-7 and TLR-9 and have the capacity to produce large amounts of type I IFNs (IFN- α/β) in response to viruses or host derived nucleic acid containing complexes. pDCs are normally absent in skin but accumulate in the skin of psoriasis patients where their chronic activation to produce IFN- α/β drives the disease formation. Whether pDCs and their activation to produce IFN- α/β play a functional role in healthy skin is unknown. Here we show that pDCs are rapidly and transiently recruited into healthy human and mouse skin upon epidermal injury.

Infiltrating pDCs were found to sense nucleic acids in wounded skin via TLRs, leading to the production of IFN- α/β . The production of IFN- α/β was paralleled by a short lived expression of cathelicidins, which form complexes with extracellular nucleic acids and activated pDCs to produce IFN- α/β in vitro. In vivo, cathelicidins were sufficient but not necessary for the induction of IFN- α/β in wounded skin, suggesting redundancy of this pathway. Depletion of

pDCs or inhibition of IFN- α/β R signaling significantly impaired the inflammatory response and delayed re-epithelialization of skin wounds.

Thus we uncover a novel role of pDCs in sensing skin injury via TLR mediated recognition of nucleic acids and demonstrate their involvement in the early inflammatory process and wound healing response through the production of IFN- α/β .

TABLE OF CONTENTS

Approval Sheet	i
Title Page	ii
Dedication	iii
Acknowledgement	iv
Abstract	v
Table of Contents	vii
List of Illustrations	ix
List of Tables	xii
Abbreviations	xiii
Chapter 1: Introduction	1
Wound healing	2
Plasmacytoid dendritic cells	22
Antimicrobial peptides	29
Clinical relevance	31
Chapter 2: Materials and Methods	35
Chapter 3: pDCs Infiltrate Skin Injury	44
Chapter 4: pDCs are Activated to Produce Type I IFNs	59
Chapter 5: Cathelicidins Activate pDCs Both in vivo and in vitro	69
Chapter 6: pDCs and Type I IFNs Promote Inflammatory Responses And Wound Healing In Injured Skin	78

Chapter 7: Human Skin Injury Induces pDC Infiltration and Expression of Type I IFNS	88
Chapter 8: Discussion	92
Bibliography:	109
Vita:	130
Publications:	132

LIST OF ILLUSTRATIONS

Figure 1	Wound healing during Inflammation phase	11
Figure 2	Wound healing during Proliferation phase	14
Figure 3	Re-epithelialization of injured porcine skin	20
Figure 4	Tape strip model	47
Figure 5	Tape stripping induces strong dermal infiltration	48
Figure 6	Sterile epidermal injury prompts a rapid and transient leukocyte infiltrate in the dermis which precedes epithelial repair	49
Figure 7	Rapid infiltration of pDCs into injured skin	53
Figure 8	Neutrophil recruitment into injured skin parallels pDC infiltrations	54
Figure 9	T cell infiltrate injured skin later than pDCs and neutrophils	55
Figure 10	Conventional DCs do not infiltrate injured skin	56
Figure 11	Time course analysis of infiltrating lymphocytes	57
Figure 12	pDCs are transiently activated to produce type I IFNs in injured skin	58
Figure 13	pDC-depleted effectively eliminates pDCs from circulation up to 5 days	63
Figure 14	pDCs are transiently activated to produce type I IFNs in injured skin	64

Figure 15	pDCs sense nucleic acid in injured skin	65
Figure 16	pDCs sense nucleic acid through TLR 7 and TLR 9 in injured skin	66
Figure 17	IL-6 and TNF- α cytokine mRNA expression in injured skin of MyD88-deficient and TLR 7/9 inhibitor treated mice	67
Figure 18	Cytokine mRNA expression in injured skin of TLR 7-deficient and TLR 9 inhibitor treated mice	68
Figure 19	Cathelicidin gene expression is upregulated in injury and Cathelicidin peptide induces pDC activation to produce IFN- α	72
Figure 20	Injection of Cathelicidin peptide induce pDC infiltration and activation in skin	74
Figure 21	Cathelicidin peptides are not necessary to induce type I IFNs in injured skin	75
Figure 22	Select mouse beta-defensin (mBD) antimicrobial peptides as well as Chemerin are expressed during skin injury	77
Figure 23	pDCs participate in the inflammatory response of skin wound healing	80
Figure 24	pDCs participate in the re-epithelialization of skin wound healing	83
Figure 25	Type I IFNs participate in the re-epithelialization	84

	of skin wound healing	
Figure 26	pDCs do not affect endothelial cell activity	86
Figure 27	pDCs do not promote upregulation of angiogenesis	87
	Genes	
Figure 28	Human skin injury induces rapid infiltration of pDCs	90
Figure 29	Human skin injury induces rapid infiltration of pDCs and their activation to produce IFN- α	91

LIST OF TABLES

Table 1	Human DC Phenotype Comparison	25
Table 2	Mouse DC Phenotype Comparison	27
Table 3	Mouse Taqman Primers	41
Table 4	Human Taqman Primers	42

ABBREVIATIONS

AMP	Anti-microbial Peptide
cDC	convention Dendritic Cell or myeloid Dendritic Cell
IFN- α	Interferon alpha
IFN- β	Interferon beta
IL-6	Interleukin 6
IL-12	Interleukin 12
IL-17	Interleukin 17
IL-23	Interleukin 23
pDCs	plasmacytoid Dendritic Cells
TH17	T-helper 17

CHAPTER 1
INTRODUCTION

WOUND HEALING

The epidermis protects the body from the outside world and enables efficient multicellular processes to occur through prevention of water loss across the body surface, protection against ultraviolet radiation emitted by the sun, and most importantly, the epidermis protects the body from daily assaults from the outside environment including bacteria, fungi, and virus. The epidermis is composed of two distinct layers, the stratified layer, which is composed of flattened, differentiated anuclear, keratinocytes, and the basal layer, which is composed of active differentiating keratinocytes and pluripotent stem cells (1, 2). The stratified layer is composed of many tiered layers of keratinocytes whose main function is to provide a very tight barrier against the outside environment. The basal layer is composed of keratinocytes with many hemidesmosomal attachments to each other as well as to the basement membrane (1, 2). The basement membrane marks the boundary between the epidermis and the underlying dermis. The dermis on the other hand is a collagen rich compartment populated by many tissue resident cells including fibroblasts, T cells, macrophages, mast cells, and endothelial cells. In addition hair follicle roots reside in the dermal compartment. The epidermis and dermis collectively form the skin. Therefore, any damage done to skin has serious ramifications if not resolved efficiently and expeditiously.

Wound healing is a multi-component process that involves many distinct cell types in an ordered and efficient manner. Broadly, wound healing takes place in distinct overlapping steps: hemostasis, inflammation, proliferation (angiogenesis, matrix remodeling), and epithelialization. Aberrant wound healing can result in chronic injury such as ulceration or can result in fibrosis such as keloid scars. Therefore, understanding the key steps as well as the molecular cues is very important to understanding the functional aspects of wound healing. Although much attention has been dedicated to wound healing research over the past decade and a lot of information has been deciphered, there remain many unknown elements in the complex process of wound healing.

Assaults to the skin can take many forms and the resulting response will depend on both the extent and nature of the trauma. For example, puncture wounds such as an incisional wound will penetrate both the epidermis and dermis. Typically puncture wounds can be stitched or stapled together and allow for healing by Primary Intention. Primary Intention involves collagen deposition, minimal migration of keratinocytes and typically heals efficiently and fast (3). However open injury such as excisional wounds requires both a new dermis and epidermis and involves new blood vessel formation, collagen deposition and migration of keratinocytes to form both a new basal layer which differentiates to form a new stratified layer. Additionally, trauma to the epidermis alone such as thermal injury and chemical injury require keratinocyte migration

from the wound margin and hair follicles to repair the injury. However, all dermal wounds repair by connective tissue matrix deposition, contraction and epithelialization.

Regardless of the nature of skin injury, I will provide a thorough, concise summary of known players in wound repair, from both the perspective of cell types involved as well as chemical mediators including cytokines, chemokines, and non-immune modulators such as ATP, hypoxia, and lipids. Again although the general scheme of wound repair has been elucidated, chronologically, the exact timing and magnitude of the response will depend upon the severity and type of injury which will not be discussed.

HEMOSTASIS

Most injuries will breach nearby blood vessel integrity causing blood components including platelets, fibrin, fibronectin, vitronectin and thrombospondin to be released into the injured tissue. Additionally, injury to the blood vessel endothelium exposes collagen within the endothelium (3-7). Platelets released from the blood bind to exposed collagen in both the dermis and blood vessel endothelium through glycoprotein Ia/IIa receptors that kick starts clot formation as well as sealing off the damaged blood vessel to prevent hemorrhaging (8). von Willebrand factor (vWF), which also circulate in the blood, binds to both platelets and collagen and this binding activates platelets

(9, 10). Activated platelets release preformed dense and alpha granules. Dense granules composed of adenine nucleotides, ionized calcium, histamine, serotonin, and epinephrine are important in amplifying platelet aggregation and vasodilation. Alpha granules are composed of chemotactic and growth factors such as insulin-like growth factor 1 (IGF), platelet-derived growth factor (PDGF), Transforming Growth Factor Beta ($\text{TGF-}\beta$), chemokine-connective tissue-activating peptide-III (CTAP-III), as well as coagulation proteins such as the chemokine platelet factor 4 (CXCL4), thrombospondin, fibronectin and von Willebrand factor (11-15).

Activated platelets also undergo morphological changes from a spherical to stellate appearance. Calcium release from dense granules activates protein kinase C and Phospholipase A2 resulting in increased affinity for fibrinogen (16). Tissue factor expressed on the surface of tissue resident fibroblasts binds to Factor VII released from the damaged blood vessel forming an activated complex which further activates Factor IX and X. Activated Factor IX then activates thrombin which then is armed to cleave soluble fibrinogen to fibrin monomers (16). Fibrin monomers then polymerize to form a dense network of insoluble fibrin fibers stabilized by Factor VIII (16).

Activated platelets bind fibrin through $\alpha_{\text{IIb}}/\beta_3$ receptors. The fibrin clot provides a provisional matrix which not only seals the exposed epidermis from the outside environment but also provides a conduit for infiltrating cells to traverse.

Moreover, fibrinogen and fibrin contain specific binding sites for vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2 or bFGF) which may play a role in facilitating homing of fibroblasts, endothelial cells, and smooth muscle cells to the injured tissue (17-21). Fibrinogen synthesis is positively regulated by both interleukin-1 beta (IL-1 β) and interleukin-6 (IL-6), cytokines important in the first few days of wound repair (22).

INFLAMMATORY PHASE

Injured keratinocytes release preformed molecules including IL-1 and defense peptides (cathelicidins, psoriasin, etc) into the extracellular matrix in addition to secreting high levels of TGF-alpha (TGF- α), IL-6, IL-8 (human) and IL-10. IL-1 acts as a mitogen on dermal fibroblasts and positively regulates fibroblast homing and proliferation (23-26). The human cathelicidin, LL-37, has been shown to possess both, direct, antimicrobial properties as well as being chemotactic for neutrophils (27, 28). Additionally, *in vitro*, LL-37 can interact with self-nucleic acids to activate both plasmacytoid and myeloid dendritic cells (29-30). TGF- α has a strong autocrine effect on wound edge and hair follicle resident keratinocytes resulting in keratinocyte hyperproliferation (25).

Further secretion of TGF- α and IL-6 by wound edge keratinocytes helps mobilize keratinocytes to the wounded epidermis early to begin the process of

re-epithelialization. KGF, IL-6, GM-CSF are also rapidly induced by fibroblasts upon exposure to keratinocyte derived IL-1. KGF binds to FGFR2_{IIIb} receptors on keratinocytes resulting in keratinocyte proliferation (25). Additionally, IL-6 has a pleiotrophic role in wound healing and acts early by stimulating keratinocyte proliferation. IL-6^{-/-} mice have a delayed wound healing response characterized by reduced re-epithelialization, leukocyte infiltration, angiogenesis and collagen accumulation (31).

Dendritic Epithelial T cells (DETC) comprise a small population of $\gamma\delta$ T cells that reside in the epidermis. Found in mouse, DETC's are in direct contact with keratinocytes and function in wound repair as well as tumor surveillance (23). In human, epidermal T cells possess $\alpha\beta$ T cell receptors but not a dendritic phenotype and are much less abundant than in mouse (32). The $\gamma\delta$ TCR recognizes an unidentified antigen released by damaged or stressed keratinocytes. DETC's produce keratinocyte growth factor-1 (KGF-1) and KGF-2, insulin-like growth factor-1 (IGF-1), interleukin-2 (IL-2), interferon-gamma (IFN- γ), and lymphotactin (23). TCR γ -deficient mice exhibit a delayed wound closure upon full thickness injury due to delayed keratinocyte hyperproliferation resulting from the absence of DETC derived KGF-1 and KGF-2 (23). Additionally, activated DETC induce keratinocytes to produce hyaluronan which is used by macrophages to migrate to the injured skin. Injured TCR γ -deficient mice failed to induce macrophage infiltration to the upper dermis (near the wound), further providing a critical role for DETC's in wound repair (23).

Concomitant with signals from injured keratinocytes and DETC's the thrombus, composed of a mesh of activated platelets and polymerized fibrin, provides a rich source of growth factors and chemotactic molecules. PDGF initiates the chemotaxis of neutrophils, macrophages, and stimulates the mitogenesis of smooth muscle cells and fibroblasts (3). CTAP-III is cleaved to neutrophil-activating peptide-2 (NAP-2; CXCL7) by neutrophils attached to the thrombus (33) and attracts blood circulating neutrophils through CXCR2 engagement. TGF- β released by activated platelets attracts macrophages and stimulates them to produce fibroblast growth factor (FGF), PDGF, tumor necrosis alpha (TNF- α) and interleukin-1 (IL-1). Additionally, TGF- β acts on fibroblast and smooth muscle cells to express collagen and collagenase (34-37).

Tissue resident mast cells cluster around blood capillaries and sense injury through the Complement proteins C3a and C5a as well as TGF- β and IL-8 (human), and release granules containing histamine, enzymes, and other active amines (38, 39). Additionally, mast cells release small lipids such as platelet activating factor (PAF), leukotrienes C₄/D₄, and prostaglandins which serve to increase vasodilation, blood vessel permeability, upregulation of adhesion molecules on endothelial cells, survival signals to tissue cells, and smooth muscle contraction which facilitates the egress of inflammatory cells, most notably, neutrophils into the damaged tissue (38, 39). Mast cells can also antagonize coagulation through release of tryptase which interferes with the ability of thrombin induced fibrin formation. Also, mast cells are recruited to the

provisional matrix through monocyte chemotactic protein-1 (MCP-1) released by tissue resident keratinocytes and endothelial cells and upregulate interleukin-4 (IL-4) expression during the later stages of the inflammatory response to shutdown expression of TNF- α and IL-8 (38-41). Moreover, mast cells play an important role in later phases of wound repair such as granulation and angiogenesis.

Neutrophils arrive to injured skin within minutes and comprise roughly 50% of all cells for the first few days post injury (Figure 1). Expression of CXCR2 by neutrophils enables them to respond to macrophage inflammatory protein-2 (MIP-2) and keratinocyte-derived chemokine (KC) in mouse (IL-8 and Melanoma growth-stimulatory activity/growth-related protein- α (MGSA/GRO α), respectively, in humans). KC produced by dermal fibroblasts and endothelial cells is the main source of neutrophil chemotaxis for the first 6 hours after injury (42). On the other hand, MIP-2 released by infiltrating neutrophils and monocytes is responsible for continued neutrophil chemotaxis. Continued release of MIP-2 occurs if an infection is present, otherwise, neutrophils cease to infiltrate after the first two days of injury (37, 42). Additionally, murine but not human neutrophils express CCR1 that recognizes MCP-1, macrophage inflammatory protein 1 alpha (MIP-1 α), and regulated on activation normal T expressed and secreted protein (RANTES). Interestingly, CXCR2-deficient mice exhibit delayed wound healing upon excisional injury, which is mainly attributed to the lack of neutrophil infiltration (37, 43). Although, expression of CCR1 by

neutrophils is thought to attract some neutrophils, CXCR2 expression is vital for a sufficient neutrophil response. However, normal levels of monocyte infiltration occur in CXCR2-deficient mice which clearly suggest that although, classically, neutrophils appear to the injured tissue prior to monocytes, their responses are independent of one another.

Although the historic function of infiltrating neutrophils was to clear bacteria and cellular debris, it has recently been shown that neutrophils contribute much more dynamically to the wound healing process. Upon arrival to the injury neutrophils are the predominant source of TNF- α , IL-1 and IL-8 in human (44, 46). Neutrophil activity is most prominent during the first 48 hours after injury and precipitously decline throughout the remainder of the wound repair process. Neutrophils stop infiltrating through many processes including negative regulation of CXCR2 expression by increasing levels of TNF- α , IL-10, interferon-g-inducible protein-10 (IP-10; CXCL10) and other inhibitory ligands expressed in high concentrations. Infiltrating macrophages phagocytose remaining neutrophils beginning around day 4.

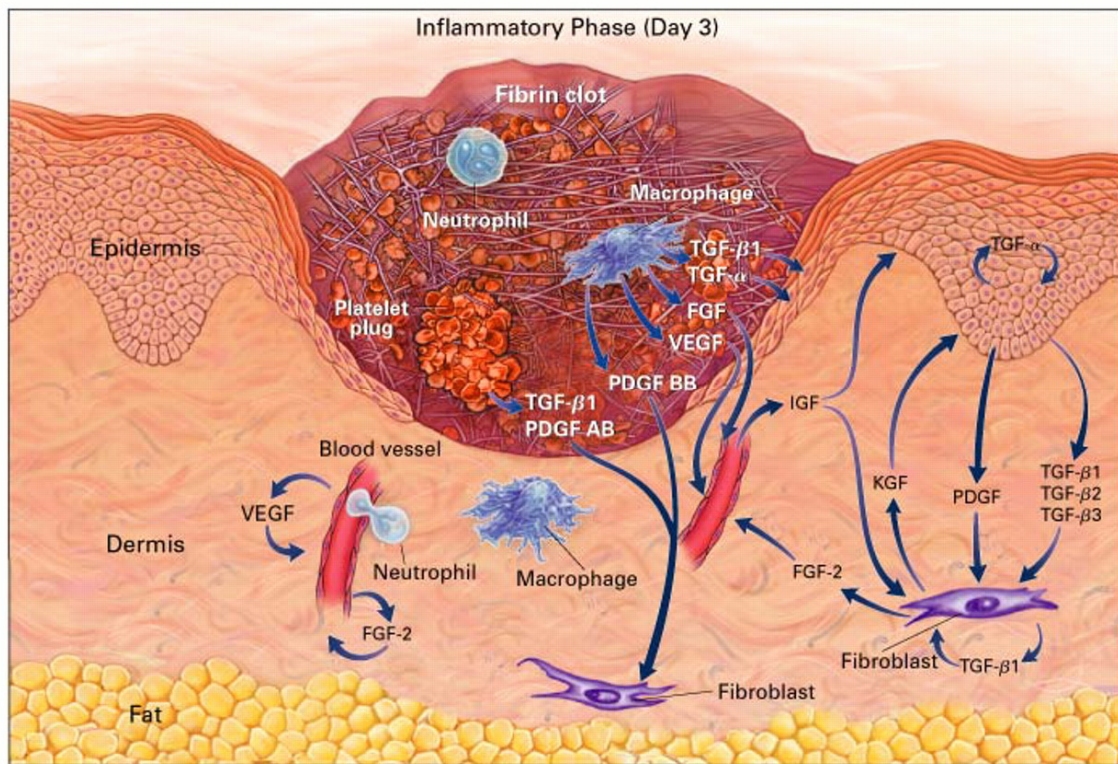


Figure 1. Wound healing during Inflammation phase (3 days after injury). Growth factors thought to be necessary for cell movement into the wound are shown (Singer, (56), Reprinted with permission from Massachusetts Medical Society).

The presence of monocytes at day 3 marks the near end of the inflammatory response and the beginning of the proliferation phase (9, 15). Circulating blood monocytes are attracted to injured tissue by degradation products of the extracellular matrix such as fragments of collagen and fibronectin as well as the chemokines MCP-1, MIP-1 α , RANTES released by endothelial cells (15). Upregulation of adhesion molecules on the blood vessel endothelium promote monocyte extravasation from circulation and monocytes differentiate into macrophages upon engagement of Mac-1 integrin receptor (CD11b) to fibronectin within the provisional matrix. Macrophages release growth factors such as TNF- α , IL-6, IL-1, IGF, and FGF in addition to facilitating breakdown of the fibrin clot by stimulating fibroblast and endothelial cell mobilization to the clot through release of TGF- β and PDGF which enable degradation of the fibrin clot and replacement with collagen. Moreover, macrophages are thought to be the primary source of monokine induced by interferon- γ (Mig, CXCL9), IP-10, and macrophage-derived chemokine (MDC, CCL22) that are associated with lymphocyte accumulation in the wound and marks the shift from a pro-inflammatory response (TNF- α /IL-1) to a IFN- γ one (15, 46).

PROLIFERATION PHASE

Concomitant with PDGF and TGF- β released in the provisional matrix, fibrin and fibronectin activate neighboring fibroblasts to upregulate α 3 β 1, α 5 β 1, α v β 1, α v β 3, and α v β 5 integrins, proliferate, and migrate to the injury (4). Collectively,

these signals cue both fibroblasts and endothelial cells to upregulate expression of the serine proteases tissue plasminogen activator (tPA, endothelial cells) and urokinase plasminogen activator (uPA, fibroblasts) to cleave plasminogen to the active plasmin (Figure 2). Fibrin is a cofactor for tPA while uPA receptor (uPAR) expression on fibroblasts serves as a cofactor for uPA. Additionally, fibroblasts must migrate to the injury and do so by clearing a way through breakdown of the provisional matrix through interstitial collagenase-1 and -3 (MMP-1 and MMP-13, respectively), the 72-kDa gelatinase A (MMP-2), and stromelysin (MMP-3) (5). Plasmin degrades the provisional matrix through fibrin proteolysis and enables fibroblasts to synthesize a new collagen matrix and endothelial cells to form new blood vessels to the injured tissue.

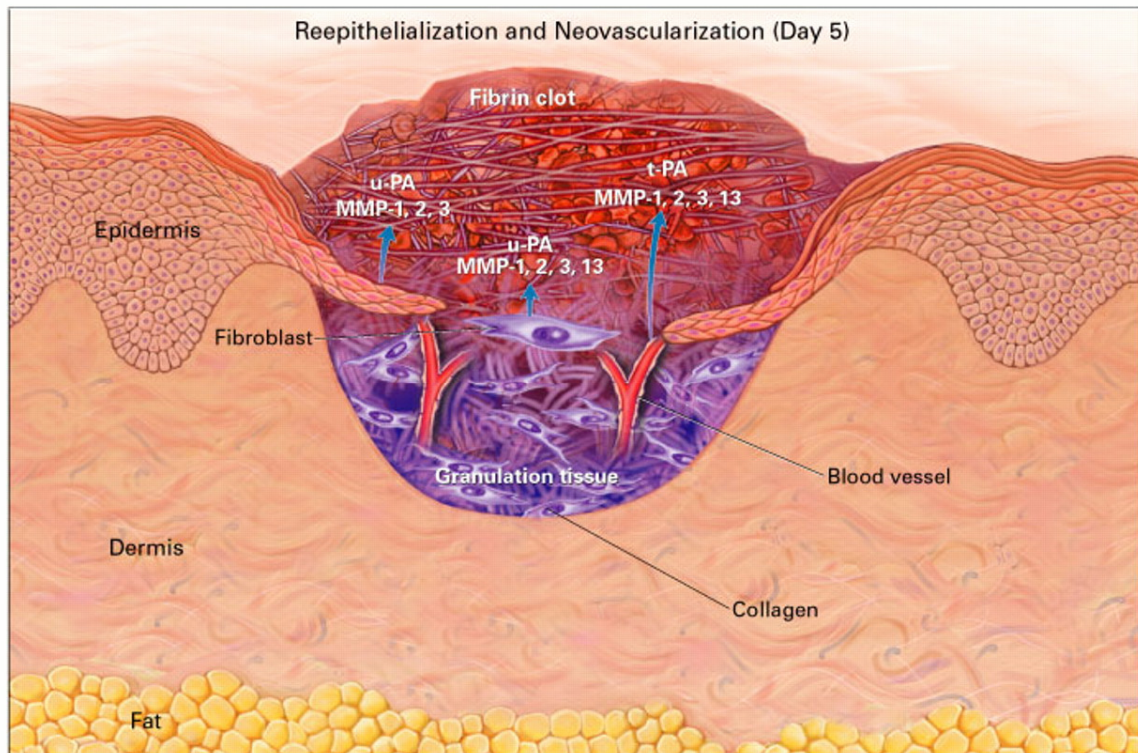


Figure 2. Wound healing during Proliferation phase (5 days after injury). Proteinases thought to be necessary for cell movement are shown (Singer (56), Reprinted with permission from Massachusetts Medical Society).

Fibroblasts are the predominant cell in the wound during the proliferative phase and play a critical role by synthesizing new collagen. TGF- β serves a vital role during the proliferative phase as it stimulates fibroblast proliferation, promotes transcription of the genes for collagen, proteoglycans and fibronectin, inhibits secretion of proteases responsible for the breakdown of the matrix and stimulates the protease inhibitor tissue inhibitor of metallo-protease (TIMP) (3). Fibroblasts attach to the provisional fibrin clot after migration to the wound and begin proteolysis of the fibrin clot and then switch functions to produce high amounts of Type I collagen. Collagen synthesis is then halted when sufficient collagen has been deposited into the extracellular matrix.

During collagen synthesis, there is an important step involving hydroxylation of proline and lysine residues (47). Hydroxylation allows collagen to form its characteristic triple helical structure and hydroxyproline residues stabilize the helical conformation. Procollagen is then secreted into the extracellular spaces where it is further processed by cleavage of the N and C- terminal peptides (47). Collagen undergoes further modifications to strengthen it. Lysyl oxidase, found in the extracellular matrix, further cross-links collagen strands to give more rigidity and tensile strength to the dermis. Additional intrastrand and interstrand cross-links form over time that further increases collagens strength and stability (47, 48). Collagen, on a gram per gram basis, has the same tensile strength as steel. Collagen remodeling also occurs during the remodeling phase which, classically, is the last phase of wound repair. Specific collagenases from

fibroblasts, neutrophils, and macrophages are capable of cleaving collagen through all three chains of the triple helix, which is further digested by other proteases (47, 48).

TGF- β 1 also stimulates fibroblasts to differentiate into myofibroblasts, key cells involved in connective tissue remodeling characterized by high expression levels of the contractile molecule alpha smooth muscle actin (α -SMA) (49). However, myofibroblast differentiation is dependent upon the fibronectin splice variant ED-A in addition to TGF- β 1 (50). Also, TGF- β can induce α -SMA synthesis through SMAD-dependent and -independent processes (51, 52). α -SMA expression and enhanced contraction are restricted to later phases of wound healing despite high levels of TGF- β 1 during the early stages due to negative regulation of α -SMA expression by the inflammatory molecules IL-1 and IFN- γ .

Although myofibroblasts serve a prominent role in wound contraction, myofibroblasts also synthesize significant levels of ECM components and matrix degrading enzymes including collagens of types I, III, IV, and V as well as the glycoprotein tenascin-C (53). Tenascin-C has been shown to recruit fibroblasts and to injured tissue and to promote their differentiation into myofibroblasts (54). Not surprisingly, myofibroblast are implicated in hypertrophic scarring and fibrosis development and persistence activity within a fibrotic lesion leads to elevated scarring and functional impairment of the

affected organ. Tight regulation of myofibroblast activity is necessary to prevent overscarring of the ECM. Recently, it has been shown that in addition to keratinocyte derived IL-1 secretion, growth hormone suppresses TGF- β induced myofibroblast development and consequently reduced fibroblast contractile activity (49).

Vascular endothelial cells in and around the injured tissue proliferate to provide new vasculature to the healing wound. Neovasculature also known as angiogenesis is a tightly regulated process and occurs concurrent with fibroblast and macrophage infiltration. Angiogenic factors released by keratinocytes and macrophages provide the necessary signal for vascular endothelial cells to form new capillaries from existing blood vessels. Sprouting capillaries express $\alpha_v\beta_3$ integrin at their tips induced by VEGF and FGF that allows capillaries to migrate through the fibrin/fibronectin rich clot (10). Fibroblast mediated proteolysis of the ECM is necessary for endothelial cell movement into the injured tissue and macrophages provide pro-angiogenic molecules. However, excess granulation and delayed macrophage infiltration inhibit angiogenesis (10).

Glu-Leu-Arg (ELR) containing CXC chemokines are potent inducers of angiogenesis (15). ELR+ CXC chemokines include IL-8, GRO- α , GRO- β (CXCL2), GRO- γ (CXCL3), CTAP-III, β -thromboglobulin, and NAP-2, which are released by macrophages and fibroblasts in the granular tissue (55).

Additional angiogenic factors include VEGF, KGF-1 KGF-2, TGF- β , angiogenin, angiopoietin, and mast cell tryptase. Angiopoietins belong to the VEGF family and are thought to be the only growth factors specific for vascular endothelium. Two important members include the angiogenesis agonist, Angiopoietin-1 and Angiopoietin-2, a natural antagonist (10). As the provisional matrix is replaced by Type I and III collagen (scar tissue) $\alpha_v\beta_3$ integrin expression is lost and angiogenesis ceases. Moreover, many of the newly formed capillaries undergo apoptosis during scar tissue formation. Interestingly, the inflammation induced molecules; Platelet factor-4 (PF4), IP-10, MIG inhibit angiogenesis and thus further demonstrate the spatio-temporal aspects of wound healing (10).

RE-EPITHELIALIZATION

Upon injury, wound edge keratinocytes as well as neighboring hair follicular keratinocytes migrate across the provisional matrix to form a monolayer over the denuded epidermis. Mitogenic signals from DETC's, dermal fibroblasts, and keratinocytes induce basal keratinocytes to dissolve $\alpha_6\beta_4$ integrin attachments to the basal layer and to induce the expression of $\alpha_5\beta_1$ and $\alpha_v\beta_6$ fibronectin/tenascin receptors, $\alpha_v\beta_5$ vitronectin receptor, and to rearrange $\alpha_2\beta_1$ collagen receptors (1). Upregulation of these integrins allows leading edge keratinocytes to migrate across or through the injured dermis. Once the migrating keratinocytes form a monolayer over the injured dermis they

downregulate expression of $\alpha_5\beta_1$ and $\alpha_v\beta_5$ integrins, stop migrating, and undergo differentiation to form a new stratified epidermis (Figure 3) (1).

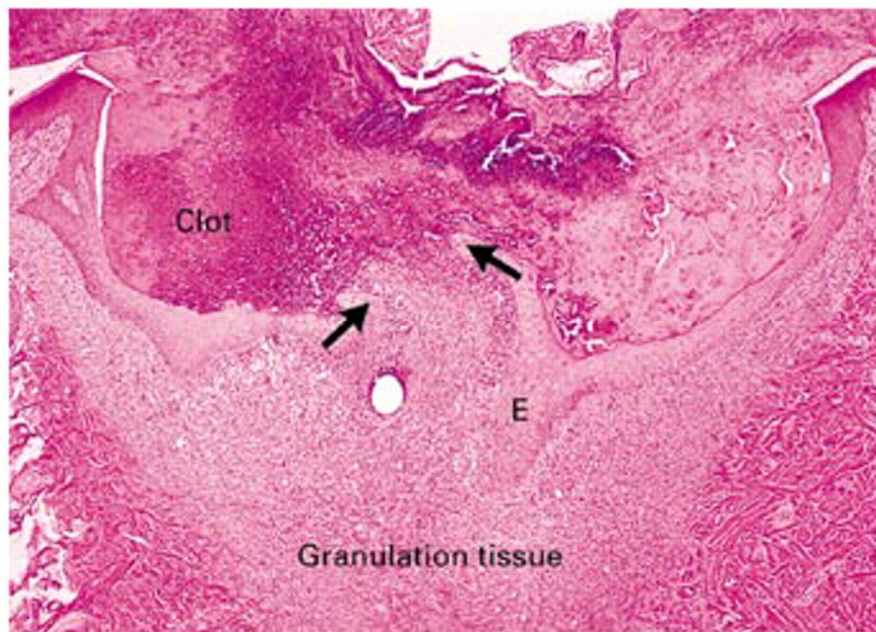


Figure 3. Re-epithelialization of injured porcine skin. Leading edge keratinocytes at wound margin forming epidermal monolayer (basal layer, black arrows), E: epidermis, clot: fibrin provisional matrix, granulation tissue: neo-collagen rich dermis. (Singer et al. (56), Reprinted with permission from Massachusetts Medical Society).

CONCLUSION

Wound healing is a highly dynamic process, includes the participation of many diverse cell types, and dependent upon many factors. Too much inflammation can lead to chronic ulceration while too little can lead to excessive scarring. Although, wounds heal remarkably, fast healing is imperfect and the resultant scar tissue is both weaker and less organized than undamaged dermis. In summary, epidermal injury causes both epidermal constituents, namely, keratinocytes and DETCs, as well as dermal resident cells, principally, platelets, fibroblasts, endothelial cells, neutrophils, monocytes/macrophages and mast cells to undergo cooperative activation to repair the injury to limit trauma as well as to resupply nutrients and blood flow.

PLASMACYTOID DENDRITIC CELLS

pDCs have a fascinating history beginning with their initial identification in 1958 by Lennert and Rammele (57) who reported plasma like cells located within the T cell area in human lymph nodes. These cells lack B cell and plasma cell markers and were initially called T-associated plasma cells. Later, research efforts by Feller in 1983 determined that these cells expressed CD4 but lacked immunoglobulin and B-cell antigen (58) and were renamed plasmacytoid T cell. It was postulated that these cells performed T cells functions and produce T cell cytokines. However, in 1988 Facchetti determined that these cells do not express CD3, a vital component of the T cell receptor complex expressed by all T cells. Moreover, these cells expressed myeloid antigens CD36 and CD68 as well as MHC Class II molecules. Based on these observations it was thought that these cells were more like monocytes than T cells and again, these cells were renamed to plasmacytoid monocytes (59). Much chaos ensued as the field was divided as to whether these cells were a subset of monocytes or were a new cell type. This led to additional efforts which were directed at identifying these cells both phenotypically and functionally. In 1994, O'Doherty identified a CD11c⁻ HLA-DR⁺ dendritic cell population from human peripheral blood characterized by low MHC Class II expression and low T cell stimulatory capacity (60). However, upon culture with monocyte conditioned medium these cells upregulated MHC Class II expression and assumed a dendritic cell morphology (60). Concomitantly, research efforts led by Liu identified two types

of CD4+CD3- cells located in different areas of human tonsil tissue (61, 62). Intriguingly, CD4+CD3- cells located within the germinal center expressed CD11c and were potent at activating naïve CD4 T cells and germinal center B cells. Conversely, CD4+CD3- cells located near high endothelial venules within T cell rich areas lacked CD11c expression and were matured when stimulated with IL-3 or IL-3 plus CD40 ligand (63). CD4+CD3-CD11c+ cells were classified within the DC lineage while CD4+CD3-CD11c- cells were classified as pre-DC (61-63). In 1997 Liu's lab successfully isolated pure CD11c- pre-DC from tonsillar tissue (63). Isolation of pure CD11c- pre-DC allowed further characterization and classification of these cells. CD11c- pre-DC were subsequently found to be unrelated to monocytes as they lacked phagocytic activity in addition to not expressing monocyte antigen and were renamed plasmacytoid dendritic cells (pDCs). pDCs were further classified as type 2 precursor DC (DC2) based on their ability to promote TH2 T cell differentiation, whereas monocytes or myeloid DCs were classified as type 1 DC (DC1) based on their ability to favor TH1 T cell differentiation (64). However, later studies showed that both DC1 and DC2 cells exhibit flexibility in inducing both TH1 and TH2 responses and that the particular T cell response is mediated by both the environmental cytokine milieu as well as DC interaction (65).

While the identification and investigation of CD11c+ and CD11c- DCs were being studied, an additional field of immunological research was focused on the identification of a Type I Interferon producing cell. In the late 1970's it was

discovered that upon viral challenge there was a peripheral blood cell responsible for strong Type I Interferon induction (59). Although most cells are capable of producing Type I Interferon, this unidentified cell was able to produce 10-1000 times more Interferon-alpha (IFN- α) than any other cell (66) and was called Professional Interferon Producing Cell or IPC. These cells were initially thought to be NK cells as NK cells were found to be activated by IFN- α in response to viral infection (67, 68). With the development of specific monoclonal antibodies for NK cells, T cells and monocytes/macrophages it was found that IPC did not express classical markers for these cells (69, 70). Investigation over the next few years revealed little insight into the cell identity of IPC. However, a breakthrough study in 1996, demonstrated that HSV challenge prompted strong intracellular IFN- α induction in CD4+DR+CD45RA+CD11c-CD11b-CD14-CD13-CD33-CD16-CD80-CD86-surface expressing cells (71). In 1999, Siegel (72) demonstrated that pDC are IPC (73, Table 1) that was confirmed by Cella (74).

	pDC/IPC	Monocytes	CD11c+ immature DC
<u>Phenotype</u>			
CD11b	—	+	+
CD11c	—	+	+
CD13	—	+	+
CD14	—	+	+
CD4	++	+	+
TLR7	++	+	++
TLR9	++	—	—
BDCA2	+	—	—
IL-3R	+++	+	+
<u>Function</u>			
IFN- α/β	+++	+	+
IL-12	—	++	++
Phagocytosis	—	++	++

Table 1. Human DC Phenotype Comparison. pDCs can be differentiated from monocytes and other DC based on the absence of CD11c expression, common myeloid marker expression as well as having strong expression of BDCA-2 and IL-3 Receptor. Functionally, pDC produce large amounts of Type I IFN, lack IL-12 production and are unable to phagocytose (73).

In mouse, pDCs were identified as having different surface marker expression than human pDC. Murine pDCs were classically defined as CD11c+B220+Gr-1+CD45Rb^{high}CD11b⁻ cells (75-77). However, murine pDCs also express PDCA-1 (78) and Siglec H (79) (73, Table 2).

Under steady state conditions, pDCs are present in the blood stream and secondary lymphoid organs, but are normally absent from most peripheral tissues including the skin (80, 81). pDCs can, however, infiltrate the skin infected by viruses including varicella zoster virus (82), human papillomavirus (83), and herpes simplex virus (84). These skin-infiltrating pDCs were found to produce IFN- α/β , which is consistent with the ability of viruses to infect pDCs and deliver their nucleic acid cargo into intracellular TLR 7/9 compartments of pDCs.

	pDC/IPC	CD8a ⁺ CD4 ⁻	CD8a ⁻ CD4 ⁻	CD8a ⁻ CD4 ⁺
Phenotype				
CD8	+/-	+	-	-
CD11b	-		+	+
CD11c	+	+	+	+
CD4	+/-	-	+	+
B220	+	-	-	-
Siglec H	+	-	-	-
Ly6c	+	-	-	-
PDCA-1	+	-	-	-
DEC-205	-	+	+/-	-
Function				
IFN- α/β	+++		-	-
IL-6	+		-	-
IL-12	+	+++	-	-
IFN- γ	-	++	-	-
CD8 T cell	-	+	-	-

Table 2. Mouse DC Phenotype Comparison. pDCs can be differentiated from other DC based on strong expression of B220, Siglec H, PDCA-1 and Ly6c. Functionally, pDC produce large amounts of Type I IFN and generate low IL-12 production (73, 78, 79).

pDCs are a rare population of circulating cells comprising only 0.2 to 0.8% of peripheral cells (85). pDCs selectively express endosomal restricted Toll-like receptors (TLR)7 and TLR9 (86), which recognize single-stranded viral RNA and DNA transported into endosomal compartments by the virus infecting the cell (87-90). IFN- α/β produced by pDCs was shown to be critical in inhibiting viral replication but also to contribute to the induction and expansion of an antiviral immune response by activating memory T cells, B cells, and NK cells (91, 92).

Surprisingly, large numbers of pDCs have also been found in the skin of patients with psoriasis (80, 81, 93), a chronic inflammatory disease of the skin mediated by autoimmune T cells. Here, pDCs are chronically activated to produce IFN- α/β , a process that triggers the activation and expansion of autoimmune T cells leading to the epidermal hyperproliferation and the formation of psoriasis. We have recently found that pDC activation in psoriatic skin is driven by the human cathelicidin antimicrobial peptide known as LL37 (29). LL37 was found to convert otherwise inert extracellular host-derived (self) nucleic acids, into a potent trigger of pDC activation by forming a complex with the self-RNA and self-DNA and by transporting them into intracellular TLR 7 and TLR 9 compartments (30, 29). The cathelicidin peptide is usually not expressed in healthy skin but is continuously overexpressed by keratinocytes of psoriatic skin, providing an explanation for the chronic activation of pDCs in psoriasis (29). Interestingly, the expression of cathelicidin peptides can be transiently induced in keratinocytes by common skin injury (94, 95).

ANTI-MICROBIAL PEPTIDES (AMPS)

AMPs are evolutionarily conserved host defense peptides which possess potent, broad spectrum antibiotic properties (96). Cationic AMPs are relatively short peptides (12-50 AA) and comprise two families, the cathelicidins and the defensins. Only one member of the cathelicidins has been identified to date; LL37 in human and CRAMP in mouse (97-99). The defensins, composed of alpha and beta families are produced by neutrophils, keratinocytes and paneth cells in human and epithelial cells, respectively in mice (96). Cationic AMPs have a net positive charge and are amphipathic while possessing either a predominant alpha helical or β -sheet structure (96). Cathelicidins and defensins are expressed by keratinocytes and are important contributors to host defense (100-104). Upon epidermal injury keratinocytes release pro- peptides which are subsequently cleaved to reveal active peptides (105). Moreover, these peptides are capable of forming pore-forming complexes in microbial cell wall resulting in cell lysis. Additionally, host cells are protected from autolysis as the cationic AMPs selectively bind to microbial cell membranes (96). Cathelicidins has been found to contain chemotactic properties as cathelicidins can directly mediate neutrophil, macrophage, and T cell migration (104).

AMP release is also positively regulated by a subset of T cells (104). In psoriasis, continued inflammation amplifies cathelicidin, defensin, and psoriasin peptide production by keratinocytes (104, 105). Extremely elevated levels of LL-

37 and psoriasin have been measured in chronic psoriatic plaques compared to healthy or uninvolved skin (105). This dysregulated response helps maintain the inflammatory state and recent evidence suggests that continued LL-37 production can provide a potent stimulator for pDC activation mediated through associated with nucleic acids and uptake and triggering of TLR 7 and TLR 9 (30, 29).

CLINICAL RELEVANCE

There are numerous skin diseases which are due to or result in dysregulated immune responses such as psoriasis, contact dermatitis and atopic dermatitis (106). Psoriasis is the most understood skin disorder and provides a good model to evaluate pDC involvement in the pathogenesis of this inflammatory disease. Psoriasis is a chronic inflammatory disease affecting 1-3% of the global population (107, 108). Phenotypically, psoriasis is characterized by raised, scaly, erythematous plaques. Immunologically, psoriasis is characterized by chronic activation of myeloid dendritic cells and autoreactive T cells (106). mDC once activated present self-antigens to T cells which then drives the pathogenesis of psoriasis by differentiating into TH1 and TH17 effector cells (106, 108). The exact timing and location of this process is still under debate. One school of thought is that mDC and other dermal DC migrate to skin draining lymph nodes upon activation and present self-antigens to naïve T cells (107). Auto-reactive T cells then migrate into the psoriatic dermis. However, Th1 differentiation requires IL-12 and TH17 requires IL-23 along with the appropriate MHC ligands. Sources of IL-12 and IL-23 in the lymph node may be attributed to the activated DC presenting the antigen to the T cell. Alternatively, activated DC present antigen to dermal resident T cells, which then differentiate into TH1 or TH17 cells (107). Recent reports have demonstrated that activated mDC can produce IL-12 and IL-23 in the dermis. TH1 cells produce IFN- γ while TH17 cells produce IL-17 and IL-22. TH1

and TH17 cells then propagate the inflammatory cycle by stimulating proliferation and activation of keratinocytes to produce antimicrobial peptides such as LL-37, B-defensin 1, B-defensin 2, S100A7 and S100A9. Keratinocytes also produce IL-1 β and neutrophil chemotactic molecules CXCL1, CXCL3, CXCL5 and CXCL8 (107, 109).

Recent progress in understanding the initiation of the inflammatory reaction witnessed in psoriasis has elucidated pDC as an important trigger. Local epidermal or dermal injury triggers the Koebner phenomenon that results in new lesions where the injury occurred. It is thought that the initial injury disrupts keratinocytes at the site of injury. In response to the injury keratinocytes then release antimicrobial peptides such as LL-37 as well as additional cytokines. Local injury also causes cell damage resulting in apoptosis which releases apoptotic DNA into the extracellular space. Recently it was shown that pDCs are important mediators in the initiation of the inflammatory cascade in psoriasis (93). pDCs become activated in the injured skin and release IFN- α . Neutralization of pDCs through BDCA-2 specific antibodies as well as anti-IFN- α antibody completely abolished development of the psoriatic plaque. More recently, our group demonstrated that pDCs can bind LL-37/self-DNA and LL-37/self-RNA to become activated to produce large amount of IFN- α (29, 30). Potentially, LL-37 released by damaged keratinocytes can bind endogenous DNA which then becomes a potent stimulus for pDC activation. Additionally, LL-37/self-RNA can activate mDC to produce IL-12 and IL-23 (30). IFN- α then

drives mDC maturation which promotes more efficient antigen presentation to auto-reactive T cells. Interestingly, pDC are absent from chronic psoriatic plaques indicating that once the inflammatory cycle is initiated positive feedback loops between mDC, T cells and keratinocytes maintains psoriatic pathogenesis (107, 109). In human pDC constitute less than 1% of dermal lymphocytes whereas pDC are absent in murine dermis under steady-state conditions.

Basis for Dissertation

Some of main questions yet to be answered in autoimmune skin disorders such as psoriasis are 1) what drives pDCs infiltration into psoriatic skin, 2) why don't pDCs infiltrate into uninvolved skin in psoriasis patients, 3) why do psoriatic patients have unabridged pDCs activation whereas non-psoriatic patients don't develop psoriasis, 4) what role do pDCs play in skin injury in non-psoriatic skin and 5) what mechanism counters pDCs activation to prevent uncontrolled inflammation?

My research addresses the role of pDCs in normal skin injury. By normal I mean non-diseased, steady-state skin. My research has shed light onto the basis of pDC involvement in skin injury as well as the consequence of wound healing without pDC involvement. I uncovered a new role for pDC in sensing injury that will hopefully provide new strategies for therapeutic intervention in autoimmune skin disorders such as psoriasis.

CHAPTER 2
MATERIALS AND METHODS

MICE

Wild-type BALB/cJ, C57Bl/6, or 129SV/J mice and TLR7^{-/-} (B6.129S1-Tlr7tm1Flv/J) mice were purchased from The Jackson Laboratory. MyD88^{-/-} mice were kindly provided by S. Akira (110), IFNAR^{-/-} mice (111) were provided by W. Overwijk. Cramp^{-/-} mice were from the lab of Richard L Gallo (112). All animal experiments were conducted on 6-14 week old mice. Animals were maintained and bred in specific pathogen free facilities. All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of Texas M.D. Anderson Cancer Center.

REAGENTS

The synthetic mouse cathelicidin peptide CRAMP (GLLRKGGGEKIGEKLLKKIGQKIKNFFQKLVPQPEQ), and the corresponding scrambled control (LLGQNGKFKIREPPIQKVKGIQEKEFGLKQKLKG) were obtained from AnaSpec. For in vivo experiments 200 µg of the peptides were injected into the dermis of the upper dorsum of shaven and depilated mice. For in vitro pDC stimulation 30 µM of the peptides were used. The TLR 7/9 inhibitor IRS 954 (5'-TGC TCC TGG AGG GGT TGT-3', phosphorothioate oligodeoxynucleotide) was a kind gift of F. Barrat, Dynavax Technologies. The TLR 9 inhibitor IRS 869 (5'-TCC TGG AGG GGT TGT-3', phosphorothioate oligodeoxynucleotide) was purchased from Integrated DNA Technologies. For inhibition of TLR9 or TLR7 and TLR9 in vivo, intradermal administration of IRS 869 and 954 were used 24 hours and 4 hours prior to the skin injury.

MECHANICAL INJURY OF MOUSE SKIN

Mice backs were shaven and depilated (Veet, Reckitt Benckiser) immediately prior to injury. Mechanical injury was then applied by tape stripping, using 20 strokes of transparent tape (Scotch, 3M) across the back. For full-thickness injury, mice dorsal skin was shaven, cleaned with 70% ethanol, and a 3 mm punch biopsy (Acuderm) was applied to remove skin (care was taken to ensure excision was restricted to a depth of the fascia layer). Calipers were used to monitor wound closure over a 14-day period.

DEPLETION EXPERIMENTS

pDC depletion was carried out using a combination of two anti-BST antibodies (PDCA1, Miltenyi Biotec, Clone: JF05-1C2.4.1) and mAb 927 (kindly provided by Marco Colonna, Washington University School of Medicine. 0.5 mg of each antibody was injected, intraperitoneally, 48 hours and 24 hours before injury. Rat IgG antibodies were injected into control mice. Depletion of pDCs was monitored in the spleen by flow cytometry and was found to be most efficient when the combination of the antibodies was used. Neutrophil depletion was conducted using an anti-Ly6G specific antibody (Bioxcell, clone 1A8). 1 mg of anti-Ly6G or whole rat IgG was injected, intraperitoneally, into mice at 24 hours and 4 hours before injury.

MECHANICAL AND CHEMICAL INJURY OF HUMAN SKIN

Mechanical injury of human skin was induced by application of 10 strokes of cellophane tape across healthy skin of 7 human volunteers. Biopsies were taken before (uninjured) and 24 hours after tape stripping (injured). The specimen was snap frozen and stored at -80°C before immunohistochemical and gene expression analysis was performed. Chemical injury of human skin was induced by treatment of healthy skin of human volunteers with the chemical irritant Sodium Lauryl Sulphate (SLS) (Merck KGaA, Darmstadt, Germany) at 1% in water as described previously (113). Briefly, SLS was applied in large Finn Chambers to the skin on the back of the patients, before biopsies were taken. Specimens were immediately snap frozen and stored at -80°C before immunohistochemical and gene expression analysis was performed. All human studies were performed at the Skin and Allergy Hospital, Helsinki University Central Hospital, Helsinki, Finland and approved by the local ethics committee (Helsinki-Uusimaa Hospital District Ethics Committee).

GENERATION AND ANALYSIS OF DERMAL SINGLE CELL SUSPENSIONS

Injured skin was excised, minced and digested with 1 mg/ml Dispase (Sigma) for one hour at 37°C and the epidermis was manually removed with forceps. The dermis was removed to a clean culture plate and incubated with 1 mg/ml Collagenase (Gibco) for two hours to generate a single-cell suspension. Leukocytes were counted using trypan blue exclusion. Cells were treated for 20 min with anti-CD16/CD32 to block non-specific binding, followed by the addition

of the following antibodies at 10 µg/ml final concentration for 20 min: anti-PDCA-1 FITC (Miltenyi Biotec), anti-Siglec-H-FITC (eBioscience) anti-CD11c-PE, anti-B220-APC, anti-IA/IE-PE, anti-CD11b-FITC, anti-Gr-1-PerCp-Cy5.5 and anti-CD3e-APC (all BD). Cells were washed twice and acquired on a FACSCaliber and analyzed using FlowJo software.

IMMUNOHISTOCHEMISTRY AND IMMUNOFLUORESCENCE

Mouse skin tissue was excised, embedded in OCT (Tissue-Tek) and frozen immediately on dry ice. 8 µM frozen sections were cut and stained with anti-Siglec H (3H3 clone) followed by Horseradish Peroxidase labeled goat anti-rat IgG and a color development step with Aminoethylcarbazole. For K6 staining, frozen sections were stained with a purified rabbit anti-mouse keratin 6 antibody (Covance) and subsequently stained with Alexa 546 labeled goat anti-rabbit IgG (H+L) (Molecular Probes). For detection of pDCs in human skin tissue we used an anti-BDCA2 antibody (Miltenyi Biotec) according to the previously described protocol (29).

REAL-TIME PCR ANALYSIS

All excised tissue was immediately saturated in RNAlater (Ambion) and stored at -20°C until RNA was isolated. Skin was homogenized using a Tissue-Miser device (Fisher) in 1 ml Trizol reagent (Ambion). The homogenate was transferred to a clean RNase free 1.5 ml microtube and 100 µl of BCP (MRC) was added and the sample was vortexed. The tube was centrifuged at 4°C at

13k RPM (15g) for 10 min. The aqueous phase (400 μ l) was removed and precipitated with 200 μ l of 200 proof ethanol. The mixture was placed into a filter insert and centrifuged at 13k RPM for 20 seconds. The supernatant was discarded and the filter unit was washed twice with 500 μ l of wash solution (RiboPure Kit, Ambion). The sample was eluted in 100 μ l of elution buffer (RiboPure) and immediately mixed with 350 μ l of Lysis buffer (RNAqueous Kit, Ambion). 250 μ l of 200 proof ethanol was added and mixed. The sample was applied to a new filter insert and centrifuged at 13k RPM for 20 seconds. The sample was washed twice with 500 μ l of 80% ethanol and eluted in 100 μ l of elution solution (RNAqueous) and quantitated using UV/Vis absorbion at 260 nm. The 260/280 ratio was consistently above 1.7, signifying very little protein contamination. 2 μ g of RNA was used to make cDNA using the High Capacity cDNA kit (Applied Biosystems) on a Bio-Rad C1000 thermocycler. 40 ng of cDNA was used for each individual gene expression using Taqman based amplification on an ABI 7500 Fast system using the default Standard protocol. Mouse Taqman probes used were: *Gapdh*, *Ifna2*, *Ifnb*, *Cramp*, *Il6*, *Tnfa*, *Il23p19*, *Il12p35*, *Il12p40*, *Il22*, *Il17a*, *Vegfa*, *Vegfb*, *Egf* and *Ifng* (Table 3). Human Taqman probes used were: *Gapdh*, *Ifna2*, and *Ifnb* (Table 4).

Primer	Assay I.D.
Beta defensin 1	Mm00432803_m1
Beta defensin 2	Mm00657074_m1
Beta defensin 3	Mm04214158_s1
Beta defensin 4	Mm00731768_m1
Beta defensin 14	Mm00806979_m1
chemerin	Mm00503579_m1
CRAMP	Mm00438285_m1
Egf	Mm00438696_m1
Gapdh	Mm99999915_g1
IFN-a	Mm00833961_s1
IFN-b	Mm00439552_s1
IFN-g	Mm00801778_m1
IL6	Mm00446191_m1
IL12p35	Mm00434165_m1
IL12p40	Mm01288991_m1
IL17A	Mm00439619_m1
IL-22	Mm00444241_m1
IL23p19	Mm00518984_m1
TNF-a	Mm00443258_m1
Vegf-a	Mm00437304_m1
Vegf-b	Mm00442102_m1

Table 3. Mouse Taqman Primers. Taqman primers generated by Applied Biosystems for the indicated genes are provided as well as their unique sequence ID.

Primer	Assay I.D.
IFN-a	Hs02621172_s1
IFN-b	Hs01077958_s1
Gapdh	Hs03929097_g1

Table 4. Human Taqman Primers. Taqman primers generated by Applied Biosystems for the indicated genes are provided as well as their unique sequence ID.

PDC ISOLATION AND IN VITRO STIMULATION

pDCs were isolated from spleens of 6-12 week female BALB/cJ mice using the pDC Isolation Kit II (Miltenyi Biotec) with a purity of > 90%. 50,000 cells were cultured overnight in RPMI based media (10% FCS, 50 μ M beta mercapto ethanol, pen/strep, glutamine, sodium pyruvate, Hepes). The supernatants were used to determine Interferon- α protein secretion using a commercial Elisa kit (PBL). As a source of DNA, we used synthetic phosphodiesteric CpG-containing oligonucleotides, hybridized to its complementary strand to mimic natural mammalian DNA fragments. 0.3 μ M dsDNA was mixed with 30 μ M cathelicidin peptides in a volume of 20 μ l for 30 minutes at RT.

CHAPTER 3

PDCS INFILTRATE SKIN INJURY

TAPE STRIP INJURY MODEL

To establish an injury model we focused on the endogenous response rather than the aggregate stimuli encompassing both host driven injury mediators and external triggers of immunity (e.g. bacterial cell components, virus' or other environmental factors). Our aim was to elucidate the immune response to a "sterile injury." Most wound studies employ full thickness injuries wherein a dermal biopsy punch is applied across the epidermis into and through the dermis. This method was unsuitable for my research as it completely breaches the skin barrier and exposes the skin to foreign immunogens. To this end we primarily utilized an epidermal stripping approach in which the outer layers of the epidermis, composed of flattened anuclear keratinocytes, is removed through use of cellophane tape. Tape stripping not only removes cells of the epidermis but also kills keratinocytes at the epidermal/dermal border which form the basement membrane.

Unlike the full thickness injury model, the basement membrane cells remain intact, although transepidermal barrier functionality is breached and a marked increase in transepidermal water evaporation occurs. However, the damaged basement layer generates a danger signal and activates the wound healing response. Tape stripping using 20 strokes across the dorsal flank of shaven and depilated Balb/c mice results in acute erythema (Figure 4) characterized by a large but transient increase in cellularity in the underlying dermis (Figure 5).

Pathologically, the bulk of cellular infiltration occurs at 24 to 48 hours characterized by dense granularity. By 72 hours to 5 days post injury, the basement membrane is replaced by a new keratinocyte layer which further differentiates to form flattened anuclear layers characteristic of the stratified epidermis (Figure 6).

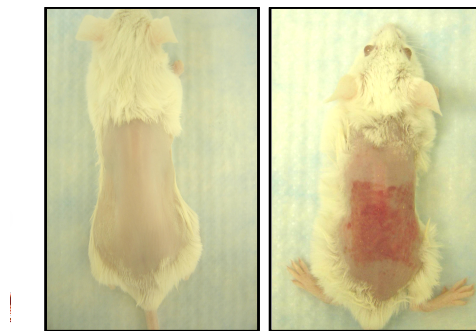


Figure 4. Tape strip model. Murine epidermis before and after tape stripping with 20 strokes of cellophane tape

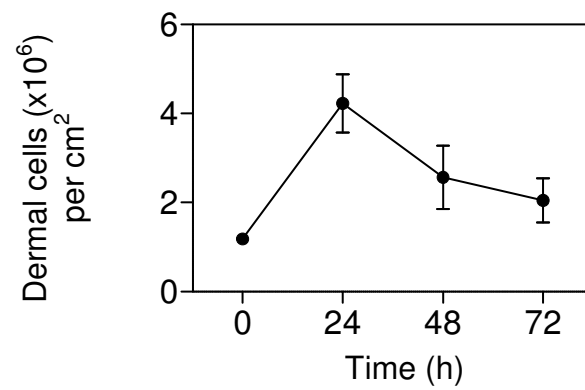


Figure 5. Tape stripping induces strong dermal infiltration. Dermal cell suspensions isolated from skin at various times after injury and viable cells were counted. The mean number of cells \pm SEM per cm^2 of injured skin is given and represent data from five independent mice over a 3-day time course.

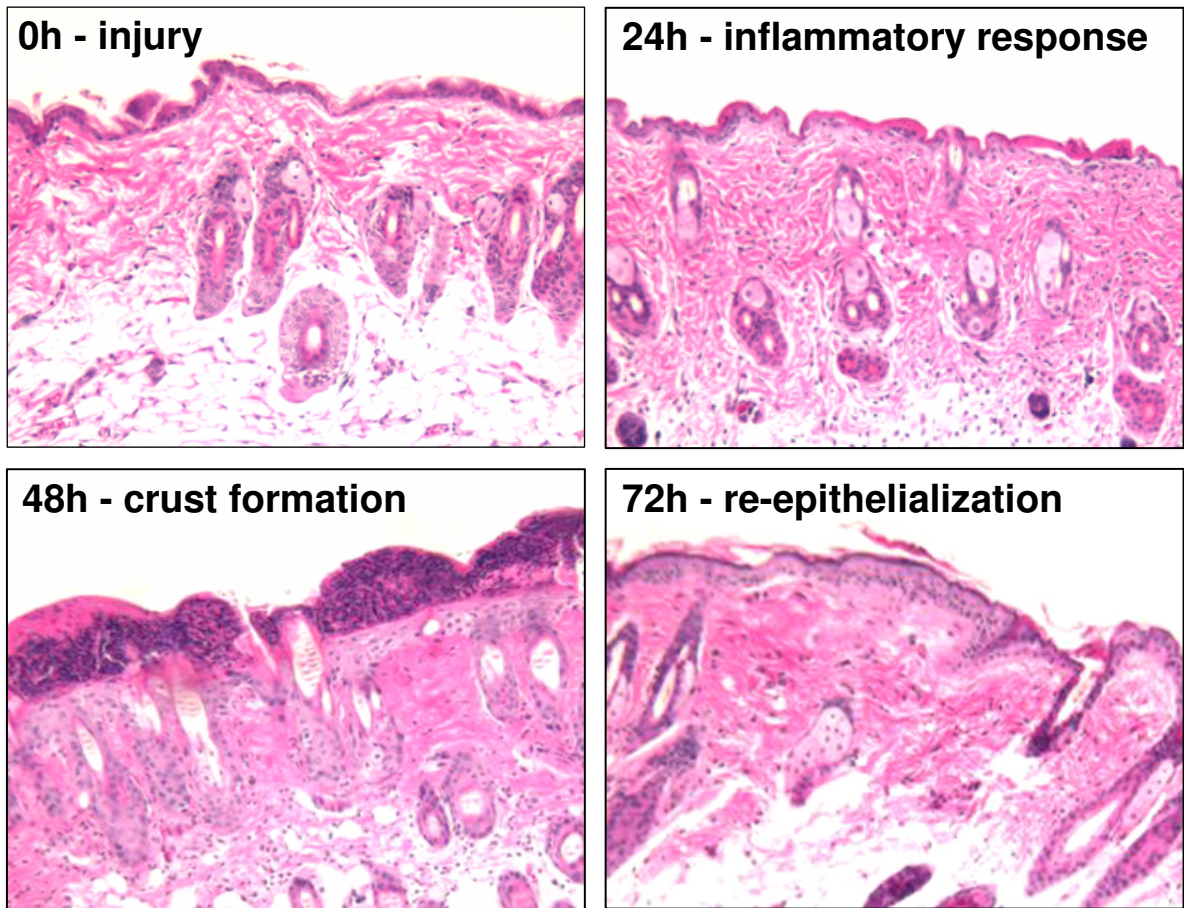


Figure 6. Sterile epidermal injury prompts a rapid and transient leukocyte infiltrate in the dermis which precedes epithelial repair. Frozen sections of injured skin were stained with H&E to identify infiltrating cells. There are few dermal cells in nascent injured skin. However, by 24h a strong inflammatory response can be seen. By 48h there is strong granulation tissue formation in the epidermis characterized by a thick crust like appearance. By 72h, the injured epidermis is repaired by migrating keratinocytes which proliferate to form a new stratified layer.

SKIN INJURY INDUCES A RAPID INFILTRATION OF PDCS

Mice were shaven, depilated, tape stripped and sacrificed at various time points including 6, 24, 48, and 72 hours. The injured skin was excised and a single cell suspension was generated using a Dispase/Collagenase treatment scheme. Cells were stained for leukocyte populations with fluorescent-labeled antibodies and analyzed by flow cytometry. Interestingly, there was a large but transient increase in pDCs (B220+PDCA1+) after injury (Figure 7 A). Remarkably, the infiltration was brief as pDC levels returned to pre-injury levels by 48 hours. The pDC population was co-stained for MHC Class II (IA/IE), CD11c, and Siglec H to confirm pDC phenotype (IA/IE^{low}CD11c+Siglec-H+, Figure 7 B). Moreover, the B220+PDCA1+ population lacked expression of common myeloid markers CD3, CD11b, and CD19 (data not shown). Additionally, immunohistological staining of Siglec H (3H3 clone) of injured skin at 24h confirmed leukocyte morphology (Figure 7 C).

NEUTROPHILS INFILTRATE SKIN INJURY EARLY WHILE T CELLS INFILTRATE LATER

Concomitant with pDC infiltration there was a large and robust neutrophil (Gr-1+CD11b+) infiltration at 24 hours (Figure 8). Surprisingly, the bulk neutrophil infiltrate was gone by 48 hours, mirroring pDC kinetics. However, there was a delay in T cell (CD3+) infiltration (Figure 9). There was a substantial baseline T

cell population (17%) in the skin which increased to 55% by 48 hours and was back to baseline levels by 72 hours. Similarly, there was a resident population of conventional dendritic cells (mDC, CD11c+CD11b+PDCA1-) which did not show an increase but rather decreased in population after injury (Figure 10). The mDC baseline population was gradually restored by 48 hours. Cumulative experiments are shown depicting infiltration time course kinetics of pDCs, neutrophils, and T cells (Figure 11).

SKIN INJURY INDUCES TRANSIENT INCREASE IN TYPE I IFN AND PRO-INFLAMMATORY CYTOKINES

pDCs are the main producers of IFN- α/β , so we sought to investigate whether Type I IFN were involved in wound repair. Tape stripped mice were euthanized and skin was harvested at 6, 24, 48, and 72 hours. Pre-injury skin was used as a baseline control. Excised skin was stored in RNAlater until RNA was extracted. RNA was isolated from skin using a specialized protocol (see Materials and Methods).

Gene expression for IFN- α , IL-6, and TNF- α was performed. There was a rapid but transient induction of IFN- α with peak expression at 24 hours which paralleled maximal pDC skin infiltration. IL-6 expression peaked at 6 hours with significant expression at 24 hours before returning to baseline levels. Neutrophils are known to be early producers of IL-6 and have been reported to

infiltrate skin as early as 3 hours after injury. TNF- α expressed reached maximum levels at 48 hours before declining. It has been reported that neutrophils are the chief TNF- α producers during the first 48 hours after injury whereas monocytes/macrophages take over TNF- α production after 48 hours.

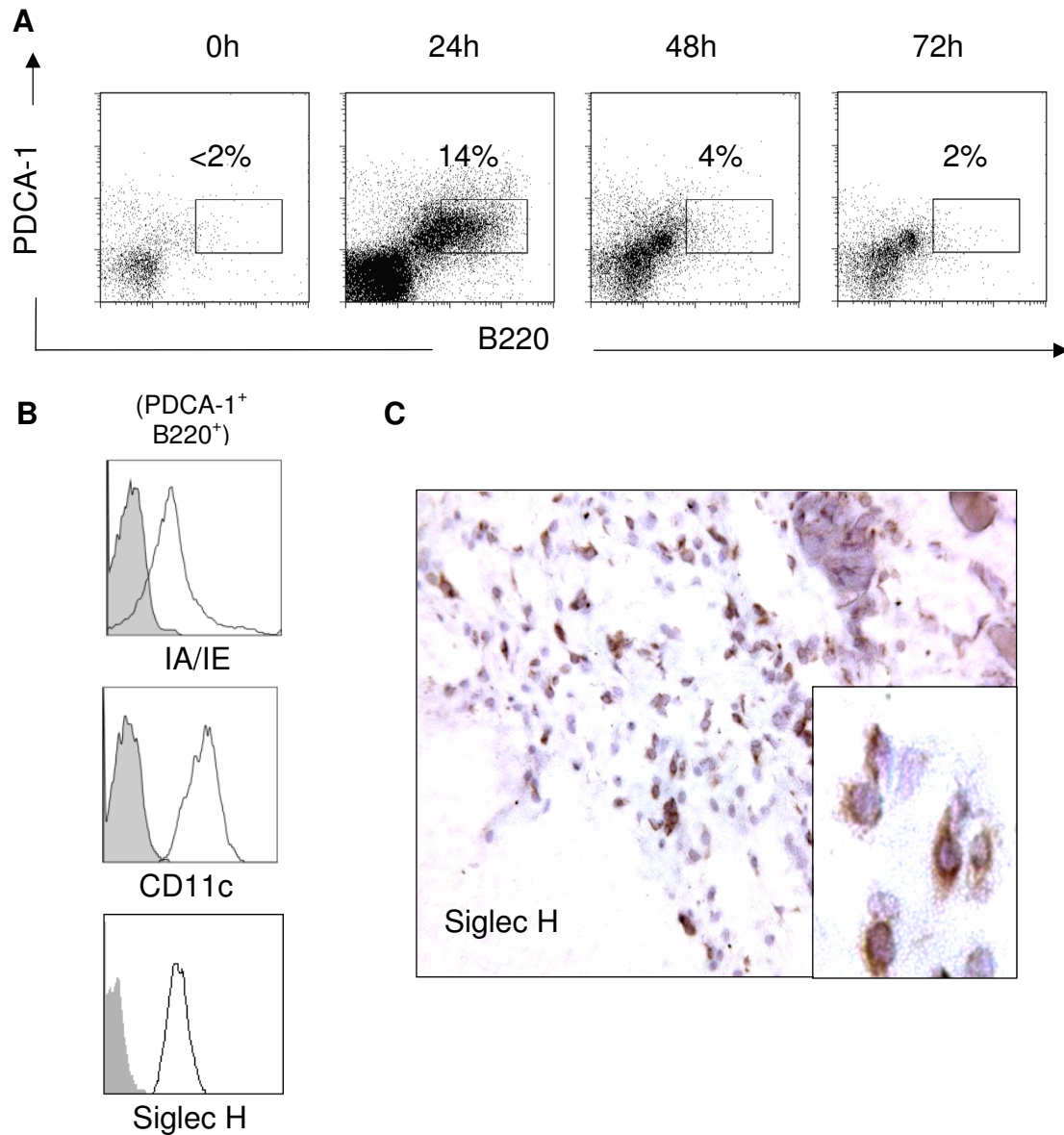


Figure 7. Rapid infiltration of pDCs into injured skin. (A) The percentage of pDCs (B220+PDCA-1+) in dermal single cell suspensions isolated from injured skin was measured by flow cytometry. Representative flow cytometry plot of pDC in injured skin over a 3-day time course. The percentage of each population is shown in the plots. (B) Flow cytometry for CD11c, IA/IE, Siglec H surface expression on B220+PDCA-1+ pDC 24 hours after skin injury. (C) Representative immunohistochemical staining for Siglec H in injured skin collected 24 hours after tape stripping.

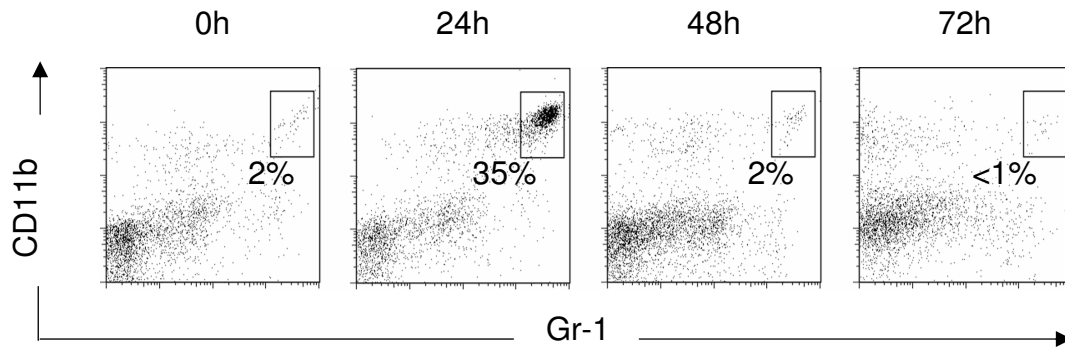


Figure 8. Neutrophil recruitment into injured skin parallels pDC infiltration. The percentage of neutrophils (CD11b+Gr-1+) in dermal single cell suspensions isolated from injured skin was measured by flow cytometry. Representative flow cytometry plot of neutrophils in injured skin over a 3-day time course. The percentage of each population is shown in the plots.

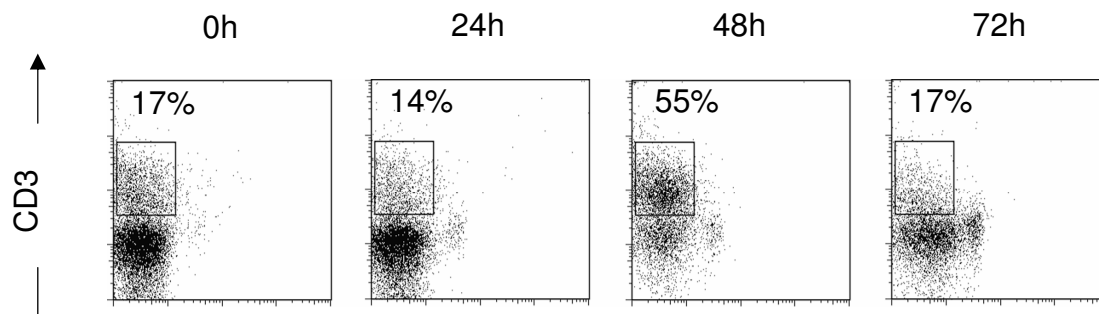


Figure 9. T cell infiltrate injured skin later than pDCs and neutrophils. The percentage of T cells (CD3+) in dermal single cell suspensions isolated from injured skin was measured by flow cytometry. Representative flow cytometry plot of neutrophils in injured skin over a 3-day time course. The percentage of each population is shown in the plots.

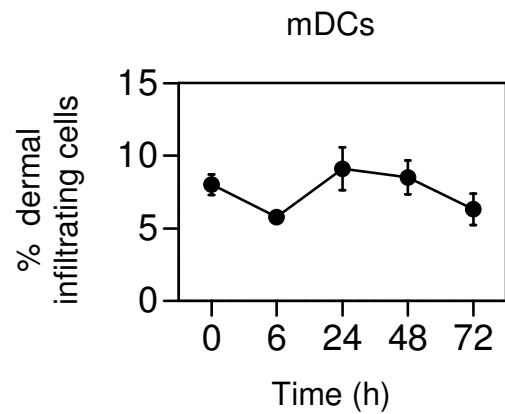


Figure 10. Conventional DCs do not infiltrate injured skin. Time course analysis of cDCs (CD11c+CD11b+PDCA-1-) in dermal single cell suspensions isolated from injured skin was measured by flow cytometry. Data are representative of 3 mice.

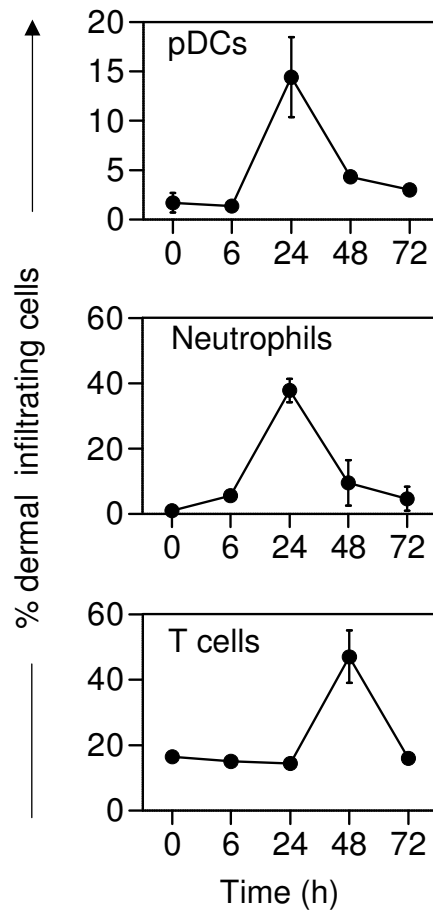


Figure 11. Time course analysis of infiltrating lymphocytes. The percentage of pDCs (B220+PDCA-1+), neutrophils (Gr1+CD11b+), and T cells (CD3+) in dermal single cell suspensions isolated from injured skin was measured by flow cytometry. Data are the mean \pm SEM of five independent mice per each timepoint.

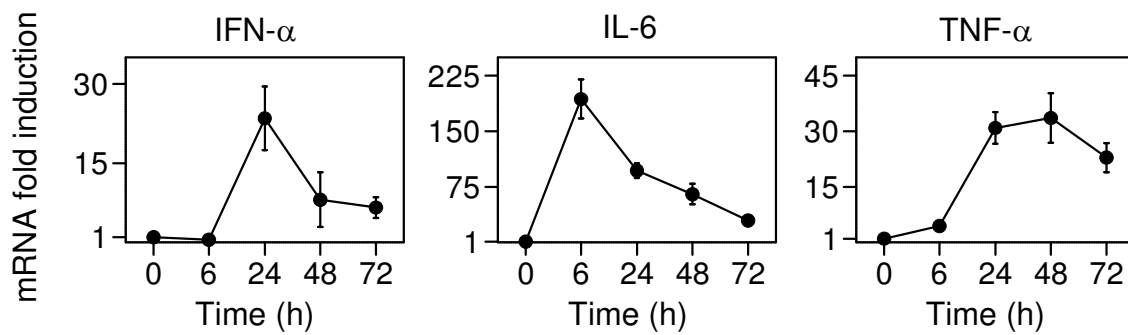


Figure 12. pDCs are transiently activated to produce type I IFNs in injured skin. Time course analysis of IFN- α , IL-6 and TNF- α mRNA tissue expression in injured skin. The data are given as fold induction over time 0 and represent the mean \pm SEM of five mice per timepoint.

CHAPTER 4

PDCS ARE ACTIVATED IN SKIN INJURY TO PRODUCE TYPE I IFNS

PDCS ARE ACTIVATED TO PRODUCE TYPE I IFN

To determine whether pDCs were the main producers of IFN- α seen in skin injury, pDCs were depleted using a BST depleting antibody cocktail composed of α -PDCA1 (Miltenyi) and mAB 927 (kind gift from Marco Colonna, Figure 13). Similar effects in skin were observed. Neutrophils were depleted using a Ly6G specific antibody (1A8 clone, Bioxcel). 500 μ g of each pDC depleting antibody was injected, intraperitoneally, at -48 and -24 hours prior to tape stripping. 1 mg of α -Ly6G antibody was applied, intraperitoneally, -24 and -4 hours prior to tape stripping. Depleted and control (whole rat IgG) injected mice were tape stripped and the skin was excised 24 hours later. Gene expression was performed using Taqman chemistry. Complete inhibition of IFN- α was observed in pDC depleted mice (Fig 14) with no significant reduction in neutrophil depleted mice. Additionally, IL-6 but not TNF- α gene expression was significantly reduced in pDC depleted mice. Conversely, TNF- α was significantly reduced in neutrophil but not pDC depleted mice (Figure 14).

PDC ARE ACTIVATED TO PRODUCE TYPE I IFN THROUGH NUCLEIC ACID RECOGNITION PATHWAY

Next we wanted to determine whether pDC activation was induced through TLR recognition of nucleic acids that are released from dead and dying cells in the injured tissue. First we evaluated Type I IFN signaling in tape stripped MyD88-

deficient mice. MyD88 is a downstream adapter molecule of both TLR 7 and TLR 9 receptor signaling (114). TLR 7 recognizes single strand RNA and TLR 9 is responsible for single and double stranded DNA recognition (114). MyD88-deficient mice were tape stripped and gene expression analysis was performed on injured skin at 24 hours after injury. Remarkably, both IFN- α and IFN- β gene expression were suppressed in MyD88-deficient mice signifying that pDC activation was Toll-Like Receptor dependent. To further elucidate the roll of nucleic acids in providing the stimulus for pDC activation in skin injury we injected Balb/c mice with IRS 954, a combined a TLR-7/9 inhibitory compound (115, 116). IRS 954 is a phosphorothioate oligodeoxynucleotide molecule whose sequence, 5'-TGC TCC TGG AGG GGT TGT-3' is capable of mediating both TLR 7 and TLR 9 suppression (115). TLR 7 suppression occurs through the 5'-TGC sequence whereas TLR 9 suppression derives from the adjacent TCC TGG AGG GGT TGT-3' sequence. IRS 954 or saline control was injected 24 and 6 hours prior to tape stripping. Consistent with the MyD88-deficient mice data, IRS 954 was capable of completely abrogating both IFN- α and IFN- β gene expression following skin injury. These data conclusively demonstrate that infiltrating skin pDC are activated through TLR engagement of nucleic acids.

Skin injury is marked by dying keratinocytes which release genomic nucleic acid into the extracellular space. To understand whether infiltrating skin pDC activation is RNA, DNA or both RNA/DNA mediated we evaluated Type I IFN gene expression in TLR7-deficient and TLR 9 inhibitor treated mice after tape

strip injury. The specific TLR 9 inhibitor IRS 869, 5'-TCC TGG AGG GGT TGT-3', was used (116). IRS 869 or saline control was injected 24 and 6 hours prior to tape stripping. IFN- α and IFN- β gene expression was significantly reduced in TLR7-deficient mice (Figure 16 A). Additionally, pretreatment of IRS 869 reduced both IFN- α and IFN- β gene expression following injury (Figure 16 B). These data demonstrate that pDC activation is mainly driven through TLR 7 engagement and that RNA is the predominant nucleic acid mediating such activation.

Furthermore, the pro-inflammatory cytokine, IL-6 gene expression was significantly repressed in MyD88-deficient and IRS 954 treated mice following injury (Figure 17 A and B) whereas TNF- α gene expression was unaffected by TLR 7/9 abrogation. Similar responses were observed in TLR7-deficient and IRS 869 treated mice (Figure 18 A and B). These data corroborated our observations made in pDC depleted mice (Figure 14) which showed strong IFN- α and IL-6 reduction.

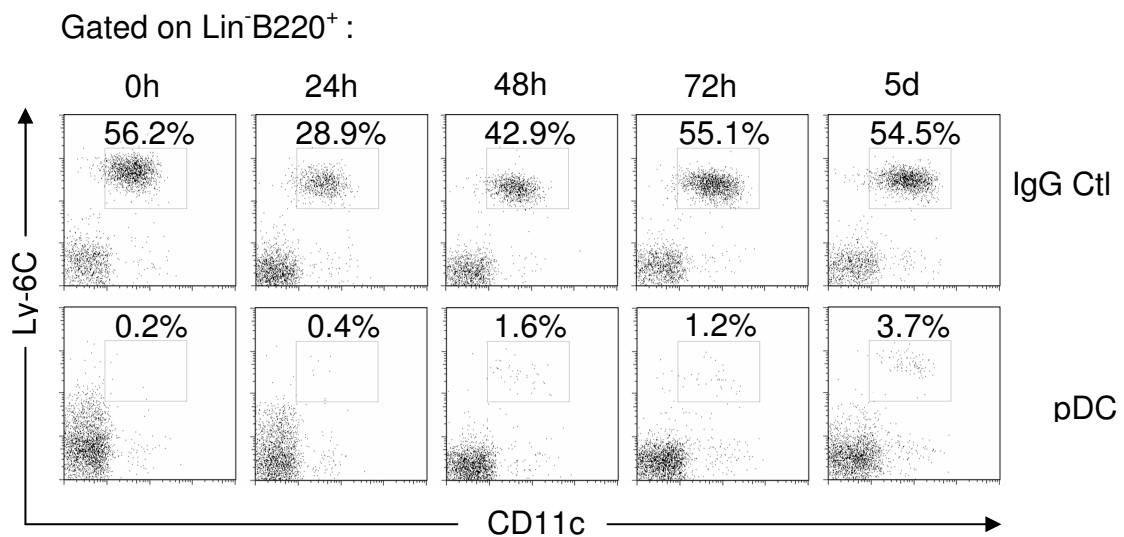


Figure 13. pDC-depletion effectively eliminates pDCs from circulation up to 5 days. Time course analysis of pDCs (lineage-B220-CD11c+Ly6C+) in spleens of either pDC-depleted or control IgG treated mice. Combined BST antibody cocktail injection efficiently depletes pDC from spleens. One representative experiment of 3 is shown.

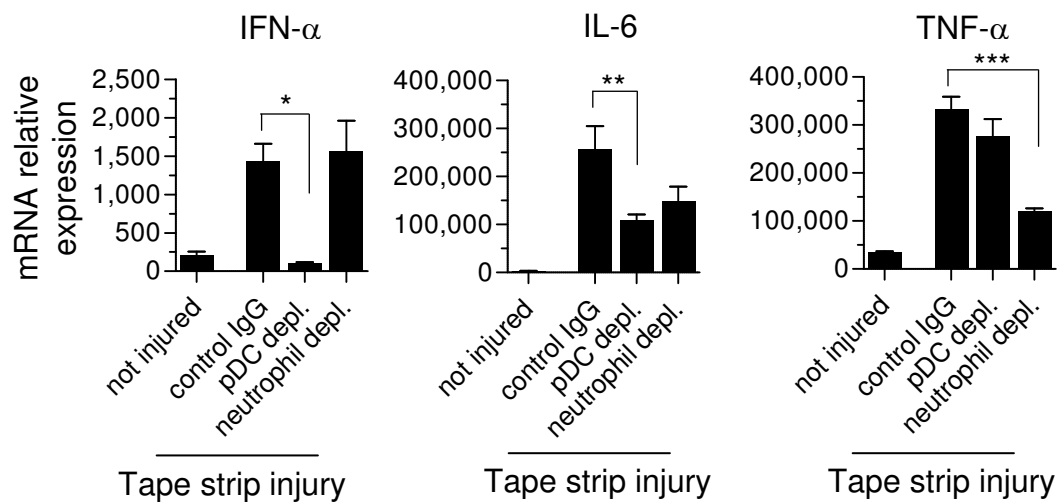


Figure 14. pDCs are transiently activated to produce type I IFNs in injured skin. Relative IFN- α , IL-6 and TNF- α mRNA tissue expression of uninjured skin or injured skin collected 24 hours after tape stripping of either pDC-depleted, neutrophil-depleted, or control IgG treated mice. Data represent the mean \pm SEM of five mice per group. *, $P = 0.001$; **, $P = 0.02$; ***, $P < 0.001$, unpaired Student's t test.

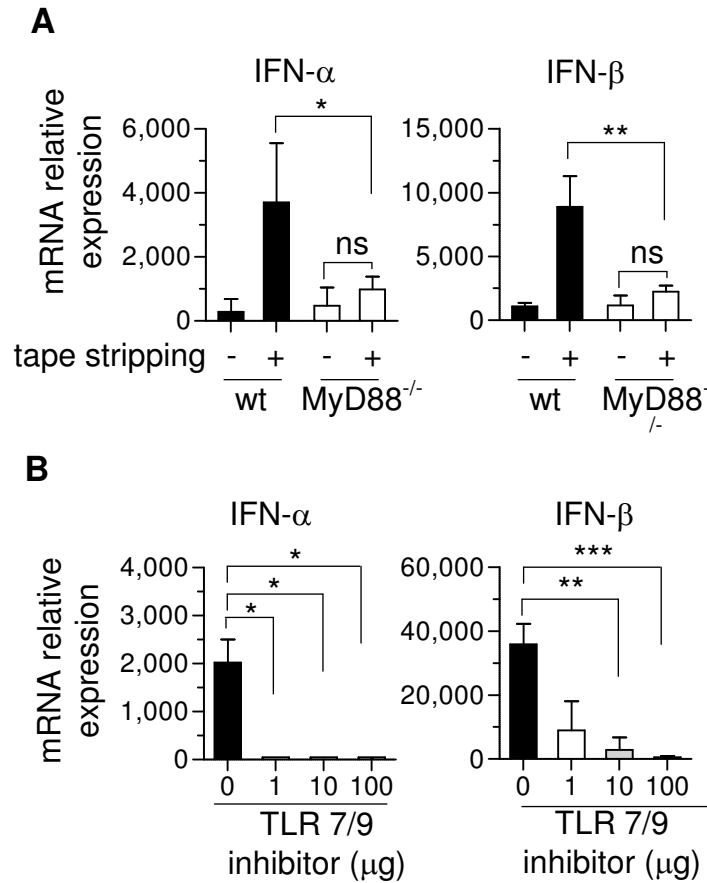


Figure 15. pDCs sense nucleic acid in injured skin. (A) Relative IFN- α and IFN- β mRNA tissue expression in injured skin collected 24 hours after tape stripping of MyD88^{-/-} or control mice. Data represent the mean \pm SEM of five mice per group. *, $P=0.01$; **, $P=0.02$, unpaired Student's t test. (B) Relative IFN- α and IFN- β mRNA tissue expression in injured skin (24h) of mice pretreated with 0, 1, 10, or 100 μ g of TLR 7 and TLR 9 inhibitor IRS 954. Data represent the mean \pm SEM of three mice per group. *, $P=0.002$; **, $P=0.02$; ***, $P=0.01$, unpaired Student's t test. Data in (A) and (B) are representative of at least two independent experiments.

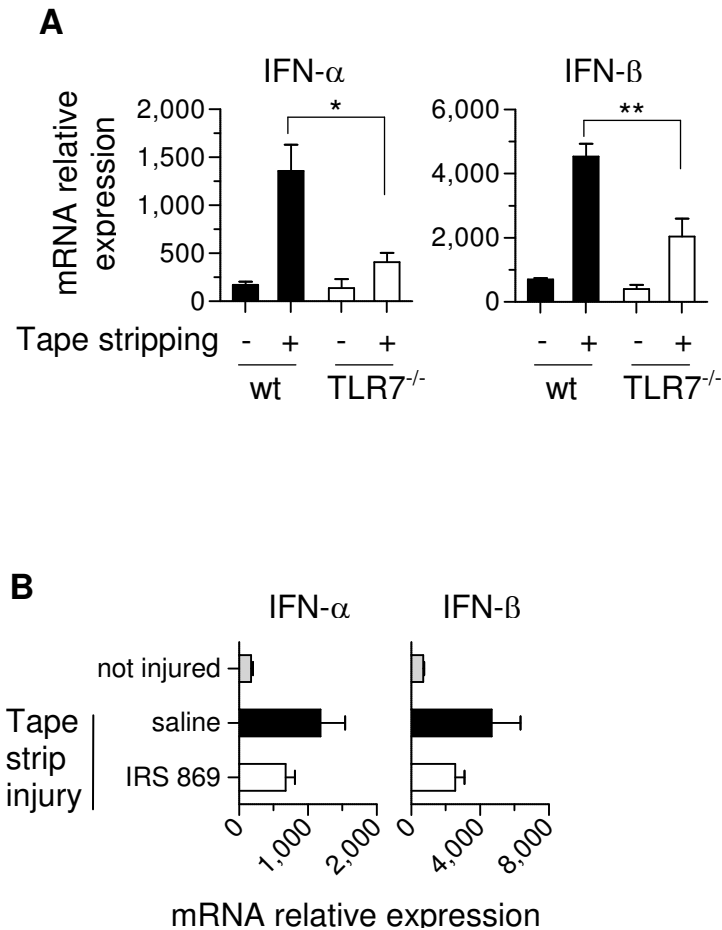


Figure 16. pDCs sense nucleic acid through TLR 7 and TLR 9 in injured skin. (A) Relative IFN- α and IFN- β mRNA tissue expression of uninjured skin or injured skin collected 24 hours after tape stripping of TLR7^{-/-} or control mice. Data represent the mean \pm SEM of four mice. *, $P = 0.01$; **, $P = 0.02$, unpaired Student's t test. (B) Relative IFN- α and IFN- β mRNA tissue expression in uninjured skin and injured skin (24h) of mice pretreated with saline or 100 μ g of TLR 9 inhibitor IRS 869. Data represent the mean \pm SEM of seven mice.

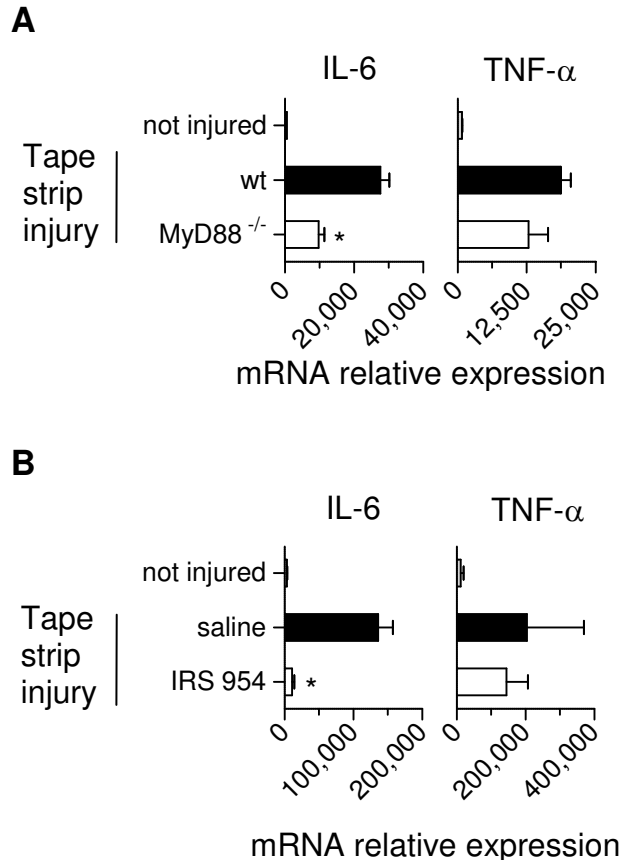


Figure 17. IL-6 and TNF- α cytokine mRNA expression in injured skin of MyD88-deficient and TLR 7/9 inhibitor treated mice. (A) Relative IL-6 and TNF- α mRNA tissue expression of uninjured skin or injured skin collected 24 hours after tape stripping of MyD88^{-/-} or control wild-type mice. Data represent the mean \pm SEM of five mice per group: *, $P < 0.001$, unpaired Student's t test. (B) Relative IL-6 and TNF- α mRNA tissue expression in uninjured skin and injured skin (24h) of mice pretreated with saline or 1 μ g of TLR 7/9 inhibitor (IRS 954). Data represent the mean \pm SEM of three mice. *, $P < 0.004$, unpaired Student's t test. Data in (A) and (B) are representative of at least two independent experiments.

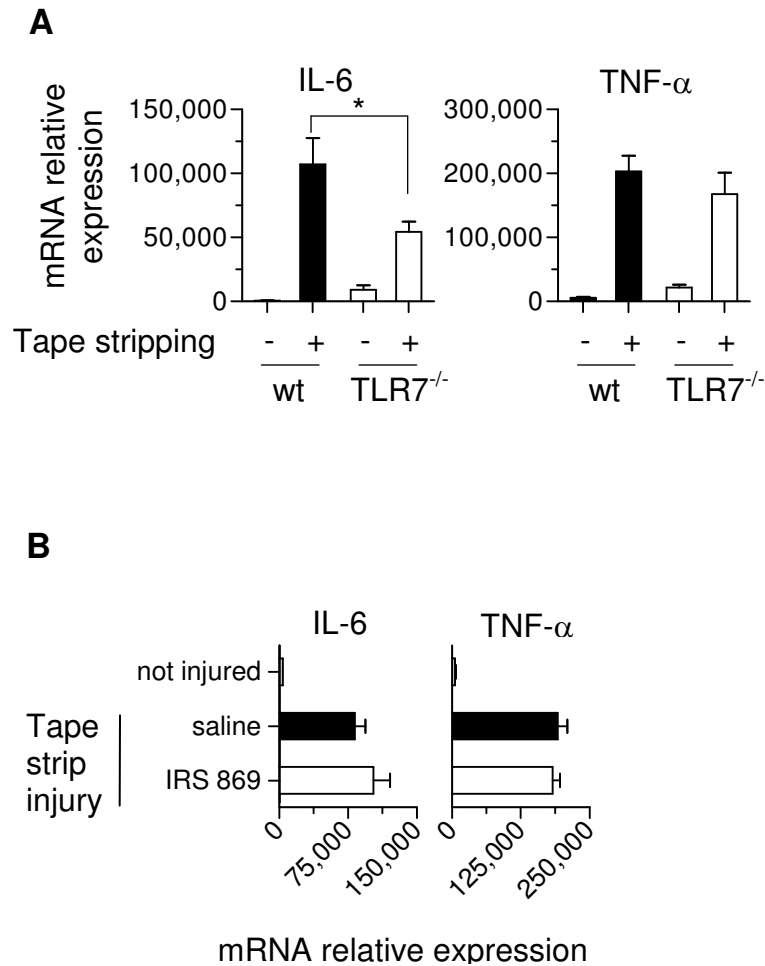


Figure 18. Cytokine mRNA expression in injured skin of TLR 7-deficient and TLR 9 inhibitor treated mice. (A) Relative IL-6, and TNF- α mRNA tissue expression of uninjured skin or injured skin collected 24 hours after tape stripping of TLR7^{-/-} or control mice. Data represent the mean \pm SEM of four mice. *, P=0.05, unpaired Student's t test. (B) Relative IL-6, and TNF- α mRNA tissue expression in uninjured skin and injured skin (24h) of mice pretreated with saline or 100 μ g of TLR 9 inhibitor IRS 869. Data represent the mean \pm SEM of seven mice.

CHAPTER 5

CATHELICIDINS ACTIVATE PDCS BOTH *IN* *VITRO* AND *IN VIVO*

CATHELICIDINS ARE EXPRESSED UPON SKIN INJURY

Host derived-nucleic acids released by damaged cells are normally inert but can be converted into triggers of TLR7 and TLR9 in the presence of the human cathelicidin peptide LL-37 (30, 29). We therefore sought to determine whether the murine cathelicidin ortholog of LL-37, called CRAMP, is induced in our skin injury model. The expression of CRAMP mRNA in mouse skin was not detectable prior to injury, but was rapidly induced upon tape stripping, reaching a peak at 24 hour and declining thereafter (Figure 19 A). This time course closely paralleled the infiltration of pDCs into injured skin and their activation to produce IFN- α/β , suggesting a potential role of cathelicidins in breaking innate tolerance to self-nucleic acids injured skin.

MURINE CATHELICIDIN BIND NUCLEIC ACIDS

Next, we wanted to evaluate whether CRAMP could bind dsDNA. In our previous work (30, 29) we demonstrated that LL-37 efficiently binds dsDNA and RNA and can activate human pDC to produce robust levels of IFN- α . We developed a technique to measure the efficiency of peptide binding to nucleic acid utilizing an ultra-sensitive fluorescent DNA binding probe. PicoGreen fluorescent dye, which predominantly binds to dsDNA, is excited at 480 nm and has primary emission spectra at 520 nm. The principle of the assay is that DNA bound to peptides are inaccessible to PicoGreen binding. Hence, strong

peptide-DNA interactions lead to lower fluorescence whereas weak peptide-DNA interactions do not decrease the high DNA-PicoGreen fluorescence. CRAMP at various concentrations: 0.01 μ M, 1 μ M, 50 μ M and 100 μ M were mixed with 3 μ g/ml dsDNA for 30 min and analyzed for complex formation. PicoGreen was added to each mixture and PicoGreen fluorescence emission was measured. CRAMP was able to bind DNA at 1 μ M with increased binding to 100 μ M of CRAMP (Figure 19 B).

CRAMP-DNA COMPLEXES ACTIVATE PDCS TO PRODUCE IFN- α

Finally, to investigate whether CRAMP-DNA complexes could activate murine pDCs, we isolated fresh splenic pDCs using a negative magnetic beads approach (pDC Isolation Kit II, Miltenyi). 50,000 pDCs (>90% purity) were stimulated overnight with either DNA alone, CRAMP alone, or DNA mixed with CRAMP. As a control we also used a scrambled form of CRAMP alone or mixed with DNA. We found that only DNA mixed with CRAMP induced IFN- α production in pDCs (Figure 19 C).

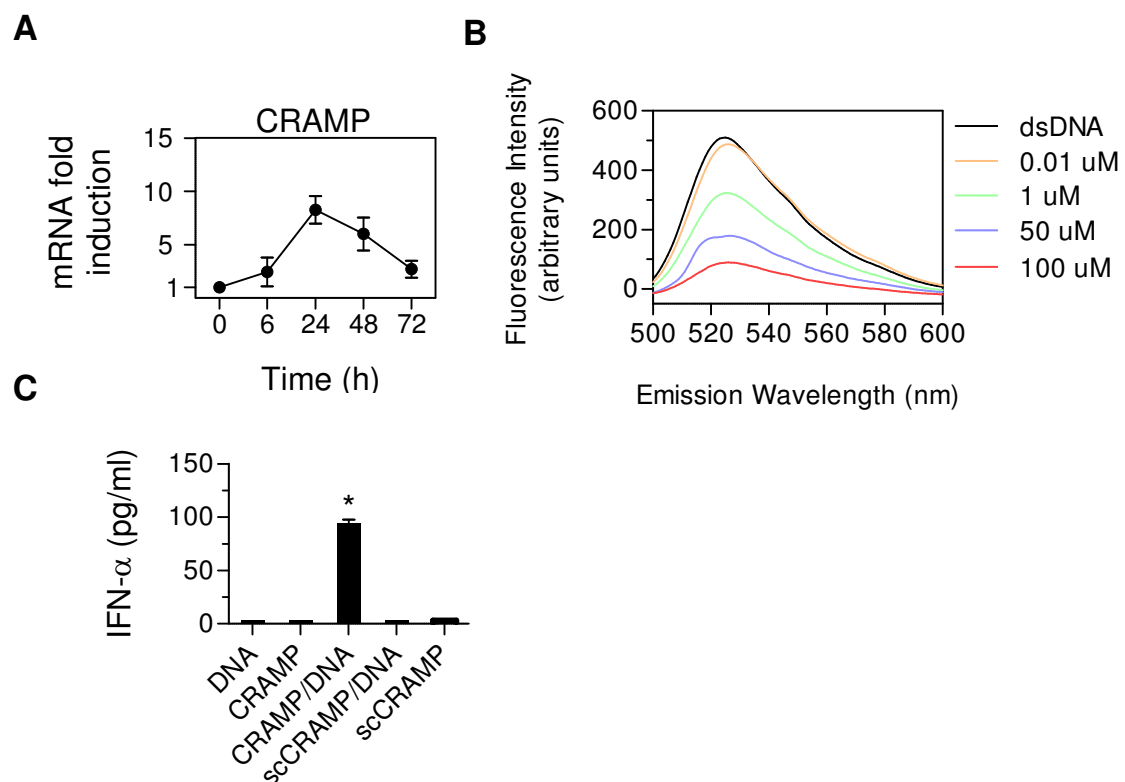


Figure 19. Cathelicidin gene expression is upregulated in injury and Cathelicidin peptide induces pDC activation to produce IFN- α . (A) Time course analysis of cathelicidin mRNA tissue expression in injured skin. The data are given as fold induction over time 0 and represent the mean \pm SEM of four mice per timepoint. (B) Cathelicidin-DNA binding studies using 3 μ g/ml dsDNA and 0.01, 1, 50 or 100 μ M cathelicidin peptide using PicoGreen DNA exclusion. 480 nm excitation wavelength. (C) IFN- α produced by purified splenic pDC after overnight stimulation with either DNA alone, CRAMP alone, scrambled (sc) CRAMP alone, CRAMP plus DNA, or scCRAMP plus DNA. Data are representative of two independent experiments, error bars represent the SEM of triplicate wells. *, $P=0.001$.

IN VIVO INJECTED CRAMP PROMOTES PDC INFILTRATION AND ACTIVATION TO PRODUCE TYPE I IFNs

To test whether CRAMP would also break innate tolerance to nucleic acids and activate pDCs *in vivo*, we injected CRAMP, scrambled CRAMP or saline into mouse skin. We found that CRAMP but not the scrambled peptide or saline injection induced a rapid and transient infiltration of pDCs and the expression of IFN- α/β (Figure 20 A and B). These findings indicate that CRAMP is sufficient to break innate tolerance to induce activation of pDCs to produce IFN- α/β in skin *in vivo*.

CRAMP IS SUFFICIENT BUT NOT REQUIRED FOR PDC INFILTRATION IN SKIN INJURY

To determine whether CRAMP is necessary for pDC activation to injured skin, we tape-stripped the skin of cathelicidin-deficient mice (112) and analyzed gene expression for IFN- α/β . Surprisingly, we found that IFN- α and IFN- β produced by pDCs were still induced in injured skin even in the absence of cathelicidins (Figure 21). Thus, cathelicidins are sufficient but not necessary to break innate tolerance to nucleic acids and induce IFN- α/β production by pDCs in injured skin.

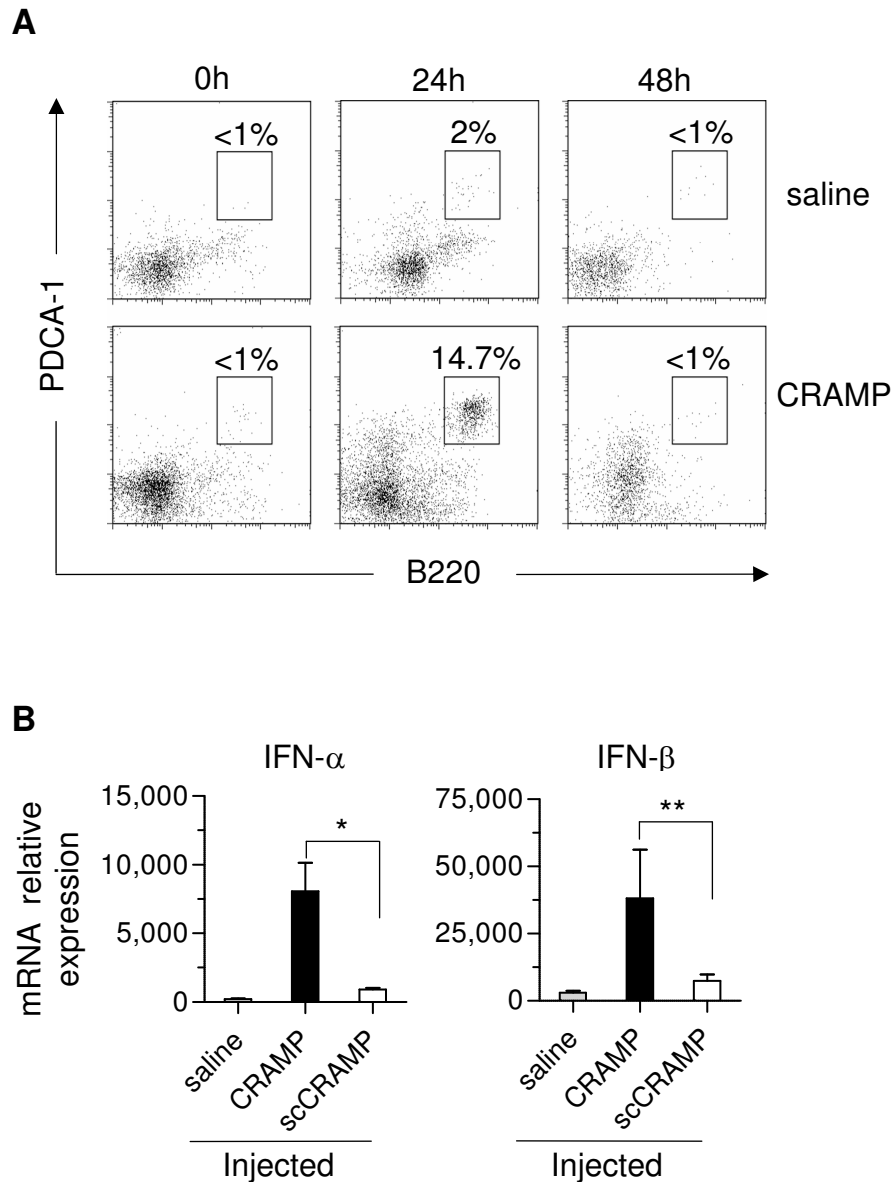


Figure 20. Injection of Cathelicidin peptide induces pDC infiltration and activation in skin. (A) Flow cytometry time course analysis of pDCs (B220+PDCA-1+) in dermal single cell suspensions derived from skin injected with either saline or CRAMP. The percentage of each population is shown in the plots. Data are representative of three mice. (B) Relative IFN- α and IFN- β mRNA tissue expression in the skin injected with either saline, CRAMP, or scCRAMP and collected after 24 hours. Data represent the mean \pm SEM of five mice per group. *, $P = 0.02$; **, $P = 0.1$, unpaired Student's t test.

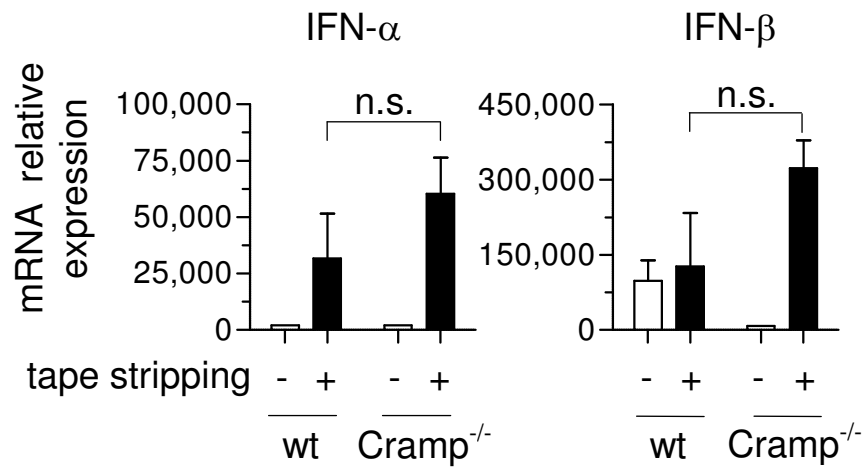


Figure 21. Cathelicidin peptides are not necessary to induce type I IFNs in injured skin. Relative IFN- α and IFN- β mRNA tissue expression in uninjured skin or injured skin collected after tape stripping of Cramp^{-/-} or control wild-type mice. Data represent the mean \pm SEM of five mice per group. n.s., not significant, unpaired Student's t test.

ADDITIONAL MOLECULES MAY MEDIATE PDC INFILTRATION TO INJURED SKIN

It is possible that there is a redundant signaling pathway which can mediate pDC infiltration and activation in the skin. Other antimicrobial peptides such as the beta-defensins have been shown in human psoriasis to be overexpressed in involved tissue (105,107). To investigate whether beta-defensins may play a role in skin injury we performed gene expression analysis for mouse beta-defensins -3, -4, and -14 which correlate to human beta-defensins -2, -3, and -4. As a control, we analyzed mouse beta-defensins -1, and -2 which have been shown to be expressed under homeostasis and are downregulated upon injury (Figure 22). Interestingly, we found that beta-defensin-3, -4, and -14 were all upregulated upon injury which indicate that pDC may be activated through nucleic acid-Beta-defensin complexes.

Alternatively, chemerin has recently been shown to play a pivotal role in pDC recruitment to the skin especially in psoriasis (117). We analyzed chemerin gene expression in injury skin and found a drastic increase in chemerin gene expression (Figure 22).

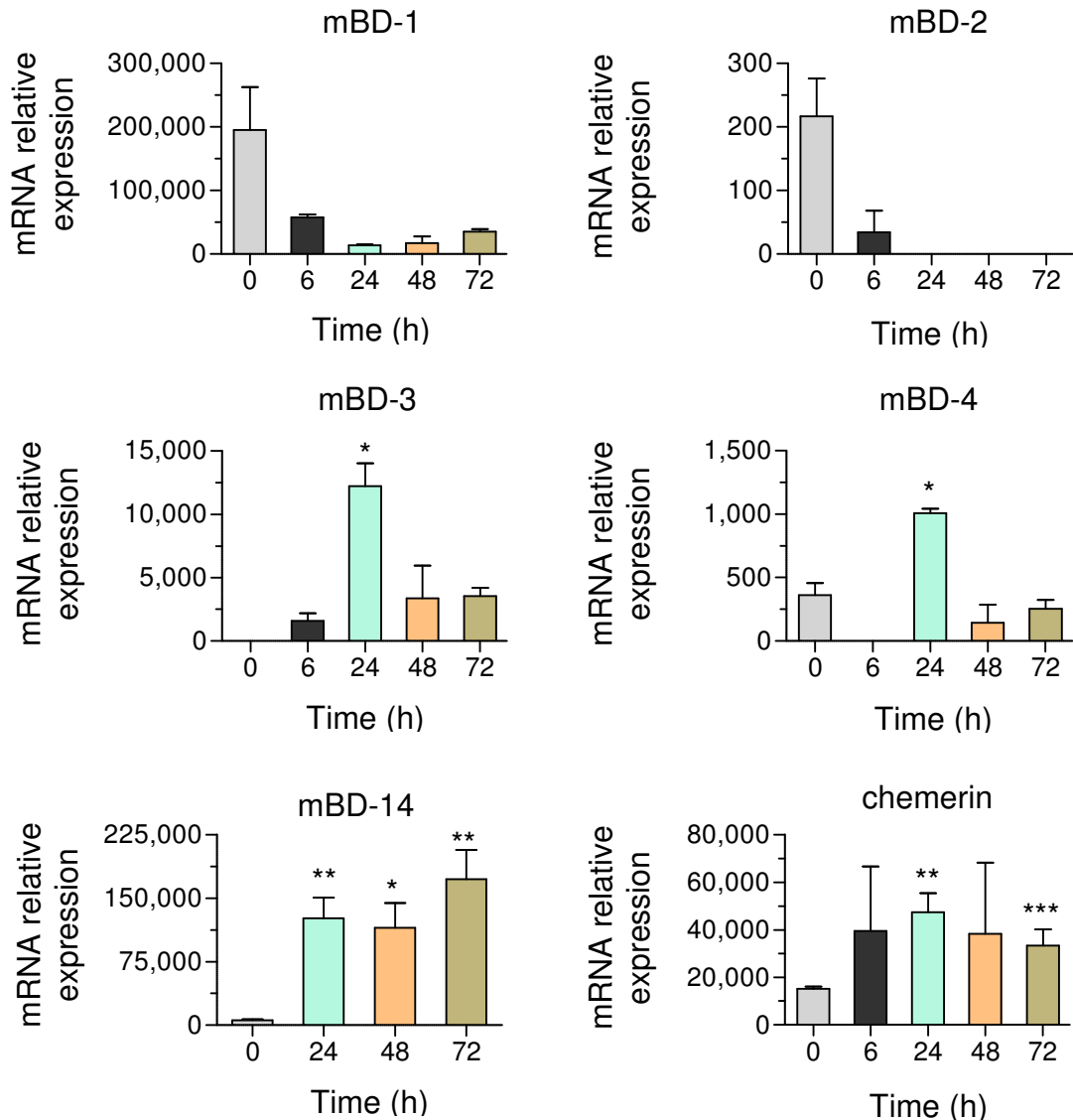


Figure 22. Select mouse beta-defensin (mBD) antimicrobial peptides as well as Chemerin are expressed during skin injury. mBD-3 and mBD-4, are transiently expressed during skin injury whereas there was sustained mBD-14 expression. Additionally, chemerin was highly induced early in skin injury with sustained expression. Data represent the mean \pm SEM of at least two mice per group. *, $P = 0.02$; **, $P = 0.007$; ***, $P = 0.005$ unpaired Student's t test.

CHAPTER 6

PDCS AND TYPE I IFNS PROMOTE INFLAMMATORY RESPONSES AND WOUND HEALING IN INJURED SKIN

PDCS DRIVE EXPRESSION OF DC, TH1 AND TH17 CYTOKINES

Because pDCs are potent stimulators of immune responses through their production of IFN- $\alpha\beta$, we next sought to investigate the role of pDCs in the induction of inflammatory responses in injured skin. We found that, along with IL-6 and TNF- α (Figure 14), skin injury induced a rapid expression of DC-derived cytokines IL-12 and IL-23, TH1 cytokine IFN- γ , and TH17 cytokines IL-17 and IL-22 (Figure 23 A), but not IL-4 or IL-10 (not shown), reaching a peak between 24 and 48 hours after injury. pDC depletion, which reduced the expression of IL-6 in injured skin (Figure 14), was also found to decrease the expression of IL-12 and IL-23 (Figure 23 A). Intriguingly, pDC depletion was found to profoundly inhibit the induction of IL-17 and IL-22 without affecting the expression of IFN- γ (Figure 23). These findings demonstrate that skin-infiltrating pDCs play an important role in the induction of inflammatory immune responses in injured skin, in particular the induction of IL-6 and TH17 cytokines.

PDCS ARE REQUIRED FOR EFFICIENT RE-EPITHELIALIZATION

Since the inflammatory process is directly linked to the wound healing response, we next sought to determine whether pDCs also play a role in the re-epithelialization of tape stripped skin. Keratin 6 (K6), expressed by early differentiating and proliferating keratinocytes but not by fully differentiated

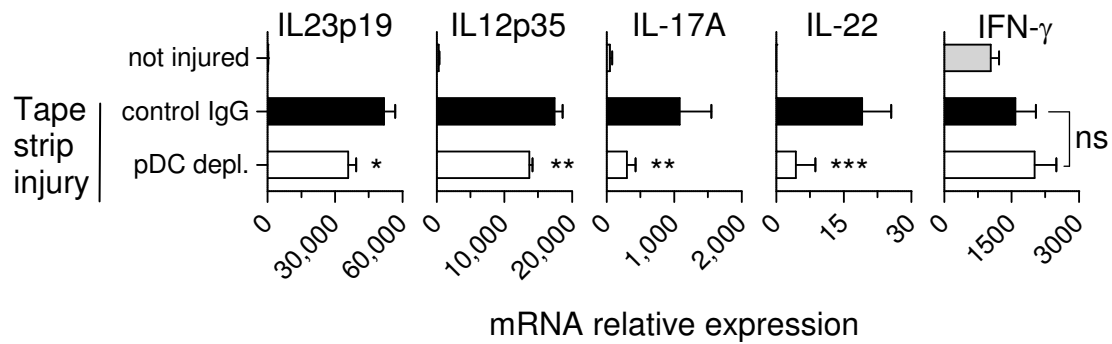


Figure 23. pDCs participate in the inflammatory response of skin wound healing. Relative IL-23p19, IL-12p35, IL-17A, IL-22, and IFN- γ mRNA tissue expression of uninjured skin or injured skin collected 24 hours after tape stripping of either pDC-depleted or control IgG treated mice. Data represent the mean \pm SEM of five mice per group. *, $P = 0.03$; **, $P = 0.05$; ***, $P = 0.04$, unpaired Student's t test.

keratinocytes, was used as a marker to quantify re-epithelialization of skin wounds (118). In normal mice, K6-positive keratinocytes appeared 24 hours after injury and repopulated the entire injured skin surface within 72 hours (Figure 24 A). In pDC depleted mice, K6-positive keratinocytes appeared only around 72 hours (Figure 24 B) and complete re-epithelialization lagged behind control mice by three days (not shown). These data indicate that the infiltration of pDCs in skin wounds plays a key role in promoting early wound re-epithelialization. To confirm these data we adopted another skin injury model, in which a full-thickness skin defect is placed by a 3 mm punch biopsy and the wound closure is measured over time. Similarly, pDC-depleted mice showed a significant delay in wound closure when compared to control mice, (Figure 24 C).

TYPE I IFN PROMOTE EFFICIENT RE-EPITHELIALIZATION AND INDUCE DC AND TH17 CYTOKINES

To determine the role of pDC-derived IFN- $\alpha\beta$ in wound healings, we used IFN- $\alpha\beta$ -receptor-deficient mice and repeated similar experiments performed with pDC-depleted mice. Like pDC-depleted mice, IFN- $\alpha\beta$ -receptor-deficient mice showed a significant delay in wound re-epithelialization (Figure 25 A) and displayed a profound deficiency in IL-6, IL-17 and IL-22 expression levels in injured skin (Figure 25 B). A similar deficiency of IL-6, IL-17 and IL-22 expression was observed in MyD88-deficient mice. These data suggest that the

ability of pDCs to trigger early inflammatory responses and promote wound repair is related to their nucleic acid mediated TLR activation and production of IFN- $\alpha\beta$.

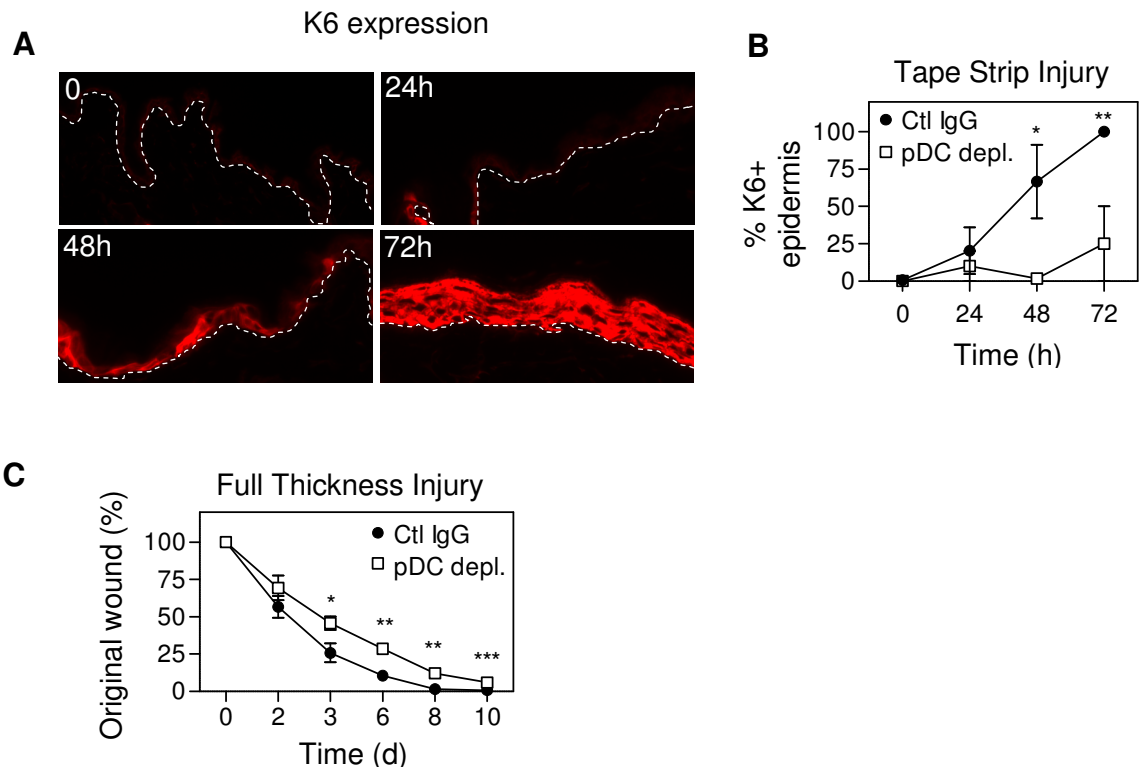


Figure 24. pDCs participate in the re-epithelialization of skin wound healing. (A) Representative time course of Keratin 6 (K6) expression in injured skin measured by immunofluorescence. (B) Percentage of K6 expression in injured skin in pDC-depleted and control IgG treated mice. Data represent the mean \pm SEM of five mice per group. *, $P = 0.01$; **, $P = 0.05$, unpaired Student's t test. (C) Time course of wound closure after full-thickness injury of the skin pDC depleted or IgG treated mice (right panel). Data represent the mean \pm SEM of at least three mice per group. *, $P = 0.02$; **, $P < 0.001$; ***, $P = 0.005$, unpaired Student's t test. Data in (A)-(C) are representative of at least two independent experiments.

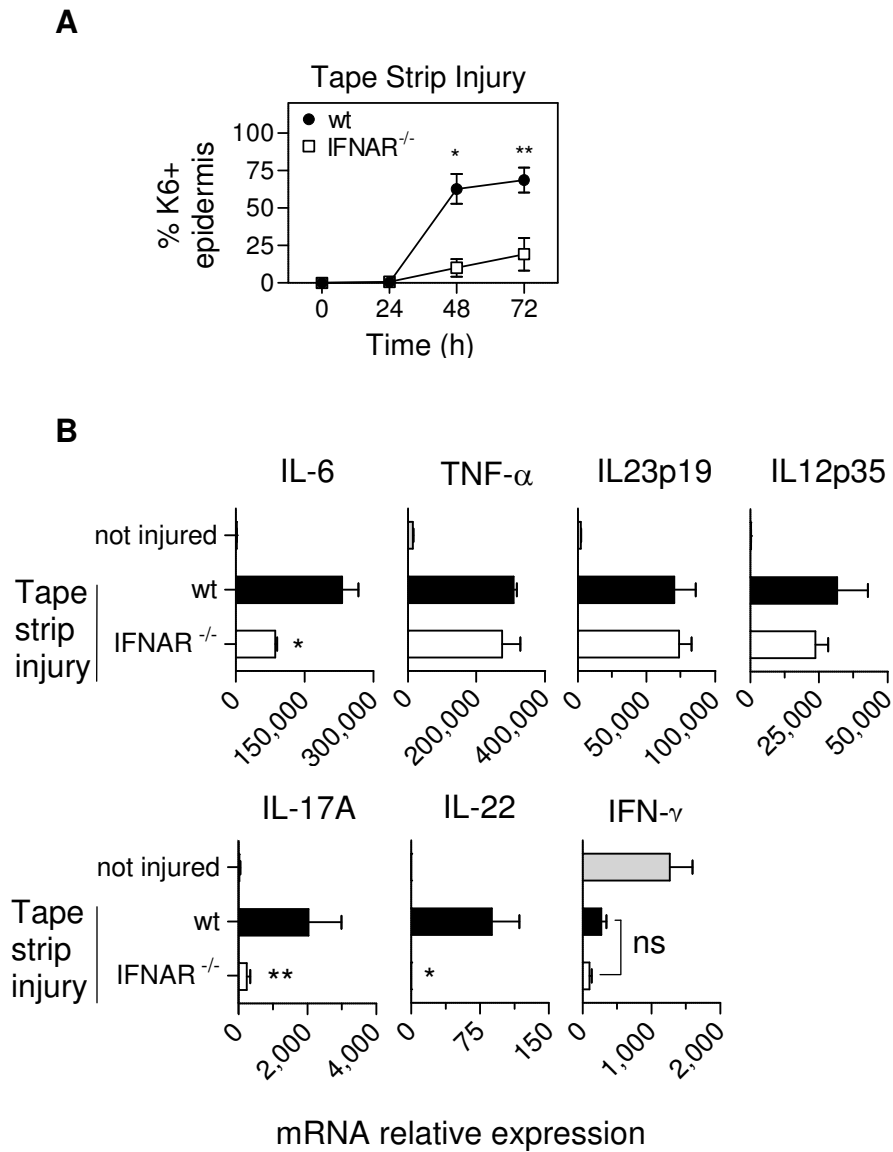


Figure 25. Type I IFNs participate in the re-epithelialization of skin wound healing.

(A) Percentage of K6 expression in injured skin in IFNAR^{-/-} or control mice. Data represent the mean \pm SEM of three mice per group for each timepoint. *, $P = 0.002$; **, $P < 0.001$, unpaired Student's t test. (B) Relative TNF- α , IL-6, IL-23p19, IL-12p35, IL-17A, IL-22, and IFN- γ mRNA tissue expression of uninjured skin or injured skin collected 24 hours after tape stripping of IFNAR^{-/-} or control mice. Data represent the mean \pm SEM of five mice per group: *, $P = 0.003$; **, $P = 0.04$, unpaired Student's t test. Data in (A)-(B) are representative of at least two independent experiments.

PDCS DO NOT AFFECT ANGIOGENESIS

To evaluate whether pDCs also participated in other processes of wound healing in addition to re-epithelialization we measured angiogenesis related markers. We evaluated endothelial cell activity using CD31 as a marker for neovasculature. CD31 also known as PECAM-1 is highly expressed by endothelial cells and is also weakly expressed by peripherhal lymphoid cells. Importantly, CD31 is down regulated by neutrophils upon extravasation from circulation. pDC-depleted mice did not show reduced CD31 expression compared to control mice in tape stripped mice (Figure 26). Additionally, we performed gene expression for *Vegfa*, *Vegfb*, and *Egf*, key genes involved in angiogenesis in pDC-depleted mice to determine whether pDCs may exert an effect upstream from endothelial cell proliferation. However, we did not observe any significant differences between pDC-depleted and control mice in expression of these genes (Figure 27).

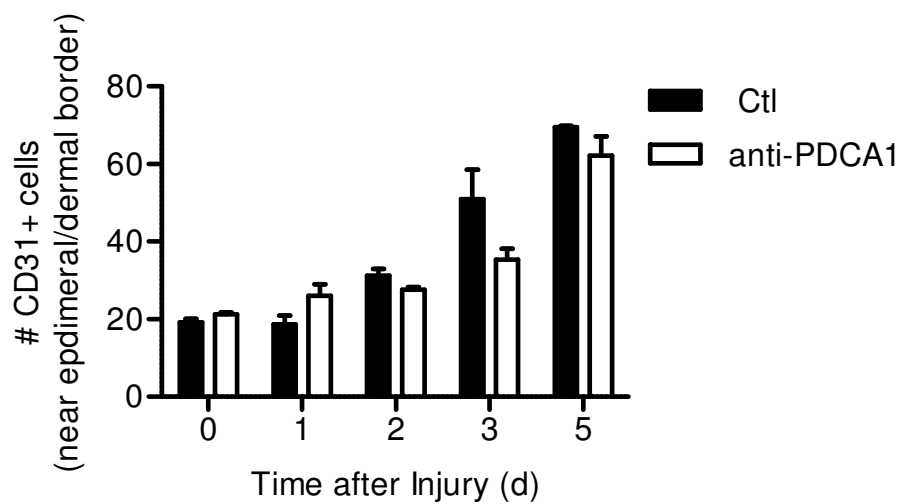


Figure 26. pDCs do not affect endothelial cell activity. Percentage of CD31 expression in the skin (epidermal/dermal border area) of injured control or pDC-depleted. Data represent the mean \pm SEM of five mice per group.

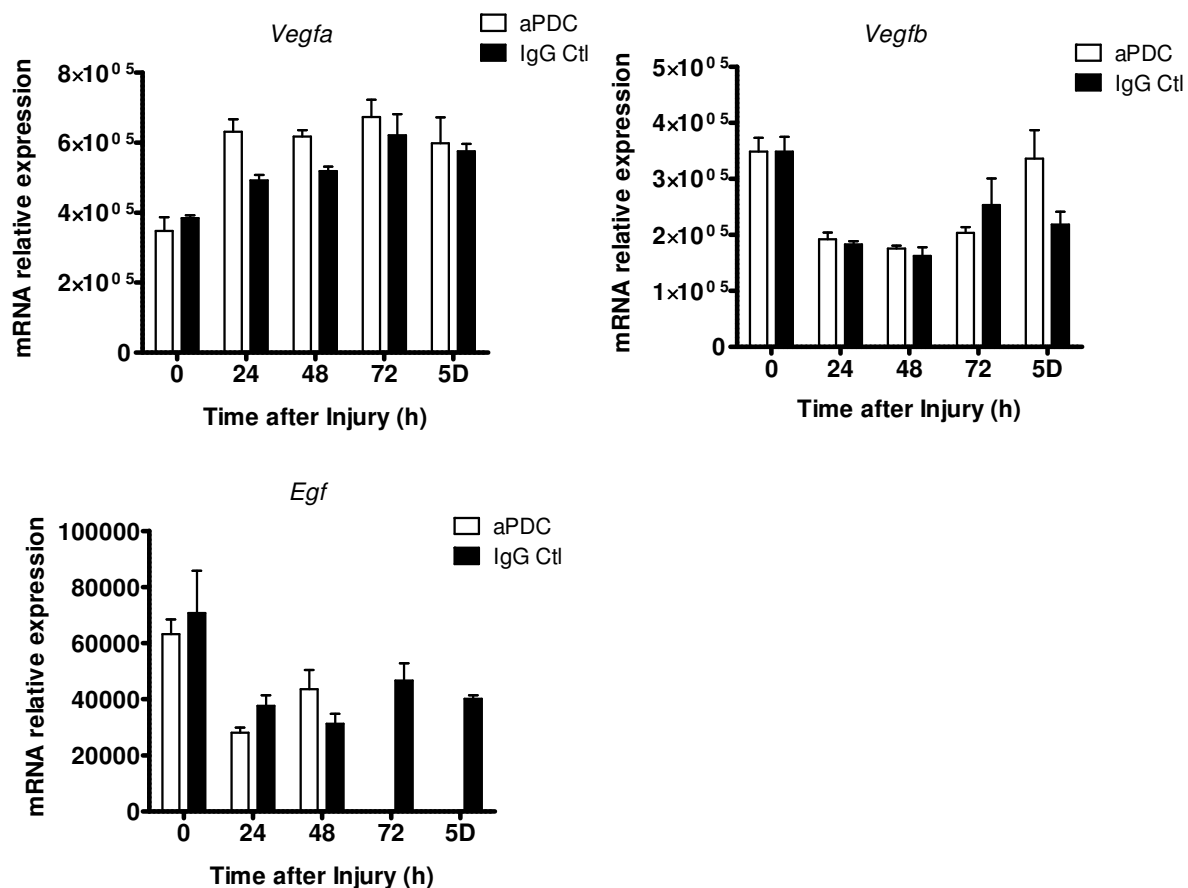


Figure 27. pDCs do not promote upregulation of angiogenesis genes. mRNA relative tissue expression of *Vegfa*, *Vegfb* and *Egf* in injured skin of pDC-depleted and control mice. Data represent the mean \pm SEM of three mice per group.

CHAPTER 7

HUMAN SKIN INJURY INDUCES PDC INFILTRATION AND EXPRESSION OF TYPE I IFNS

HUMAN SKIN INJURY INDUCES PDC INFILTRATION AND EXPRESSION OF TYPE I IFNS

To determine whether pDCs also infiltrate and sense human skin wounds, we performed skin biopsies in healthy human volunteers prior and 24 hours after tape stripping. Immunohistochemistry for BDCA2, a specific marker for human pDCs, revealed that large numbers of pDCs infiltrate human skin 24h after mechanical injury (Figure 28). We also found a significant induction of IFN- α mRNA expression in injured skin (Figure 29 A), suggesting that pDCs are activated to produce IFN- α similar to our finding in the murine models. We also confirmed these data in another model of skin injury induced by Sodium Lauryl Sulfate (SLS) treatment, which induces a chemical disruption of the epidermal barrier by perturbing the lipid bilayer structure in the stratum corneum, leading to damages to the basal keratinocytes (119). Like mechanical-induced skin injury, this chemical induced skin injury induced rapid pDC infiltration and significant induction of IFN- α mRNA expression in injured skin (Figure 28, Figure 29 B).

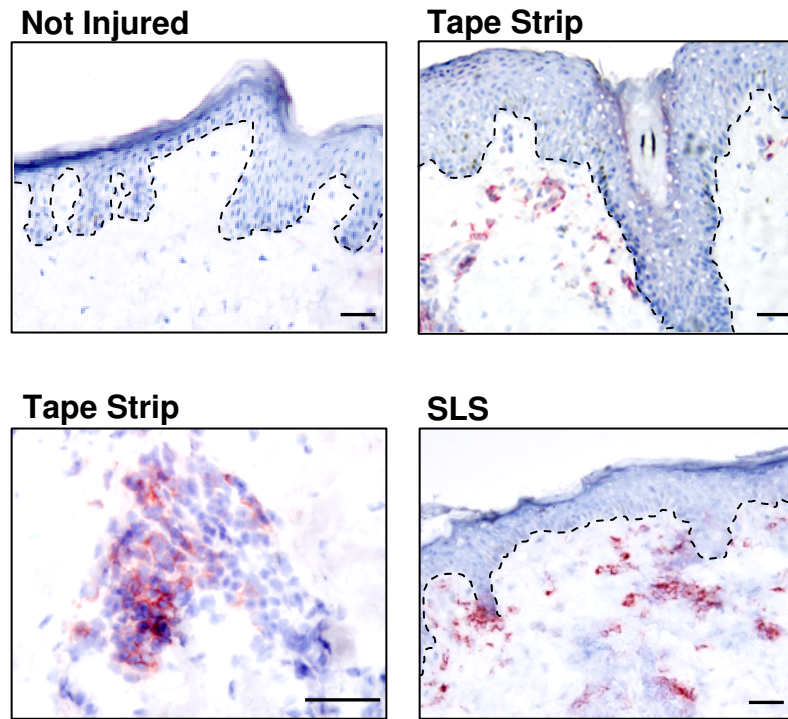


Figure 28. Human skin injury induces rapid infiltration of pDCs. Representative immunohistochemical staining of human skin for the pDC-specific marker BDCA2 reveals absence of pDCs in normal skin prior to injury (upper left panel), the rapid infiltration of pDCs in injured skin by tape stripping (upper right and lower left panels) or by treatment with SLS (lower right panel), scale bar: 10 μ M. The data are representative of at least five independent healthy individuals.

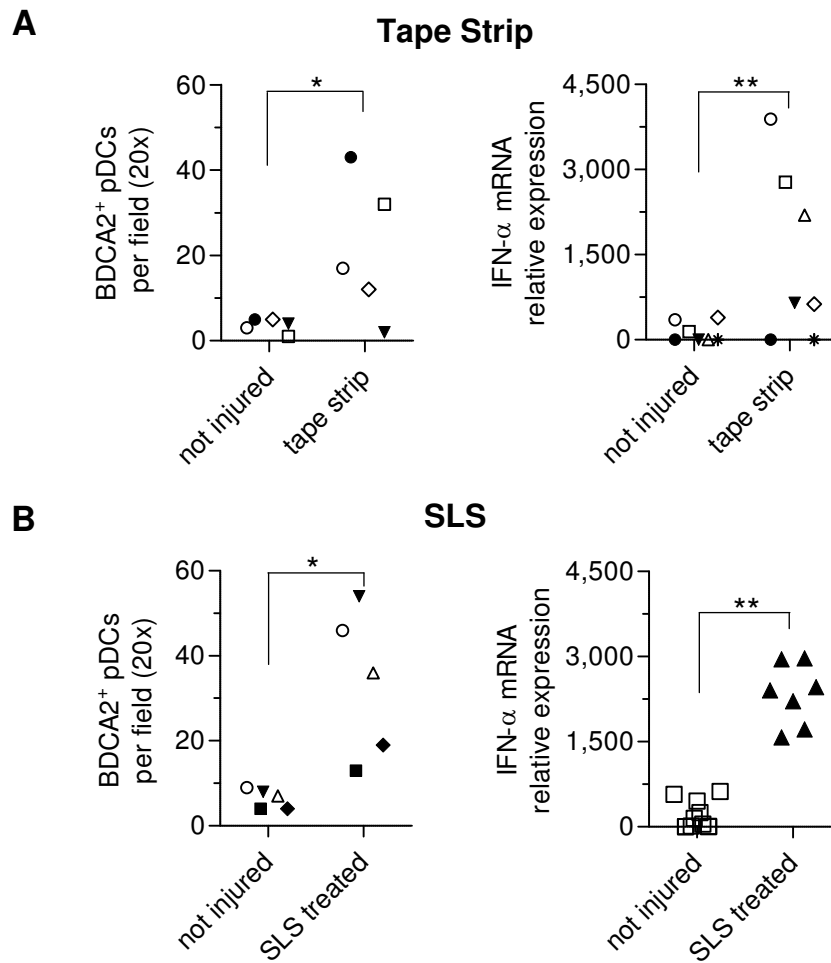


Figure 29. Human skin injury induces rapid infiltration of pDCs and their activation to produce IFN- α . (A) Quantification of BDCA2-positive pDC numbers in human skin before and 24 hours after tape stripping of five independent healthy individuals (left panel). Relative IFN- α mRNA tissue expression in healthy human skin before and 24 hours after tape stripping (right panel). Each symbol represents an independent donor. *, $P = 0.05$; **, $P = 0.03$, unpaired Student's t test. (B) Quantification of BDCA2-positive pDC numbers in human skin before and 24 hours after chemical injury with SLS of five independent healthy individuals (left panel). Relative IFN- α mRNA tissue expression in human skin before and 24 hours after chemical injury with SLS of at least 7 independent healthy individuals (right panel). Each symbol represents the independent individual. *, $P = 0.04$; **, $P < 0.001$, unpaired Student's t test.

CHAPTER 8
DISCUSSION

Injury is the most common assault that an organism must confront. It is vital to the existence of the host to limit exposure of compromised tissue in order to avoid infection as well as to limit inflammatory mediated tissue destruction. Mammalian systems have evolved a very highly ordered repair process to heal wounds. The process is very intricate and the efficiency is dependent upon many factors such as the health status of the host, nature and scope of the injury, and most importantly, the degree of pathogen inoculation. Many aspects of skin injury have been characterized over the past few decades, yet many processes are not yet understood. We know from the past decade of skin research that keratinocytes play a much more dynamic role in maintaining homeostasis than just by providing a barrier to the external environment.

Keratinocytes play a vital immunological role in maintaining barrier protection as well as sensing injury and alerting the body of problems. In fact, it has recently been shown that keratinocytes can “sense” bacteria through TLR expression which can then elicit an antimicrobial response (120). Keratinocytes are armed with evolutionary conserved defense peptides which can kill bacteria directly through perturbing the cell wall resulting in lysis. Moreover, the cathelicidin antimicrobial peptide can act as in a chemotactic fashion and has been shown to recruit neutrophils, T cells, and macrophages to the skin (121-123). Furthermore, injured keratinocytes release growth factors and pro-inflammatory cytokines such as IL-1 β , IL-8, IL-6, and TNF- α (105-107). Recently our group has uncovered a novel role for keratinocyte-derived cathelicidin in activating

pDCs through association of cathelicidin with self-nucleic acid (29, 30). These studies suggest that keratinocytes may work in concert with other cell types in both initiating an immune response as well as eliciting an effective barrier against pathogen infection.

pDCs provide a unique role in immunity. On one hand they are capable of promoting a strong inflammatory response through generation of vast amounts of Interferon alpha in response to viral pathogens. On the other hand, they can promote tolerance through induction of ICOSL and interaction with ICOS+ expressing T cells (124). Typically pDCs lack efficient mechanisms to uptake and bear MHC Class II antigens but they can cross-present antigens via MHC Class I. Additionally, pDCs originate from the lymphoid lineage unlike other dendritic cells which are myeloid derived. Many functions of pDC have been well characterized such as robust viral sensors, peripheral tolerance, MHC cross-presentation and most notably, producers of high Type I Interferon levels.

pDCs lack the ability to recognize specific molecules but instead are armed to recognize evolutionarily conserved patterns such as prokaryotic DNA, viral proteins, etc. This ability enables pDCs to be one of the early sensors of viral infection. However, the exact nature of pDC activity in non-infectious roles has scarcely been evaluated. Here, we demonstrate a novel but logical role for pDC in detecting skin injury. This study was driven by the observation that in psoriasis, pDCs are present in the early lesion. Studies by Gilliet and others

have shown that pDCs are in fact early infiltrators and initiators of the psoriatic lesion. Remarkably, pDCs promote inflammation as well as mediating the recruitment and activation of mDC and T cells. However, the trigger of pDC infiltration into the skin as well as the stimulus for pDC activation has not been elucidated. Coupled with the recent observations that pDCs can become activated to produce large amounts of IFN- α in response to self-DNA associated with the self-skin peptide, LL-37, along with the findings that LL-37 is released by keratinocytes in psoriasis we desired to understand the physiological role of pDCs in normal wound healing.

There is scant evidence for a physiological role for pDCs in injury response. Moreover, there have been conflicting reports on the effect of Type I IFN in promoting more efficient wound repair. To this end, we developed an murine injury model based on the study by Sano which utilized tape stripping the dorsal flank of mice with constitutive expression of Stat3 (125). In that study, the authors observed increased dermal inflammation in as little as 12 hours. In our experiments, taped stripped mice exhibited a strong inflammatory infiltrate characterized by abundant and transient levels of pDCs and neutrophils. Interestingly, both pDCs and neutrophils were absent by 48 hours. It is well known that neutrophils infiltrate injury (tape stripping, full-thickness, and burn) early with maximal infiltration lasting up to 3 days. pDCs are absent in skin under homeostatic conditions in mice whereas they constitute a very minor population in human.

Evidence of pDCs playing a role in a physiological setting prompted us to examine their functional role in wound healing. First, we observed that Type I IFN gene expression was upregulated transiently after injury. Furthermore, IFN- α gene expression studies in wild type, pDC-depleted, MyD88-deficient, and TLR7-deficient demonstrated that pDCs were the primary source of IFN- α . Pro-inflammatory genes were evaluated to understand the exact contributions of pDC in the injured skin. Interestingly, IL-6 was significantly pDC dependent while TNF- α was unaffected by pDC depletion. On the other hand, neutrophils contributed the bulk of TNF- α while exerting modest effects on IL-6 production. Previous studies have shown that IL-6-deficient mice have severe defects in wound healing, in particular, delayed re-epithelialization, impaired angiogenesis, and reduced macrophage infiltration (126).

TLR signaling through MyD88 was requisite for pDC activation. This finding is line with the known molecular recognition pathway for pDC which selectively express TLR 7 and TLR 9. Furthermore, in a recent skin injury study, MyD88-deficient mice exhibited delayed wound contraction, decreased and delayed granulation tissue formation, and reduced new blood vessel density (127). Our recent studies that demonstrated pDC activation through cathelicidin/nucleic acid associated complexes fully supports the notion that in vivo skin infiltrating pDC can sense these complexes to trigger endosomal activation of TLRs to produce IFN- α . Intriguingly, studies performed in TLR7-deficient mice

demonstrated that TLR 7 may be the preferred mechanism of MyD88 signaling as there was very little induction of IFN- α and IFN- β after injury. However, TLR 9 signaling may also be important in pDC activation in skin injury. Our studies directed at the role of TLR 9 signaling were limited to the use of TLR 9 specific inhibitor. Although, no significant differences in IFN- α and IFN- β gene induction between control and inhibitor treated mice were observed in these mice we cannot rule out the possibility that the inhibitor was not applied at the optimal time or was ineffective. Moreover, TLR9-deficient mice were recently found to be unable to induce Type I IFN responsive genes (in press). Additional research efforts should be directed at probing the dynamic contributions of TLR 7 and TLR 9 signaling in greater detail in order to fully understanding the molecular basis of pDC activation in skin injury.

Mechanisms underlying pDC infiltration and activation in skin injury were evaluated to understand how pDCs potentially can provoke inflammation in psoriasis. Previously we showed that, in human, pDCs can produce robust levels of IFN- α upon stimulation with mammalian DNA complexed with the human cathelicidin peptide, LL-37. This mechanism may be responsible for pDC activation during a psoriatic episode in which a surface wound triggers keratinocytes to produce LL-37. Damaged cells may release DNA which can complex with LL-37 and provide a potent stimulus for pDC activation. I confirmed our earlier findings which demonstrated the ability of LL-37 to bind to nucleic acid in mice using the CRAMP, the murine ortholog of LL-37. CRAMP

was capable of binding murine DNA although requiring higher molar concentrations to fully quench PicoGreen fluorescence compared to LL-37.

Additionally, when complexed with DNA, CRAMP was able to induce significant amounts of IFN- α protein from fresh splenic murine pDCs. Moreover, intradermally injected CRAMP was able to solicit a strong pDC infiltrate to the skin in vivo compared to controls (PBS and scrambled CRAMP). Gene expression studies from CRAMP-injected mice revealed strong Type I IFN induction. CRAMP is clearly capable of mediating a strong pDC response in vivo. Although CRAMP was injected without associated DNA, DNA released from cells damaged by the injection may provide DNA necessary to bind to the injected CRAMP. CRAMP is not however the sole player responsible for pDC activation, as mice deficient in cathelicidin produced similar levels of both IFN- α and IFN- β upon tape stripping.

pDC infiltration and activation in skin injury is unlikely to be driven by autoantibodies and the formation of immune complexes, as we did not find an increase in anti-nuclear antibodies after skin injury (not shown). Candidate factors that trigger this process are HMGB1 (128), heat shock proteins (129) and other cationic antimicrobial peptides as they are all expressed in damaged skin and they have the ability to form self-nucleic acid-containing complexes that activate pDCs. Investigations by others have demonstrated that additional AMPs belonging to the beta defensin family are involved in skin defense and

are upregulated upon injury. We investigated gene expression of five beta-defensins (beta defensins 1-4, 14) shown to be important in skin defense. Importantly, some of these defensins in humans have been shown to bind DNA and activate pDC (beta defensin 3, 4, 14). In mice, beta defensin 3, 4 and 14 were all upregulated upon injury and may potentially provide additional stimuli for pDC infiltration and activation in injured tissue. Further evaluations of the ability of beta defensins to induce pDC infiltration and activation *in vivo* need to be conducted as well as the consequence of their deficiency using knockout models.

Recent investigations in psoriasis have uncovered Chemerin, a novel chemokine substantially expressed during peak pDC activity. pDC migration to the skin has been attributed to the effect of chemerin, an agonist for chemokine like receptor 1 (CMKLR1) specifically expressed by pDCs (117, 130, 131). Chemerin is constitutively expressed in healthy skin by endothelial cells and fibroblasts as an inactive propeptide that requires activation through C-terminal cleavage by serine proteases. It is possible that during skin injury the release of proteases by damaged keratinocytes allows the formation of the active chemerin that recruits pDCs to the injury site. In addition, skin injury induces the expression of CXCR3-ligands (not shown), a set of chemokines which are typically induced in structural cells of the skin as a result of IFN- α/β expression and which have been shown to promote recruitment pDCs into sites of their

activation (132). Evaluation of chemerin in our model showed a strong induction of Chemerin which persisted for at least 3 days post injury.

To investigate whether pDCs were exerting its effects on wound healing through Type I IFN we analyzed wound healing responses in tape stripped IFN- α/β receptor knockout mice (IFNAR^{-/-}). Recent reports investigating the use of exogenous IFN- α applied topically to full-thickness injuries have resulted in mixed conclusions (133, 134). Pammer et al. found that 1000 IU/ml of human recombinant IFN- α 2b (INTRON-A or ROFERON) was able to inhibit apoptosis of endothelial cells, in vitro, but induced senescence upon continued treatment (133). However, Bhartiya et al observed that 20,000 Units (murine) injected intramuscularly over a 5 day time course in both full-thickness and incisional injuries enhanced re-epithelialization and increased fibroblast migration to the wound bed (134). No studies have evaluated the role of IFN- α , neither using physiological levels of IFN- α , nor have looked at physiologically produced IFN- α . In gene expression studies we found that Type I IFN was important for IL-6 induction. Similarly, both pDC-depleted and IFNAR^{-/-} mice induced significantly lower amounts of IL17A and IL22 mRNA transcripts. However, there was one main exception, IL23p19 and IL12p35 gene induction was significantly reduced in pDC-depleted but not IFNAR^{-/-} mice. This striking finding suggests that pDCs contribution to wound healing is much more dynamic than simply producing Type I IFNs.

As mentioned in the introduction wound healing is comprised of four overlapping phases. Phase one involves triggers hemostasis involving both the release chemotactic and pro-inflammatory molecules by keratinocytes and platelet activation. pDCs infiltrate the injury during the second phase (inflammatory phase). Therefore, we examined whether pDCs played a role in downstream injury responses. Phase three consists of neo angiogenesis to supply damaged skin nutrients and oxygen vital for tissue repair. Injured skin was stained for CD31, a marker for endothelial cells, to see whether abrogation of the pDC response impaired angiogenesis. No differences in CD31 expression were observed suggesting that pDCs do not influence endothelial cell division. Moreover, gene expression for angiogenic markers *Vegfa*, *Vegfb*, and *Egf* did not reveal any differences throughout a 5 day time course between wt and pDC-depleted mice. Phase 3 also includes matrix remodeling which consists of metalloprotease activity to remove the fibrin clot and collagen synthesis to provide a structured dermis was evaluated. No differences in Mmp3, Mmp8 and Mmp9 gene expression were observed. pDCs do not contribute to angiogenesis or matrix remodeling.

Phase four of wound healing encompasses the mobilization of keratinocytes to re-epithelialize the injured epidermis. Re-epithelialization characterized by migration of keratinocytes from both the wound edge and hair follicles to form a new basal layer was evaluated to evaluate the overall effects of wound healing. We utilized K6 expression as a surrogate marker for re-epithelialization. K6 is

expressed by early hyperproliferating keratinocytes and is downregulated upon after a new basement membrane is generated. Interestingly, both pDC-depleted and IFNAR^{-/-} mice demonstrated delayed wound healing compared to control mice. Wound healing defects persisted through day 3 post injury and eventually resolved by day 5 (data not shown). Additionally both pDC-depleted and IFNAR^{-/-} mice exhibited reduced wound closure after full-thickness injuries. These data show that both pDCs and Type I IFN are important in resolving skin injury. However, the nature of pDC contributions to wound healing independent of Type I IFN is not fully understood.

pDCs may potentially exert their influence in wound healing through keratinocyte mobilization. Keratinocytes from the wound edge are required to detach anchoring attachment molecules such as desmosomes from neighboring cells and migrate to the injured tissue to form a new basement membrane. This process involves both mitogenic and proliferative cues. Potentially pDC act on keratinocytes ability to navigate through and across the fibrin clot. Keratinocytes upregulate proteolytic enzymes necessary for their movement across the injured tissue. Once they reach the denuded epidermis keratinocytes must proliferate in order to re-establish both the basement membrane as well as the the stratum corneum. As evidenced by K6 which is expressed only by early hyperproliferating keratinocytes, keratinocytes rapidly undergo proliferation when they form a new basal layer. In my studies, the most pronounced effect on pDC depletion was compromised K6 expression. Future

studies evaluating the role of pDC and Type I IFN should be performed to determine whether keratinocyte mobility and proliferative ability are affected.

Our investigation was directed at understanding the role of pDCs in a physiologically relevant process which may influence potential therapeutic strategies for skin disorders such as psoriasis, melanoma, and burn trauma. We evaluated whether pDCs, in addition to being present in murine skin injury, were present in human skin injury. pDCs infiltrated both mechanical (tape stripped) as well as chemical (Sodium Lauryl Sulfate) mediated human skin injury. Moreover, pDCs in both conditions were activated to produce substantial levels of IFN- α and IFN- β mRNA. These results demonstrate that pDCs response to injury is potentially an evolutionarily conserved response to both aid in injury recovery as well as to potentially thwart a pathogenic response. Whether pDCs were programmed to respond only to danger cues such as host cell degradation products or viral antigens it is clear that pDCs participate in non-infectious related processes.

Another striking result of our studies was that pDC involvement in wound healing is important for upregulation of cytokines vital for T cell differentiation into TH17 cells. IL-23 is a heterodimeric cytokine composed of p19 and p40 subunits. p19 is exclusive to IL-23 while p40 is shared with IL-12. IL-23 has been shown to be important in host inflammatory response to infection. In addition to being important in driving CD4 T cell differentiation to TH17 cells, IL-

IL-23 also promotes the upregulation of MMP9 (important for keratinocyte mobilization through granulation tissue) and angiogenesis (107). IL-23 in combination with IL-6 and TGF- β 1 drives differentiation of CD4 T cells into effector TH17 cells which secrete IL-17 and IL-22. Interestingly, abrogation of pDC response to wound healing significantly impaired IL23p19, IL17A, and IL-22 gene induction. No evidence suggests that pDCs produce IL23p19 directly. So the likely mechanism is that pDC activation induces a bystander response, possibly by resident mDC, to induce IL-23 gene expression. Another important observation that we made was that IL23p19 gene induction was not suppressed in IFNAR^{-/-} mice suggesting an IFN- α/β independent mechanism. IL-23 injected into the skin of mice was found to induce psoriasis features such as erythema, acanthosis, dermal inflammatory infiltrates, dermal papillary blood vessels and IL-22 dependent psoriasisform changes.

pDCs also affect the production of TH17 cytokines, as the induction of IL-17 and IL-22 is abrogated in pDC-depleted mice. This is in line with recent studies showing that pDCs can drive the differentiation of IL-17 and IL-22-producing T cells (135-137), and that IL-6 is implicated in this process (135). IL-22 appears to be particularly important in epidermal regeneration as this cytokine directly promotes keratinocyte migration and proliferation (138-140). Surprisingly, mice deficient in IFN- α/β -receptors display a similar inhibition in IL-6 and TH17 cytokine induction with delayed re-epithelialization of skin wounds. Because IFN- α/β are potent stimulators of immune responses but do not exert a direct

activity on keratinocytes (141), these findings suggest that IFN- α/β in skin wounds promote epidermal regeneration and wound repair through the induction of TH17-biased inflammatory responses. The exact mechanism that links IFN- α/β production to pDC-mediated TH17 responses is still unclear. However, there is additional evidence that IFN- α/β produced by pDCs drives TH17 responses and epidermal proliferation in a therapeutic model of skin treated with the TLR 7 agonist imiquimod (142). Furthermore, IFN- α/β produced by pDCs triggers psoriasis (93), a disease characterized by large numbers of pathogenic TH17 cells that trigger epidermal hyperproliferation (143).

Our studies were centered on understanding what role, if any, pDCs play in a normal wound assault in the skin at aims of further understanding why and how pDCs facilitate psoriasis pathogenesis. We have uncovered a novel role for pDCs in wound healing and future efforts should be directed at understanding both chemotactic and regulatory cues involving pDC infiltration and activation in skin injury. A broader appreciation for the role of keratinocytes in immunological settings could be useful in understanding pDC function. Keratinocytes produce pro-inflammatory cytokines in addition to antimicrobial peptides which may be important for pDC activity. TH17 cells may provide a positive feedback loop which acts on keratinocytes to further release cationic antimicrobial peptides. Sustained activation of pDCs by cationic antimicrobial peptides complexed with extracellular nucleic acid may lead to a dysregulated response resulting in chronic inflammation. Psoriasis does have a genetic component which may

increase the susceptibility for an uncontrolled inflammatory response to injury. This continued cycle may be involved in the initiation of a psoriatic lesion.

Many questions regarding the mechanisms of pDC infiltration and regulation as well as the role of pDC induced IL-23, IL-17, and IL-22 gene expression in skin injury remain. Future efforts at determining mediators of pDC infiltration to wounded skin should encompass the following molecules: chemerin, the CXCR3 ligands; CXCL9 (Mig), CXCL10 (IP-10), and CXCL11 (ITAC), and the CXCR4 ligand, CXCL12 (SDF-1). Chemerin is highly expressed in wounded skin as well as in psoriasis. Moreover, pDCs express CMKLR1, the cognate receptor for chemerin. Additionally, CXCL9, CXCL10, and CXCL11 are all expressed by dermal resident cells, are highly expressed in psoriasis and can be recognized by pDCs. Finally, CXCR3 requires cooperative CXCR4 engagement of CXCL12 for pDC trafficking.

We showed that the AMP, CRAMP, was able to mediate pDC infiltration to the skin and activate pDC. Yet, CRAMP-deficient mice produced equivalent levels of IFN- α and IFN- β mRNA upon skin injury. Potentially, CRAMP works in concert with additional AMP to mediate pDC infiltration and activation in skin injury. We have shown that additional cationic AMPs, beta-defensins -3, 4, and 14, are all elevated in injured skin. It is important to screen these beta-defensins when complexed with nucleic acid for the ability to activate purified splenic pDCs to produce IFN- α . Beta-defensins found to be able to activate pDCs

should then be injected, intradermally, to determine their ability to mediate pDC infiltration and activation *in vivo*. Beta-defensins that can mediate pDC infiltration and activation should be evaluated in a targeted gene knockout setting. Ideally CRAMP-deficient mice would be crossed with beta-defensin-deficient mice to determine whether complete type I IFN signaling is abrogated. Such a finding would lead to better therapeutic strategies for psoriasis in which cathelicidin and beta-defensins are both targeted.

Another important question is how pDCs and type I IFNs contribute to re-epithelialization. Our studies implicate delayed keratinocyte mobilization to the injury. However, mechanisms underlying this are poorly understood. Potentially, pDCs and type I IFNs influence the detachment of migrating keratinocytes from basement membrane or promote the proliferation of leading edge keratinocytes. Alternatively, pDCs and type I IFNs promote the migration of keratinocytes across the provisional matrix, which requires upregulation of MMPs and specific integrin expression. These possibilities should be explored in further detail.

Regulation of pDC activity in skin injury needs to be investigated. Some important questions are why is pDC activity transient? What mechanisms balance continued inflammation versus tolerance? How is this mechanism subverted in psoriasis?

Finally, it is important to understand the role of pDC induced IL-23, IL-17, and IL-22 gene expression in skin injury. Skin injury experiments using IL-23, IL-17, and IL-22-deficient mice can shed light on the role of these cytokines in wound healing. Potentially, these TH17 related cytokines are upregulated in preparation of an infection.

In conclusion, our study identifies a role of pDCs in recognizing nucleic acids released in injured skin and promoting early inflammatory responses and re-epithelialization of the wounds. These findings provide a paradigm shift in understanding the function of pDCs from the classical view of a specialized cell type in the recognition of viral infections to important sensors of tissue damage at epithelial surfaces.

BIBLIOGRAPHY

1. Martin, P. Wound Healing--Aiming for Perfect Skin Regeneration. *Science* **276**, 75-81 (2009).
2. R A F Clark, Ed., The Molecular and Cellular Biology of Wound Repair (Plenum, New York, 1996).
3. Diegelmann, RF. Wound healing: an overview of acute, fibrotic and delayed healing. *Front Biosci* **9**, 283 (2004).
4. Laurens N, Koolwijk P, de Maat MP. Fibrin structure and wound healing. *J Thromb Haemost: JTH* **4**, 932-9 (2006).
5. Clark, RA. Fibrin and wound healing. *Ann N Y Acad Sci* **936**, 355-67(2001).
6. Monaco JL, Lawrence WT. Acute wound healing an overview. *Clin Plast Surg* **30**, 1-12 (2003).
7. Tran KT, Griffith L, Wells A. Extracellular matrix signaling through growth factor receptors during wound healing. *Wound Repair Regen* **12**, 262-8
8. Estavillo D, Ritchie A, Diacovo TG, Cruz MA. Functional analysis of a recombinant glycoprotein Ia/IIa (Integrin alpha(2)beta(1)) I domain that inhibits platelet adhesion to collagen and endothelial matrix under flow conditions. *J Biol Chem* **274**, 35921-6 (1999).
9. Mutsaers SE, Bishop JE, McGrouther G, Laurent GJ. Mechanisms of tissue repair: from wound healing to fibrosis. *Int J Biochem Cell Biol* **29**, 5-17 (1997).
10. Tonnesen, MG, Feng, X, Clark, RA. Angiogenesis in wound healing. *J Investig Dermatol Symp Proc* **5**, 40-6 (2000).
11. Gharaee-Kermani M, Phan SH. Role of cytokines and cytokine therapy in

- wound healing and fibrotic diseases. *Curr Pharm Des* **7**, 1083-103 (2001).
12. Grazul-Bilska AT, Johnson ML, Bilski JJ, Redmer DA, Reynolds LP, Abdullah A, Abdullah KM. Wound healing: the role of growth factors. *Drugs Today (Barc)* **39**, 787-800 (2003).
 13. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. Growth factors and cytokines in wound healing. *Wound Repair Regen* **16**, 585-601 (2008).
 14. Parks WC, Wilson CL, López-Boado YS. Matrix metalloproteinases as modulators of inflammation and innate immunity. *Nat Rev Immunol* **4**, 617-29 (2004).
 15. Gillitzer R, Goebeler M. Chemokines in cutaneous wound healing. *J Leukocyte Biol* **69**, 513-521 (2001).
 16. Furie B, Furie BC. Thrombus formation in vivo. *J Clin Invest* **115**, 3355-3362 (2005).
 17. Lui WY, Lee WM, Cheng CY. Transforming growth factor-beta3 perturbs the inter-Sertoli tight junction permeability barrier in vitro possibly mediated via its effects on occludin, zonula occludens-1, and claudin-11. *Endocrinology* **142**, 1865-77 (2001).
 18. Midwood KS, Mao Y, Hsia HC, Valenick LV, Schwarzbauer JE. Modulation of Cell–Fibronectin Matrix Interactions during Tissue Repair. *J Invest Dermatol Symp Proc* **11**, 73-78 (2006).
 19. Stramer BM, Mori R, Martin P. The inflammation-fibrosis link? A Jekyll and Hyde role for blood cells during wound repair. *J Invest Dermatol* **127**, 1009-

- 17 (2007).
20. Martin P, Leibovich SJ. Inflammatory cells during wound repair: the good, the bad and the ugly. *Trends Cell Biol* **15**, 599-607(2005).
21. Nickoloff BJ, Bonish BK, Marble DJ, Schriedel KA, DiPietro LA, Gordon KB, Lingen MW. Lessons Learned from Psoriatic Plaques Concerning Mechanisms of Tissue Repair, Remodeling, and Inflammation. *J Invest Dermatol Symp Proc* **11**, 16-29 (2006).
22. Nguyen, MT, Simpson-Haidaris, PJ. Cell Type–Specific Regulation of Fibrinogen Expression in Lung Epithelial Cells by Dexamethasone and Interleukin-1 β . *Am J Respir Cell Mol. Bio*, **22**, 209-217 (2000).
23. Jameson J, Havran WL. Skin gammadelta T-cell functions in homeostasis and wound healing. *Immunol Rev* **215**, 114-22 (2007).
24. Mann A, Breuhahn K, Schirmacher P, Blessing M. Keratinocyte-derived granulocyte-macrophage colony stimulating factor accelerates wound healing: Stimulation of keratinocyte proliferation, granulation tissue formation, and vascularization. *J Invest Dermatol* **117**, 1382-90 (2001).
25. Werner, S, Krieg, T, Smola, H. Keratinocyte-fibroblast interactions in wound healing. *J Invest Dermatol* **127**, 998-1008 (2007).
26. Mann A, Breuhahn K, Schirmacher P, Blessing M. Keratinocyte-derived granulocyte-macrophage colony stimulating factor accelerates wound healing: Stimulation of keratinocyte proliferation, granulation tissue formation, and vascularization. *J Invest Dermatol* **117**, 1382-90 (2001).
27. Larrick JW, Hirata M, Balint RF, Lee J, Zhong J, Wright SC. Human CAP18:

- a novel antimicrobial lipopolysaccharide-binding protein. *Infect Immun* **63**, 1291-1297 (1995).
28. De Yang, Chen Q, Schmidt AP, Anderson GM, Wang JM, Wooters J, Oppenheim JJ, Chertov O. LL-37, the neutrophil granule- and epithelial cell-derived cathelicidin, utilizes formyl peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. *J Exp Med* **192**, 1069-74 (2000).
 29. Lande R, Gregorio J, Facchinetti V, Chatterjee B, Wang YH, Homey B, Cao W, Wang YH, Su B, Nestle FO, Zal T, Mellman I, Schröder JM, Liu YJ, Gilliet M. Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature* **449**, 564-9 (2007).
 30. Ganguly D, Chamilos G, Lande R, Gregorio J, Meller S, Facchinetti V, Homey B, Barrat FJ, Zal T, Gilliet M. Self-RNA-antimicrobial peptide complexes activate human dendritic cells through TLR7 and TLR8. *J Exp Med* **206**, 1983-94 (2009).
 31. Lin ZQ, Kondo T, Ishida Y, Takayasu T, Mukaida N. Essential involvement of IL-6 in the skin wound-healing process as evidenced by delayed wound healing in IL-6-deficient mice. *J Leukoc Biol* **73**, 713-721 (2003).
 32. Foster CA, Yokozeki H, Rappersberger K, Koning F, Volc-Platzer B, Rieger A, Coligan JE, Wolff K, Stingl G. Human epidermal t cells predominately belong to the lineage expressing. *J Exp Med* **171**, 997–1013 (1990).
 33. Brandt E, Petersen F, Ludwig A, Ehlert JE, Bock L, Flad HD. The beta-thromboglobulins and platelet factor 4: blood platelet-derived CXC

- chemokines with divergent roles in early neutrophil regulation. *J Leukoc Biol* **67**, 471-8 (2000).
34. Vaughan MB, Howard EW, Tomasek JJ. Transforming growth factor-beta1 promotes the morphological and functional differentiation of the myofibroblast. *Exp Cell Res* **257**, 180-9 (2000).
35. Lui WY, Lee WM, Cheng CY. Transforming growth factor-beta3 perturbs the inter-Sertoli tight junction permeability barrier in vitro possibly mediated via its effects on occludin, zonula occludens-1, and claudin-11. *Endocrinology* **142**, 1865-77 (2001).
36. Wang XJ, Han G, Owens P, Siddiqui Y, Li AG. Role of TGF β -Mediated Inflammation in Cutaneous Wound Healing. *J Invest Dermatol Symp Proc* **11**, 112-117 (2006).
37. Devalaraja RM, Nanney LB, Du J, Qian Q, Yu Y, Devalaraja MN, Richmond A. Delayed wound healing in CXCR2 knockout mice. *J Invest Dermatol* **115**, 234-244 (2000).
38. Artuc M, Hermes B, Steckelings UM, Grützkau A, Henz BM. Mast cells and their mediators in cutaneous wound healing--active participants or innocent bystanders? *Exp Dermatol* **8**, 1-16 (1999).
39. Artuc, M, Steckelings, UM, Henz, BM. Mast cell-fibroblast interactions: human mast cells as source and inducers of fibroblast and epithelial growth factors. *J Invest Dermatol* **118**, 391-5 (2002).
40. Engelhardt E, Toksoy A, Goebeler M, Debus S, Bröcker EB, Gillitzer R. Chemokines IL-8, GRO α , MCP-1, IP-10, and Mig are sequentially and

- differentially expressed during phase-specific infiltration of leukocyte subsets in human wound healing. *Am J Pathol* **153**, 1849-60 (1998).
41. DiPietro LA, Reintjes MG, Low QE, Levi B, Gamelli RL. Modulation of macrophage recruitment into wounds by monocyte chemoattractant protein-1. *Wound Repair Regen* **9**, 28-33 (2001).
 42. Armstrong DA, Major JA, Chudyk A, Hamilton TA. Neutrophil chemoattractant genes KC and MIP-2 are expressed in different cell populations at sites of surgical injury. *J Leukoc Biol* **75**, 641-648 (2004).
 43. Smith ML, Olson TS, Ley K. CXCR2- and E-selectin-induced neutrophil arrest during inflammation in vivo. *J Exp Med* **200**, 935-9 (2004).
 44. Hübner G, Brauchle M, Smola H, Madlener M, Fässler R, Werner S. Differential regulation of pro-inflammatory cytokines during wound healing in normal and glucocorticoid-treated mice. *Cytokine* **8**, 548-56 (1996).
 45. Strieter RM, Kasahara K, Allen RM, Standiford TJ, Rolfe MW, Becker FS, Chensue SW, Kunkel SL. Cytokine-induced neutrophil-derived interleukin-8. *Am J Pathol* **141**, 397-407 (1992).
 46. Mann A, Niekisch K, Schirmacher P, Blessing M. Granulocyte–Macrophage Colony-Stimulating Factor Is Essential for Normal Wound Healing. *J Invest Dermatol Symp Proc* **11**, 87-92 (2006).
 47. Peterkofsky B. Ascorbate requirement for hydroxylation and secretion of procollagen: relationship to inhibition of collagen synthesis in scurvy, *Am J Clin Nutr* **54**, 1135S-1140S (1991).
 48. Kadler KE, Hill A, Canty-Laird EG. Collagen fibrillogenesis: fibronectin,

- integrins, and minor collagens as organizers and nucleators. *Curr Opin Cell Biol* **5**, 495-501 (2008).
49. Desmoulière A, Chaponnier C, Gabbiani G. Tissue repair, contraction, and the myofibroblast. *Wound Repair Regen* **13**, 7-12 (2005).
 50. Serini G, Bochaton-Piallat ML, Ropraz P, Geinoz A, Borsi L, Zardi L, Gabbiani G. The fibronectin domain ED-A is crucial for myofibroblastic phenotype induction by transforming growth factor-beta1. *J Cell Biol* **142**, 873-81 (1998).
 51. Feng XH, Derynck R. Specificity and versatility in tgf-beta signaling through Smads. *Annu Rev Cell Dev Biol* **21**, 659-93 (2005).
 52. Derynck R, Zhang YE. Smad-dependent and Smad-independent pathways in TGF-beta family signalling. *Nature* **425**, 577-84 (2003).
 53. Stacey E Mills, Ed., Histology for Pathologists, (Lippincott Williams & Wilkins, Philadelphia, 2006), Ch 6.
 54. Chiquet-Ehrismann R, Chiquet M. Tenascins: regulation and putative functions during pathological stress. *J Pathol* **200**, 488-499 (2003).
 55. Belperio JA, Keane MP, Arenberg DA, Addison CL, Ehlert JE, Burdick MD, Strieter RM. CXC chemokines in angiogenesis. *J Leukoc Biol* **68**, 1-8 (2000).
 56. Singer AJ, Clark, RA., Cutaneous Wound Healing, *N Engl J Med* **341**, 738-746 (1999).
 57. Lennert K, Remmele W. Karyometric research on lymph node cells in man. I. Germinalblasts, lymphoblasts and lymphocytes. *Acta Haematol* **19**, 99-113

(1958).

58. Feller AC, Lennert K, Stein H, Bruhn HD, Wuthe HH. Immunohistology and aetiology of histiocytic necrotizing lymphadenitis. Report of three instructive cases. *Histopathology* **7**, 825-39 (1983).
59. Facchetti F, de Wolf-Peeters C, Mason DY, Pulford K, van den Oord JJ, Desmet VJ. 1988. Plasmacytoid T cells. Immunohistochemical evidence for their monocyte/macrophage origin. *Am J Pathol* **133**, 15-21 (1988).
60. O'Doherty U, Peng M, Gezelter S, Swiggard WJ, Betjes M, Bhardwaj N, Steinman RM. Human blood contains two subsets of dendritic cells, one immunologically mature and the other immature. *Immunology* **82**, 487-93 (1994).
61. Grouard G, Durand I, Filgueira L, Banchereau J, Liu YJ. Dendritic cells capable of stimulating T cells in germinal centres. *Nature* **384**, 364-67 (1996).
62. Dubois B, Barthelemy C, Durand I, Liu YJ, Caux C, Briere F. Toward a role of dendritic cells in the germinal center reaction: triggering of B cell proliferation and isotype switching. *J. Immunol.* **162**, 3428-36 (1999).
63. Grouard G, Rissoan MC, Filgueira L, Durand I, Banchereau J, Liu YJ. The enigmatic plasmacytoid T cells develop into dendritic cells with interleukin (IL)-3 and CD40-ligand. *J Exp Med* **185**, 1101-11 (1997).
64. Rissoan MC, Soumelis V, Kadowaki N, Grouard G, Briere F, de Waal Malefyt R, Liu YJ. Reciprocal control of T helper cell and dendritic cell differentiation. *Science* **283**, 1183-6 (1999).

65. Colonna M, Trinchieri G, Liu YJ. Plasmacytoid dendritic cells in immunity. *Nat Immunol* **12**, 1219-26 (2004).
66. Siegal FP, Kadowaki N, Shodell M, Liu YJ. Evidence for identity of the (natural) interferon (IFN) producing cell (NIPC) and the CD11c- precursor of DC2. *J Leukoc Biol* **2(Suppl.)**, 18 (1998).
67. Timonen T, Saksela E, Virtanen J, Cantell K. Natural killer cells are responsible for the interferon production induced in human lymphocytes by tumor cell contact. *Eur J Immunol* **10**, 422-27 (1980).
68. Djeu JY, Stocks N, Zoon K, Stanton GJ, Timonen T, Herberman RB. Positive self regulation of cytotoxicity in human natural killer cells by production of interferon upon exposure to influenza and herpes viruses. *J Exp Med* **156**, 1222-34 (1982).
69. Yamaguchi T, Handa K, Shimizu Y, Abo T, Kumagai K. Target cells for interferon production in human leukocytes stimulated by Sendai virus. *J Immunol* **118**, 1931-35 (1977).
70. Peter HH, Dallugge H, Zawatzky R, Euler S, Leibold W, Kirchner H. Human peripheral null lymphocytes. II. Producers of type-1 interferon upon stimulation with tumor cells, Herpes simplex virus and *Corynebacterium parvum*. *Eur J Immunol* **10**, 547-55 (1980).
71. Svensson H, Johannisson A, Nikkila T, Alm GV, Cederblad B. The cell surface phenotype of human natural interferon- α producing cells as determined by flow cytometry. *Scand J Immunol* **44**, 164-72 (1996).
72. Siegal FP, Kadowaki N, Shodell M, Fitzgerald-Bocarsly PA, Shah K, Ho S,

- Antonenko S, Liu YJ. The nature of the principal type 1 interferon-producing cells in human blood. *Science* **284**, 1835-7 (1999).
73. Cella M, Jarrossay D, Facchetti F, Alebardi O, Nakajima H, Lanzavecchia A, Colonna M. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat Med* **5**, 919–23 (1999).
74. Liu YJ. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol* **23**, 275-306 (2005).
75. Nakano H, Yanagita M, Gunn MD. CD11c+B220+Gr-1+ cells in mouse lymph nodes and spleen display characteristics of plasmacytoid dendritic cells. *J Exp Med* **194**, 1171-78 (2001).
76. Asselin-Paturel C, Boonstra A, Dalod M, Durand I, Yessaad N, Dezutter-Dambuyant C, Vicari A, O'Garra A, Biron C, Brière F, Trinchieri G. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat Immunol* **2**, 1144-50 (2001).
77. Bjorck P. Isolation and characterization of plasmacytoid dendritic cells from Flt3 ligand and granulocyte-macrophage colony-stimulating factor treated mice. *Blood* **98**, 3520-26 (2001).
78. Blasius AL, Giurisato E, Cella M, Schreiber RD, Shaw AS, Colonna M. Bone Marrow Stromal Cell Antigen 2 Is a Specific Marker of Type I IFN-Producing Cells in the Naïve Mouse, but a Promiscuous Cell Surface Antigen Following IFN Stimulation. *J Immunol* **177**, 3260-3265 (2006).
79. Blasius AL, Cella M, Maldonado J, Takai T, Colonna M. Siglec-H is an IPC-specific receptor that modulates type I IFN secretion through DAP12. *Blood*

- 107**, 2474-6 (2006).
80. Gilliet M, Conrad C, Geiges M, Cozzio A, Thürlimann W, Burg G, Nestle FO, Dummer R. Psoriasis triggered by toll-like receptor 7 agonist imiquimod in the presence of dermal plasmacytoid dendritic cell precursors. *Arch Dermatol* **140**, 1490-5 (2004).
81. Wollenberg A, Wagner M, Günther S, Towarowski A, Tuma E, Moderer M, Rothenfusser S, Wetzel S, Endres S, Hartmann G. Plasmacytoid dendritic cells: a new cutaneous dendritic cell subset with distinct role in inflammatory skin diseases. *J Invest Dermatol* **119**, 1096-102 (2002).
82. Gerlini G, Mariotti G, Bianchi B, Pimpinelli N. Massive recruitment of type I interferon producing plasmacytoid dendritic cells in varicella skin lesions. *J Invest Dermatol* **126**, 507-9 (2006).
83. Vanbervliet B, Bendriss-Vermare N, Massacrier C, Homey B, de Bouteiller O, Brière F, Trinchieri G, Caux C. The inducible CXCR3 ligands control plasmacytoid dendritic cell responsiveness to the constitutive chemokine stromal cell-derived factor 1 (SDF-1)/CXCL12. *J Exp Med* **198**, 823-30 (2003).
84. Donaghy H, Bosnjak L, Harman AN, Marsden V, Tying SK, Meng TC, Cunningham AL. Role for plasmacytoid dendritic cells in the immune control of recurrent human herpes simplex virus infection. *J Virol* **83**, 1952-61 (2009).
85. Lande R, Gilliet M. Plasmacytoid dendritic cells: key players in the initiation and regulation of immune responses. *Ann N Y Acad Sci* **1183**, 89-103

(2010).

86. Kadowaki N, Ho S, Antonenko S, Malefyt RW, Kastelein RA, Bazan F, Liu YJ. Subsets of human dendritic cell precursors express different toll-like receptors and respond to different microbial antigens. *J Exp Med* **194**, 863-9 (2001).
87. Diebold SS, Kaisho T, Hemmi H, Akira S, Reis e Sousa C. Innate antiviral responses by means of TLR7 - mediated recognition of single - stranded RNA. *Science* **303**, 1529-31 (2004).
88. Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, Lipford G, Wagner H, Bauer S. Species - specific recognition of single - stranded RNA via toll - like receptor 7 and 8. *Science* **303**, 1526-9 (2004).
89. Hemmi H, Kaisho T, Takeuchi O, Sato S, Sanjo H, Hoshino K, Horiuchi T, Tomizawa H, Takeda K, Akira S. Small anti - viral compounds activate immune cells via the TLR7 MyD88 - dependent signaling pathway. *Nat Immunol* **3**, 196-200 (2002).
90. Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S. A Toll - like receptor recognizes bacterial DNA. *Nature* **408**, 740-5 (2000).
91. Gilliet M, Cao W, Liu YJ. Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. *Nat Rev Immunol* **8**, 594-606 (2008).
92. Theofilopoulos AN, Baccala R, Beutler B, Kono DH. Type I interferons

- (alpha/beta) in immunity and autoimmunity. *Annu Rev Immunol* **23**, 307-36 (2005).
93. Nestle FO, Conrad C, Tun-Kyi A, Homey B, Gombert M, Boyman O, Burg G, Liu YJ, Gilliet M. Plasmacytoid predendritic cells initiate psoriasis through interferon - alpha production. *J Exp Med* **202**, 135-43 (2005).
94. Dorschner RA, Pestonjamas VK, Tamakuwala S, Ohtake T, Rudisill J, Nizet V, Agerberth B, Gudmundsson GH, Gallo RL. Cutaneous injury induces the release of cathelicidin anti-microbial peptides active against group A Streptococcus. *J Invest Dermatol* **117**, 91-7 (2001).
95. Schaubert J, Dorschner RA, Coda AB, Büchau AS, Liu PT, Kiken D, Helfrich YR, Kang S, Elalieh HZ, Steinmeyer A, Zügel U, Bikle DD, Modlin RL, Gallo RL. Injury enhances TLR2 function and antimicrobial peptide expression through a vitamin D-dependent mechanism. *J Clin Invest* **117**, 803-11 (2007).
96. Lai Y, Gallo RL AMPed up immunity: how antimicrobial peptides have multiple roles in immune defense. *Trends Immunol* **30**, 131-41 (2009).
97. Frohm M, Agerberth B, Ahangari G, Stahle-Backdahl M, Liden S, Wigzell H, Gudmundsson GH: The expression of the gene coding for the antibacterial peptide LL-37 is induced in human keratinocytes during inflammatory disorders. *J Biol Chem* **272**, 15258-15263 (1997).
98. Zaiou M, Nizet V, Gallo RL. Antimicrobial and protease inhibitory functions of the human cathelicidin (hCAP18/LL-37) prosequence. *J Invest Dermatol* **120**, 810-816 (2003).

99. Sørensen O, Cowland JB, Askaa J, Borregaard N. An ELISA for hCAP-18, the cathelicidin present in human neutrophils and plasma. *J Immunol Methods* **206**, 53–59 (1997).
100. Dorschner RA, Lin KH, Murakami M, Gallo RL. Neonatal skin in mice and humans expresses increased levels of antimicrobial peptides: Innate immunity during development of the adaptive response. *Pediatr Res* **53**, 566–572 (2003).
101. Dorschner RA, Lopez-Garcia B, Gallo RL. Skin microenvironment dictates susceptibility of bacteria to antimicrobial peptides. *J Invest Dermatol* **122**, A133 (2004).
102. Braff, MH, Bardan A, Nizet V, Gallo RL. Cutaneous defense mechanisms by antimicrobial peptides. *J Invest Dermatol* **125**, 9-13 (2005).
103. Schaubert J, Gallo RL. Antimicrobial peptides and the skin immune defense system. *J Allergy Clin Immunol* **122**, 261-6 (2008).
104. Lai Y, Gallo RL. Toll-like receptors in skin infections and inflammatory diseases. *Infect Disord Drug Targets* **8**, 144-55 (2008).
105. Büchau AS, Gallo RL. Innate immunity and antimicrobial defense systems in psoriasis. *Clin Dermatol* **25**, 616-24 (2007).
106. Nestle FO, Di Meglio P, Qin JZ, Nickoloff BJ. Skin immune sentinels in health and disease. *Nat Rev Immunol* **9**, 679-91 (2009).
107. Nestle FO, Kaplan DH, Barker J. Psoriasis. *N Engl J Med* **361**, 496-509 (2009).
108. Conrad C, Meller S, Gilliet M. Plasmacytoid dendritic cells in the skin: to

- sense or not to sense nucleic acids. *Semin Immunol* **21**, 101-9 (2009).
109. Di Cesare A, Di Meglio P, Nestle FO. The IL-23/Th17 axis in the immunopathogenesis of psoriasis. *J Invest Dermatol* **129**, 1339-50 (2009).
 110. Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, Nakanishi K, Akira S. Targeted disruption of the MyD88 gene results in loss of IL - 1 - and IL - 18 - mediated function. *Immunity* **9**, 143 - 50 (1998).
 111. Müller U, Steinhoff U, Reis LF, Hemmi S, Pavlovic J, Zinkernagel RM, Aguet M. Functional role of type I and type II interferons in antiviral defense. *Science* **264**, 1918-21 (1994).
 112. Nizet V, Ohtake T, Lauth X, Trowbridge J, Rudisill J, Dorschner RA, Pestonjamas V, Piraino J, Huttner K, Gallo RL. Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* **414**, 454-7 (2001).
 113. Meller S, Lauerma AI, Kopp FM, Winterberg F, Anthoni M, Müller A, Gombert M, Haahtela A, Alenius H, Rieker J, Dieu-Nosjean MC, Kubitza RC, Gleichmann E, Ruzicka T, Zlotnik A, Homey B. Chemokine responses distinguish chemical - induced allergic from irritant skin inflammation: memory T cells make the difference. *J Allergy Clin Immunol* **119**, 1470-80 (2007).
 114. Takeda K, Kaisho T, Akira S. Toll-like receptors. *Annu Rev Immunol* **21**, 335-76 (2003).
 115. Barrat FJ, Meeker T, Gregorio J, Chan JH, Uematsu S, Akira S, Chang B, Duramad O, Coffman RL. Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus

- erythematosus. *J Exp Med* **202**, 1131-9 (2005).
116. Duramad O, Fearon KL, Chang B, Chan JH, Gregorio J, Coffman RL, Barrat FJ. Inhibitors of TLR-9 act on multiple cell subsets in mouse and man in vitro and prevent death in vivo from systemic inflammation. *J Immunol* **174**, 5193-200 (2005).
117. Albanesi C, Scarponi C, Pallotta S, Daniele R, Bosisio D, Madonna S, Fortugno P, Gonzalvo-Feo S, Franssen JD, Parmentier M, De Pità O, Girolomoni G, Sozzani S. Chemerin expression marks early psoriatic skin lesions and correlates with plasmacytoid dendritic cell recruitment. *J Exp Med* **206**, 249-58 (2009).
118. Wojcik SM, Bundman DS, Roop DR. Delayed wound healing in keratin 6a knockout mice. *Mol Cell Biol* **20**, 5248-55 (2000).
119. Welzel J, Metker C, Wolff HH, Wilhelm KP. SLS-irritated human skin shows no correlation between degree of proliferation and TEWL increase. *Arch Dermatol Res* **290**, 615-20 (1998).
120. Lai Y, Di Nardo A, Nakatsuji T, Leichtle A, Yang Y, Cogen AL, Wu ZR, Hooper LV, Schmidt RR, von Aulock S, Radek KA, Huang CM, Ryan AF, Gallo RL. Commensal bacteria regulate Toll-like receptor 3-dependent inflammation after skin injury. *Nat Med* **12**, 1377-82 (2009).
121. Tjabringa GS, Ninaber DK, Drijfhout JW, Rabe KF, Hiemstra PS. Human cathelicidin LL-37 is a chemoattractant for eosinophils and neutrophils that acts via formyl-peptide receptors. *Int Arch Allergy Immunol* **140**, 103-12 (2006).

122. Kurosaka K, Chen Q, Yarovinsky F, Oppenheim JJ, Yang D. Mouse cathelin-related antimicrobial peptide chemoattracts leukocytes using formyl peptide receptor-like 1/mouse formyl peptide receptor-like 2 as the receptor and acts as an immune adjuvant. *J Immunol* **174**, 6257-65 (2005).
123. Niyonsaba F, Iwabuchi K, Someya A, Hirata M, Matsuda H, Ogawa H, Nagaoka I. A cathelicidin family of human antibacterial peptide LL-37 induces mast cell chemotaxis *Immunology* **106**, 20-6 (2002).
124. Ito T, Yang M, Wang YH, Lande R, Gregorio J, Perng OA, Qin XF, Liu YJ, Gilliet M. Plasmacytoid dendritic cells prime IL-10-producing T regulatory cells by inducible costimulator ligand. *J Exp Med* **204**, 105-15 (2007).
125. Sano S, Chan KS, Carbajal S, Clifford J, Peavey M, Kiguchi K, Itami S, Nickoloff BJ, DiGiovanni J. Stat3 links activated keratinocytes and immunocytes required for development of psoriasis in a novel transgenic mouse model. *Nat Med* **11**, 43-9 (2005).
126. Lin ZQ, Kondo T, Ishida Y, Takayasu T, Mukaida N. Essential involvement of IL-6 in the skin wound-healing process as evidenced by delayed wound healing in IL-6-deficient mice. *J Leukoc Biol* **73**, 713-21 (2003).
127. Macedo L, Pinhal-Enfield G, Alshits V, Elson G, Cronstein BN, Leibovich SJ. Wound healing is impaired in MyD88-deficient mice: a role for MyD88 in the regulation of wound healing by adenosine A2A receptors. *Am J Pathol* **171**, 1774-88 (2007).
128. Tian J, Avalos AM, Mao SY, Chen B, Senthil K, Wu H, Parroche P,

- Drabic S, Golenbock D, Sirois C, Hua J, An LL, Audoly L, La Rosa G, Bierhaus A, Naworth P, Marshak-Rothstein A, Crow MK, Fitzgerald KA, Latz E, Kiener PA, Coyle AJ. Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. *Nat Immunol* **8**, 487-96 (2007).
129. Okuya K, Tamura Y, Saito K, Kutomi G, Torigoe T, Hirata K, Sato N. Spatiotemporal regulation of heat shock protein 90-chaperoned self-DNA and CpG-oligodeoxynucleotide for type I IFN induction via targeting to static early endosome. *J Immunol* **184**, 7092-9 (2010).
130. Vermi W, Bonecchi R, Facchetti F, Bianchi D, Sozzani S, Festa S, Berenzi A, Cella M, Colonna M. Recruitment of immature plasmacytoid dendritic cells (plasmacytoid monocytes) and myeloid dendritic cells in primary cutaneous melanomas. *J Pathol* **200**, 255-68 (2003).
131. Zabel BA, Silverio AM, Butcher EC. Chemokine-like receptor 1 expression and chemerin-directed chemotaxis distinguish plasmacytoid from myeloid dendritic cells in human blood. *J Immunol* **174**, 244-51 (2005).
132. Vanbervliet B, Bendriss-Vermare N, Massacrier C, Homey B, de Bouteiller O, Brière F, Trinchieri G, Caux C. The inducible CXCR3 ligands control plasmacytoid dendritic cell responsiveness to the constitutive chemokine stromal cell-derived factor 1 (SDF-1)/CXCL12 *J Exp Med* **198**, 823-30 (2003).
133. Pammer J, Reinisch C, Birner P, Pogoda K, Sturzl M, Tschachler E. Interferon-alpha prevents apoptosis of endothelial cells after short-term

exposure but induces replicative senescence after continuous stimulation.

Lab Invest **86**, 997-1007 (2006).

134. Bhartiya D, Sklarsh JW, Maheshwari RK. Enhanced wound healing in animal models by interferon and an interferon inducer. *J Cell Physiol* **150**, 312-9 (1992).
135. Duhon T, Geiger R, Jarrossay D, Lanzavecchia A, Sallusto F. Production of interleukin 22 but not interleukin 17 by a subset of human skin-homing memory T cells. *Nat Immunol* **10**, 857-63 (2009).
136. Isaksson M, Ardesjö B, Rönnblom L, Kämpe O, Lassmann H, Eloranta ML, Lobell A. Plasmacytoid DC promote priming of autoimmune Th17 cells and EAE. *Eur J Immunol* **39**, 2925-35 (2009).
137. Yu CF, Peng WM, Oldenburg J, Hoch J, Bieber T, Limmer A, Hartmann G, Barchet W, Eis-Hübinger AM, Novak N. Human plasmacytoid dendritic cells support Th17 cell effector function in response to TLR7 ligation. *J Immunol* **184**, 1159-67 (2010).
138. Boniface K, Bernard FX, Garcia M, Gurney AL, Lecron JC, Morel F. IL-22 inhibits epidermal differentiation and induces proinflammatory gene expression and migration of human keratinocytes. *J Immunol* **174**, 3695-702 (2005).
139. Eyerich S, Eyerich K, Pennino D, Carbone T, Nasorri F, Pallotta S, Cianfarani F, Odorisio T, Traidl-Hoffmann C, Behrendt H, Durham SR, Schmidt-Weber CB, Cavani A. Th22 cells represent a distinct human T cell subset involved in epidermal immunity and remodeling. *J Clin Invest* **119**,

3573-85 (2009).

140. Zheng Y, Danilenko DM, Valdez P, Kasman I, Eastham-Anderson J, Wu J, Ouyang W. Interleukin-22, a T(H)17 cytokine, mediates IL-23-induced dermal inflammation and acanthosis. *Nature* **445**, 648-51 (2007).
141. van der Fits L, van der Wel LI, Laman JD, Prens EP, Verschuren MC. In psoriasis lesional skin the type I interferon signaling pathway is activated, whereas interferon-alpha sensitivity is unaltered. *J Invest Dermatol* **122**, 51-60 (2004).
142. van der Fits L, Mourits S, Voerman JS, Kant M, Boon L, Laman JD, Cornelissen F, Mus AM, Florencia E, Prens EP, Lubberts E. Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. *J Immunol* **182**, 5836-45 (2009).
143. Zaba LC, Cardinale I, Gilleaudeau P, Sullivan-Whalen M, Suárez-Fariñas M, Fuentes-Duculan J, Novitskaya I, Khatcherian A, Bluth MJ, Lowes MA, Krueger JG. Amelioration of epidermal hyperplasia by TNF inhibition is associated with reduced Th17 responses. *J Exp Med* **204**, 3183-94 (2007).

Vita

Josh Douglas Gregorio was born in the small California coastal town of Arcata on January 23, 1977. As the eldest of eight children Josh longed to leave the house for the city lights. As soon as he graduated from McKinleyville High School in 1995 Josh headed to Los Angeles to learn about biology and more importantly about himself at the University of Southern California. However, Josh spent enjoyed life in the big city a little too zealously and returned home the following year. After contemplating the meaning of life and whether to follow his father's footsteps into the local sawmill or work on his uncle's Salmon boat, Josh decided to go to the local University and work nights as a security guard. It was in his junior year, in organic chemistry class of all places, where Josh found his niche and the love of his life, Guin, at Humboldt State University in Arcata, CA. After completing his studies and obtaining his Bachelor of Science in Cellular & Molecular Biology and minor in chemistry Josh moved to the San Francisco Bay Area to work in the biotech industry. During his employment at Dynavax Technologies Josh attended law school at The University of the Pacific, McGeorge School of Law in Sacramento, CA and received his Juris Doctor. While working at Dynavax under the supervision of Bob Coffman Josh became very interested in Immunology and enrolled in graduate school at The University of Texas Health Science Center at Houston in 2005. In 2006, Josh joined the laboratory of Michel Gilliet in the Department of Immunology at MD Anderson Cancer Center to begin his PhD training. In his spare time Josh

enjoys teaching his son to be the next elite golfer, working out, and playing World of Warcraft.

PUBLICATIONS

Gregorio J, Meller S, Conrad C, DiNardo A, Homey B, Barrat F, Lauerma A, Gallo RL, DiGiovanni J, Gilliet M. Plasmacytoid DC sense nucleic acids in injured skin and promote the wound healing response by producing type I interferons. *J Exp Med*. in press.

Lande R, Ganguly D, Facchinetti V, Gregorio J, Frasca L, Conrad C, Chamilos G, Sebasigari R, Riccieri V, Bassett R, Amuro H, Fukuhara S, Liu YJ, Ito T, Gilliet M. Neutrophils release DNA-antimicrobial peptide complexes that activate pDC and trigger autoimmunity in SLE. Manuscript submitted.

Chamilos G, Ganguly D, Lande R, Gregorio J, Meller S, Goldman WE, Gilliet M, Kontoyiannis DP. Generation of IL-23 producing dendritic cells (DCs) by airborne fungi regulates fungal pathogenicity via the induction of T(H)-17 responses. *PLoS One*. **5**(9):e12955 (2010).

Ganguly D, Chamilos G, Lande R, Gregorio J, Meller S, Facchinetti V, Homey B, Barrat FJ, Zal T, Gilliet M. Self-RNA-antimicrobial peptide complexes activate human dendritic cells through TLR7 and TLR8. *J Exp Med* **206**, 1983-94 (2009).

Lande R, Gregorio J, Facchinetti V, Chatterjee B, Wang YH, Homey B, Cao W, Wang YH, Su B, Nestle FO, Zal T, Mellman I, Schröder JM, Liu YJ, Gilliet M.

Plasmacytoid dendritic cells sense self-DNA coupled with antimicrobial peptide. *Nature* **449**, 564-69 (2007).

Ito T, Yang M, Wang YH, Lande R, Gregorio J, Perng OA, Qin XF, Liu YJ, Gilliet M. Plasmacytoid dendritic cells prime IL-10-producing T regulatory cells by inducible costimulator ligand. *J Exp Med* **204**, 105-15 (2007).

Barrat FJ, Meeker T, Gregorio J, Chan JH, Uematsu S, Akira S, Chang B, Duramad O, and Coffman RL. Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus. *J Exp Med* **202**, 1131-39 (2005).

Duramad O, Fearon KL, Chang B, Chan JH, Gregorio J, Coffman RL, and Barrat FJ. Inhibitors of TLR-9 Act on Multiple Cell Subsets in Mouse and Man In Vitro and Prevent Death In Vivo from Systemic Inflammation. *J Immunol* **174**, 5193-200 (2005).

Marshall JD, Fearon KL, Higgins D, Hessel EM, Kanzler H, Abbate C, Yee P, Gregorio J, Cruz TD, Lizcano JO, Zolotarev A, McClure HM, Brasky KM, Murthy KK, Coffman RL, and Van Nest G. Superior Activity of the Type C Class of ISS In Vitro and In Vivo Across Multiple Species. *DNA Cell Biol* **24**, 63-72 (2005).

Marshall JD, Hessel EM, Gregorio J, Abbate C, Yee P, Chu M, Van Nest G, Coffman RL, and Fearon KL. Novel chimeric immunomodulatory compounds containing short CpG oligodeoxyribonucleotides have differential activities in human cells. *Nucleic Acids Res* **31**, 5122-33 (2003).

Fearon K, Marshall JD, Abbate C, Subramanian S, Yee P, Gregorio J, Teshima G, Ott G, Tuck S, Van Nest G, and Coffman RL. A minimal human immunostimulatory CpG motif that potently induces IFN-gamma and IFN-alpha production. *Eur J Immunol* **33**, 2114-22 (2003).

Marshall JD, Fearon K, Abbate C, Subramanian S, Yee P, Gregorio J, Coffman RL, and Van Nest G. Identification of a novel CpG DNA class and motif that optimally stimulate B cell and plasmacytoid dendritic cell functions. *J Leukoc Biol* **73**, 781-92 (2003).