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## Immune recognition of self nucleic acids driven by endogenous antimicrobial peptides: role in autoimmunity

Dipyaman Ganguly

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**Immune recognition of self nucleic acids driven by endogenous  
antimicrobial peptides: role in autoimmunity**

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**Immune Recognition of Self Nucleic Acids Driven by  
Endogenous Antimicrobial Peptides: Role in Autoimmunity**

**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**

**Dipyaman Ganguly, MBBS, PhD**

**Houston, Texas**

**May, 2010**

**Dedicated to my parents  
and  
all of you who stood by me**

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## **Immune recognition of self nucleic acids driven by endogenous antimicrobial peptides: role in autoimmunity**

Publication no.: .....

by Dipyaman Ganguly, MBBS, PhD

Supervisor: Michel Gilliet, MD

Innate immune recognition of extracellular host-derived self-DNA and self-RNA is prevented by endosomal seclusion of the Toll-like receptors (TLRs) in the dendritic cells (DCs). However, in psoriasis plasmacytoid dendritic cells have been found to be able to sense self-DNA molecules in complex with the endogenous cationic antimicrobial peptide LL37, which are internalized into the endosomal compartments and thus can access TLR9. We investigated whether this endogenous peptide can also interact with extracellular self-RNA and lead to DC activation. We found that LL37 binds self-RNA as well as self-DNA going into an electrostatic interaction; forms micro-aggregates of nano-scale particles protected from enzymatic degradation and transport it into the endosomal compartments of both plasmacytoid and myeloid dendritic cells. In the plasmacytoid DCs, the self-RNA-LL37 complexes activate TLR7 and like the self-DNA-LL37 complexes, trigger the production of IFN- $\alpha$  in the absence of induction of maturation or production of IL-6 and TNF- $\alpha$ . In contrast to the self-DNA-LL37 complexes, the self-RNA-LL37 complexes are also internalized into the endosomal compartments of myeloid dendritic cells and trigger activation through TLR8, leading to the production of TNF- $\alpha$  and IL-6, and the maturation of the myeloid DCs.

Furthermore, we found that these self nucleic acid-LL37 complexes can be found *in vivo* in the skin lesions of the cutaneous autoimmune disease psoriasis, where they are associated with mature mDCs *in situ*. On the other hand, in the systemic autoimmune disease systemic lupus erythematosus, self-DNA-LL37 complexes were found to be a constituent of the circulating immune complexes isolated from patient sera. This interaction between the endogenous peptide with the self nucleic acid molecules present in the immune complexes was found to be electrostatic and it confers resistance to enzymatic degradation of the nucleic acid molecules in the immune complexes. Moreover, autoantibodies to these endogenous peptides were found to trigger neutrophil activation and release of neutrophil extracellular traps composed of DNA, which are potential sources of the self nucleic acid-LL37 complexes present in SLE immune complexes.

Our results demonstrate that the cationic antimicrobial peptide LL37 drives the innate immune recognition of self nucleic acid molecules through toll-like receptors in human dendritic cells, thus elucidating a pathway for innate sensing of host cell death. This pathway of autoreactivity was found to be pathologically relevant in human autoimmune diseases psoriasis and SLE, and thus this study provides new insights into the mechanisms autoimmune diseases.

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## **Abbreviations:**

AMP – Antimicrobial peptide

DNA – Deoxyribonucleic acid

IFN- $\alpha$  – Interferon- $\alpha$

IL-6 – Interleukin-6

LPS – Bacterial lipopolysaccharide

MDC- Myeloid dendritic cells

NaCl – Sodium chloride

NET – Neutrophil Extracellular Trap

PDC – Plasmacytoid dendritic cells

RNA – Ribonucleic acid

SLE – Systemic lupus erythematosus

ssPolyU – Single stranded poly-uridine

ssRNA40 – Single stranded RNA40

TLR – Toll-like receptors

TNF- $\alpha$  – tumor necrosis factor- $\alpha$

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# **1. General Introduction**

### **1.1. Immune recognition of self vs. non-self: paradigmatic shifts**

The immune system in higher organisms is thought to operate based on some algorithm for discriminating self from nonself. Besides being resident in the body, the 'self' can also be depicted as any bodily molecular entity that is defined early in life and the 'nonself' as any entity that the body encounters later in life. Scads of cerebration has gone into elucidating this basic operating principle of immune system that decides whether any molecular entity will face the immune restraints or not. The experiments, which for the first time proved that the immune system do recognize self and do not mount responses against them, were reported by Paul Ehrlich (Ehrlich P and Morgenroth J, 1957). Since then the most widely accepted model had been the aforesaid discrimination of self from nonself.

The 'self-nonself' (SNS) model proposed by Burnet has been the first well-structured suggestion toward a uniform logistics for the immune operations that proposed that the lymphocytes are the principal architects for building up an immune response — each lymphocyte expresses multiple copies of a single surface receptor specific for a foreign entity; signaling through this surface receptor initiates the immune response, and lymphocytes having receptors that recognize self proteins are deleted early in life (Burnet FM, 1959). The observation that the activated B cells undergo somatic hypermutation later in life and in the process generate potentially hyperreactive clones to result in autoimmunity, required incorporation of some other immune cells for helping the lymphocytes to avoid mounting response out of newly arisen self reactive immune receptors. Thus the introduction of the 'helper T cells' by Bretscher and

Cohn brought the first modification into the SNS model in 1970 (Bretscher P and Cohn M, 1970).

Introducing the notion of 'co-stimulation' was the third and perhaps the most important makeover that the SNS model experienced. Responding to the reports that the T cells show stronger response against foreign cells from the same species than against cells from other species, Lafferty and Cunningham added another cell and another 'signal' to the prevailing model. They coined the term 'co-stimulation' for this newly envisaged signal and suggested that this is species-specific (Lafferty KJ and Cunningham A, 1975). But the suggestion of co-stimulation was not at all fully compatible with the original propositions of the SNS model. Because these second group of cells (named by Lafferty and Cunningham as the 'stimulator cells' and now called the 'antigen presenting cells') do not have antigen-specific recognition receptors.

To incorporate the concept of co-stimulation into the SNS model in a more justifiable way, Janeway suggested that the APCs might be envisaged to have their own self-nonself discrimination modules that recognize some molecular patterns on evolutionarily distant pathogens and thus serving to alert the lymphocytes about the potential pathogenicity of the invading organisms (Janeway CA Jr., 1989). He termed these pathogenic signatures, discerned by the APCs, as the Pathogen Associated Molecular Patterns or PAMPs. He named the corresponding recognition receptors as the Pattern Recognition Receptors or PRRs and proposed them to be encoded in the germ line and highly conserved across the species. According to this model the APCs, which are normally

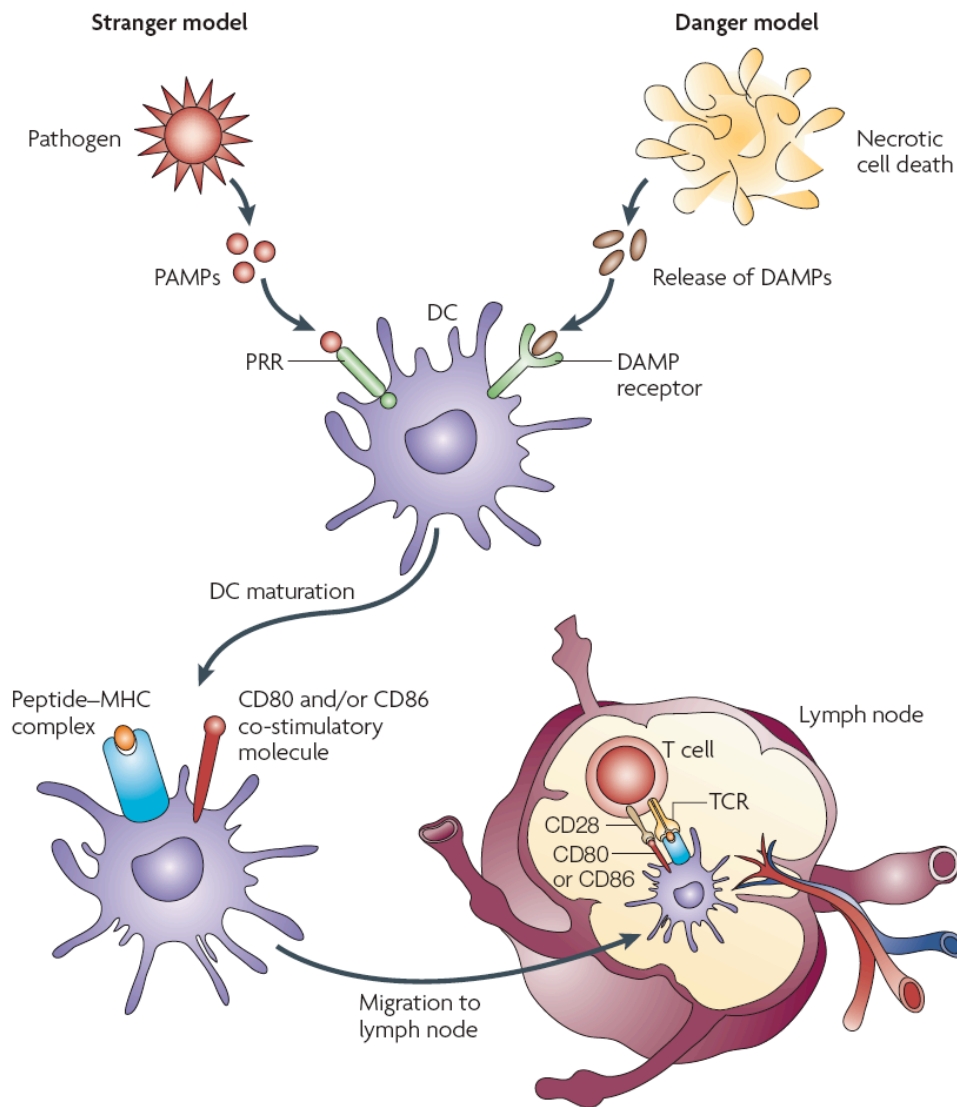
quiescent ('immature'), are activated via these PRRs on recognizing the PAMPs present on the invading organisms and upregulate the expression of the molecular determinants of 'co-stimulation' (the co-stimulatory molecules) on their surface, and offer them to activate the T cells in addition to presenting the processed antigens. Thus PRRs allow the APCs to discriminate between 'infectious non-self' and 'non-infectious self'.

Till date discreet families of pattern recognition receptors have been described, e.g. toll-like receptors (TLRs), RIG-I like receptors (RLRs), NOD-like receptors (NLRs) and C-type lectin receptors (CLRs).

More recently the 'danger' hypothesis proposed by Matzinger rethinks the logistics of immune recognition in order to address few questions left unanswered by the infectious non-self model and it proposes that the immune system is designed to react to tissue damage rather than to foreignness and is alarmed by signals from the injured tissues rather than by nonself recognition (Matzinger P, 2002; Kono H et al, 2008). The immune cells respond in the context of tissue injury (associated with microbial invasion or other endogenous causes) and this activation is triggered by some danger associated molecular patterns (DAMPs) derived from the stressed/damaged host cells (**Figure 1.1**). The potentially immunogenic DAMPs are normally hidden inside the host cells but get access to the exterior in case of host cell death followed by breach of cell membrane integrity.

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Figure 1.1



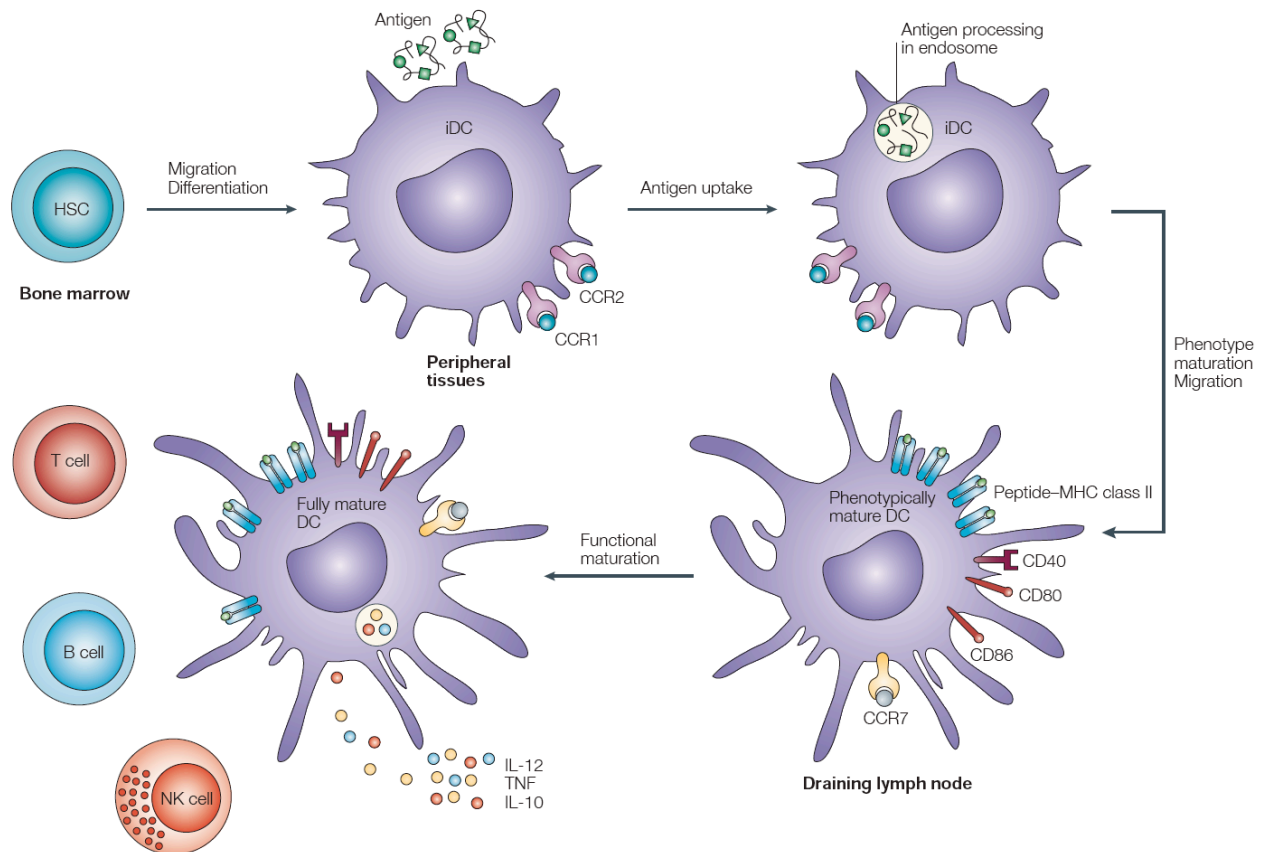
**Figure 1.1. The non-self recognition ('stranger') and 'danger' models of immune recognition.** According to the 'stranger' model of immune recognition, dendritic cells and other antigen presenting cells get activated through triggering of their pattern recognition receptors (PRRs) by different pathogen associated molecular patterns (PAMPs). This leads to expression of the co-stimulatory molecules and production of pro-inflammatory cytokines leading to an effective immune response. On the other hand the 'danger' model proposes that the immune cells only respond to a context of tissue injury (associated with microbial invasion or other endogenous causes) and this activation is triggered by some danger associated molecular patterns derived from the stressed/damaged host cells. (Kono H et al, 2008, reproduced with permission from Nature Reviews Immunology)

## **1.2. Dendritic cells: the immune sentinels**

Dendritic cells are the professional antigen presenting cells with the ability to present antigen to naïve T cells in the context of self-MHC molecules and induce primary immune response. DCs circumvent the challenges of induction of T cell immunity such as lower frequency of antigen-specific T cells and antigen-loaded major histocompatibility complex (MHC) molecules exposed on the pathogen-infected cells, and lack of co-stimulatory molecule expression. DCs are the migrating sentinels that sample antigen from peripheral tissues and carry them to secondary lymphoid organs to present them to specific T cells (**Figure 1.2**). In addition to migrating DCs, DCs that are resident in the lymph nodes can take up soluble antigens that reach the lymph nodes by diffusion through lymphatics (Itano AA et al, 2003), Upregulation of co-stimulatory molecules and chemokine receptors on the DCs in response to pathogen-derived molecules and inflammatory cytokine milieu in the tissue environment is important for the DCs to be able to perform their functions.

Two major subsets have been defined among the dendritic cells. The conventional DCs of myeloid lineage origin are called myeloid DCs (mDCs). The other subset is defined by their ability of rapid production of type I interferons in response to viral infection and because of their microscopic morphological similarity with antibody producing plasma cells they are named as the plasmacytoid DCs (pDCs).

Figure 1.2



**Figure 1.2. Dendritic cells play the immune sentinels in the body.** Dendritic cells, the professional antigen presenting cells in the body, develop from the dendritic cell precursors in the bone marrow and circulate in the blood and sample antigens from different tissue spaces. Encounter of antigens concomitantly with PAMPs is followed by antigen take-up and processing and dendritic cell maturation. Mature DCs with cell surface MHC molecules loaded with processed antigen and expression of co-stimulatory molecules migrate to the draining lymphnodes and present the antigen an co-stimulation to the adaptive immune cells. (Hackstein H et al, 2004, reproduced with permission from Nature Reviews Immunology)

Myeloid DC subset consists of further subtypes with different localization and distinct functions. The mDCs in mouse spleen have two major types that can drive T cell responses differently. The CD8 $\alpha^+$  DC subset induces helper type 1 T cell response, while the other CD8 $\alpha^-$  subset induces helper type 2 responses (Maldonado-Lopez R et al, 1999; Pulendran B et al, 1999). It has also been shown that CD8 $\alpha^+$  DCs preferentially activate antigen-specific CD8 $^+$  T cells, while CD8 $\alpha^-$  DCs preferentially induce CD4 $^+$  T-cell responses (Dudziak D, 2007). On the other hand the human mDCs have been subcategorized into three subtypes based on localization: (1) peripheral tissue resident, (2) secondary lymphoid organ resident mDCs, and (3) circulating blood mDCs.

Among the peripheral tissue resident mDCs, subsets that localize the human skin are found to be members of at least three distinguishable subsets. The first one being the Langerhans cells (LCs) that reside in the epidermis. The mDCs populating the dermis are again either CD1a $^+$  DCs or CD14 $^+$  DCs (Valladeau J et al, 2005). Epidermal and dermal DCs can be distinguished further by their expression of distinct sets of surface proteins. While the epidermal LCs express the lectins Langerin and DCIR, the dermal CD14 $^+$  DCs express a large number of surface C-type lectins, e.g. DC-SIGN, DEC-205, LOX-1, CLEC-6, Dectin-1, and DCIR, and variable TLRs recognizing bacterial PAMPs, e.g. TLR2, 4, 5, 6, 8, and 10 (Klechevsky E et al 2009). Thus the dermal CD14 $^+$  DC seem to be better equipped for bacterial recognition in the skin. Although LCs have been reported to express TLR1, 2, 3, 6, and 10 (Klechevsky E et al 2009), but other groups

have found a limited or no TLR expression on the LCs (Banchereau J et al, 2009).

Initial description of the plasmacytoid dendritic cells (pDCs) termed these cells as plasmacytoid T cells or plasmacytoid monocytes because of their plasma-cell like morphology under the microscope and expression of the T-cell marker CD4 or the myeloid-cell markers MHC class II, CD36 and CD68 (Facchetti F et al, 2000). Later on, virologists and immunologists in the 1980s defined a natural type-I-interferon-producing cell (IPC) owing to its capacity to produce large amounts of type I interferons (IFNs) following culture with viruses and simultaneous expression of class I MHC molecules and revealed them to be the same as the previously described plasmacytoid cells. In 1997 human plasmacytoid dendritic cells were for the first time isolated from peripheral blood and the purified cells were defined as CD4<sup>+</sup>CD11c<sup>-</sup> dendritic cell precursors (Grouard G et al, 1997). Later they were found to produce 200-1000 times more type I interferons compared to other blood cells after microbial challenge (Siegal FP et al, 1999). In 2001, it was reported that pDCs produced type I IFNs in response to CpG-containing DNA (as found in DNA viruses and bacteria) but not to the viral dsRNA-mimic polyinosinic-polycytidylic acid (poly I:C). In contrast, myeloid DCs (mDCs) were shown to produce type I interferons and interleukin-12 (IL-12) in response to poly I:C and they did not respond to CpG-containing DNA or CpG ODNs (Kadowaki N et al, 2001). All of the CpG DNA molecules are not able to activate pDCs to produce type I interferons equally. CpG ODNs are classified into two main types, e.g. D-type (or A-type) CpG ODNs, which induce IFN $\gamma$

IFN $\gamma$  production by natural killer (NK) cells, and K-type (or B-type) CpG ODNs, which induce proliferation and IL-6 and antibody production in B lymphocytes (Verthelyi D et al, 2001; Vollmer J et al, 2004). Now it is established that the A-type CpG ODNs more efficiently induce IFN $\alpha$  production by pDCs that in turn can activate NK cells (Hornung V et al, 2001; Gerosa F et al, 2005; Hanabuchi S et al, 2006), whereas the B type CpG ODNs are much less efficient (Kadowaki N et al, 2001; Vollmer J et al, 2004; Ito T et al, 2006). B type CpG ODNs rather induce pDCs to produce IL-6 and tumor necrosis factor (TNF) and they also can induce proliferation and antibody production in B lymphocytes (Kadowaki N et al, 2001; Vollmer J et al). These studies suggested that pDCs have different signaling pathways to respond to different types of CpG ODN.

Human pDCs circulating in the peripheral blood enter secondary lymphoid tissue through the high endothelial venules (HEV) (Siegal FP et al, 1999). They are defined as lineage-negative HLA-DR-positive cells expressing very high levels of the IL-3 receptor  $\alpha$  chain (or CD123). Some specific markers have also been characterized that are expressed by the pDCs, such as BDCA-2, BDCA-4 and ILT-7 (Cao W et al, 2006). PDCs express a different set of pattern recognition receptors from the myeloid DCs (Kadowaki N et al, 2001). PDCs can recognize viral nucleic acids through TLR7 (recognizes single stranded RNA) and TLR9 (recognizes DNA) and gets activated to rapidly produce large amounts of type I IFN (Kadowaki N et al, 2001).

Dendritic cells can and do contribute in the induction of humoral immunity as well by their capacity to directly activate B cells (Jego G et al, 2005; Qi H et al, 2006). MDCs have been shown to be able to carry captured antigens in subcellular compartments that prevent degradation of the antigens and then present the native antigens to B cells (Bergtold A et al, 2005; Batista FD et al, 2009).

Dendritic cells are also able to induce antigen-specific tolerance in both the central lymphoid organs and in the periphery. The role of thymic DCs in generating tolerance by deleting self-reactive T cells is well documented (Fairchild PJ and Austyn JM, 1990; Volkmann A et al, 1997; Brocker T et al, 1997). Several studies have reported a role for DCs in the induction of peripheral tolerance (Steinman RM et al, 2003; Dhodapkar MV et al, 2001). When immature DCs present very low dose of a soluble antigen in the absence of the specific maturation stimuli, it leads to deletion of the corresponding T cells and unresponsiveness to antigenic re-challenge even with strong adjuvants (Hawiger D et al, 2001). The similar tolerogenic property has also been reported in immature DCs in the context of cell-associated antigen (Hugues S et al, 2002) and dying cells (Liu K et al, 2002). *In vitro* experiments demonstrated that human mDCs with an immature phenotype induce differentiation of anergic regulatory T cells (Jonuleit H et al, 2000; Roncarolo MG et al, 2001). However, other studies in mouse have shown that semi-mature rather than completely immature DCs can tolerize naïve CD4<sup>+</sup> T cells (Kleindienst P et al, 2005). Although yet to be established in humans, reports have already indicated the presence of such tolerogenic DCs in mice *in vivo* (Wakkach A et al, 2003). In mice, tolerogenic

DCs could be induced by treatment of bone marrow cells with IL-10. Mouse tolerogenic DCs derived *in vitro* display plasmacytoid morphology and secrete high levels of IL-10 after stimulation (Wakkach A et al, 2003). The immunomodulatory cytokine IL-10 seems to be a critical determinant in DC-induced hypo-responsiveness in T cells. Again a feedback source of IL-10 from the regulatory T cells in turn may differentiate tolerogenic DCs.

Thus by simultaneously being the inducer of primary immune response to myriad of pathogenic invasion in the body and regulator of immune response in appropriate physio-pathological settings, dendritic cells have emerged to be the major decision making cells harboring the key nodes in the immune circuitry. The decisions involve both identifying the molecular entities they have to respond to and the type of immune response to be initiated.

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### **1.3. Toll-like receptors: discriminating self from non-self**

Toll-like receptors (TLRs) are a family of germline-encoded cell surface pattern recognition molecules containing an ectodomain with leucine-rich repeats, a transmembrane domain and a characteristic cytoplasmic domain called the TIR (Toll/IL-1 receptor) domain. This family was first described in *Drosophila* as the toll receptors, which get triggered in response to bacterial and fungal infections followed by induction of downstream signaling, leading to expression of inflammatory genes like those of the nuclear factor- $\kappa$ B (NF- $\kappa$ B) family of transcription factors and antimicrobial peptides. The first mammalian TLR

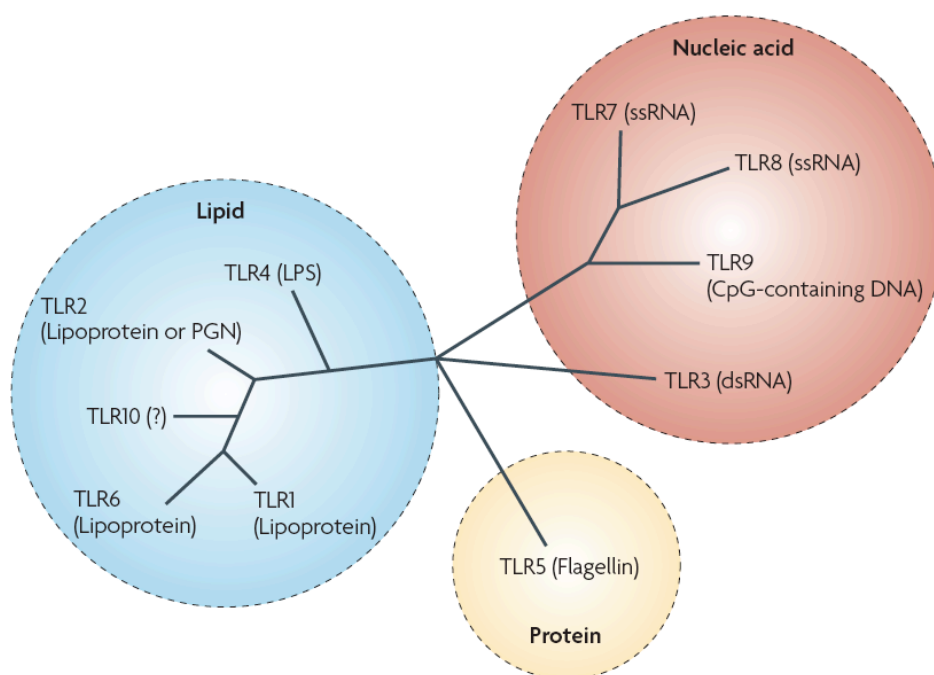
identified was TLR4 (Medzhitov R et al, 1997). Since then a number of other mammalian toll-like receptor family members (11 in human and 12 in mice) have been identified which recognize different molecular patterns on the pathogens.

Major group of the TLRs are expressed on the cell surface. The leucine-rich repeats in the ectodomains of these molecules bind to unique molecular entities on pathogens (PAMPs). For example, the bacterial cell-wall component lipopolysaccharide is a ligand of TLR4 (Medzhitov R et al, 1997; Poltorak A et al, 1998), the bacterial cell wall lipoproteins and peptidoglycans are recognized by TLR2 complexed with TLR1 or TLR6 (Aliprantis AO et al, 1999) and the bacterial flagella protein flagellin is a ligand of TLR5 (Hayashi F et al, 2001). Thus the TLRs, expressed on the host cell surface, detects and initiate responses to invading microorganisms (Akira S et al, 2004).

Another group of TLRs are located inside the cell within the endosomal-lysosomal compartments, instead of being expressed on the cell surface (Akira S et al, 2004). This group comprises of TLR3 (Alexopoulou L et al, 2001), TLR7 (Hemmi H et al, 2001; Lund JM et al, 2004), TLR8 (Heil F et al, 2004) and TLR9 (Hemmi H et al, 2000). The endosomal TLRs are specialized for detecting microbial nucleic acids after microbes get phagocytosed and reach the endosomal compartments (**Figure 1.3**).

Cognate ligands bind the TLRs and initiates downstream signaling through recruitment of TIR domain containing intracellular adaptor molecules. This leads to the formation of multi-component signal transduction complexes in the cytosol.

Figure 1.3



**Figure 1.3. The family of toll-like receptors (TLRs) in humans.** A molecular tree placing the different human TLR family members based on sequence homology and ligand specificity. (Gilliet M et al, 2008, reproduced with permission from Nature Reviews Immunology)

There are four major adaptor proteins — Myd88 (or the myeloid differentiation primary-response gene 88), TIRAP (or the TIR-domain containing adaptor protein), TRIF (or the TIRAP inducing IFN $\beta$ ) and TRAM (or the TRIF-related adaptor molecule). The adaptor molecules interact with the TLRs through the TIR domains, with different combinations of the adaptors taking part in this interaction in case of specific TLRs (Akira S et al, 2004). This TLR-adaptor molecule interactions in turn recruit other proteins to the signaling complex, which initiates multiple downstream signaling pathways, leading to activation of NF $\kappa$ B or mitogen-activated protein kinases (MAPKs) or recruitment of the IFN regulatory factors (IRFs). These different pathways in turn result in the transcription of genes encoding different cytokines, chemokines, co-stimulatory molecules or other proteins, thereby sculpting the ensuing immune response (Akira S et al, 2004).

MyD88 serves as the key adaptor molecule for almost all the TLRs and it recruits IRAKs or the IL-1-receptor associated kinases to these TLRs (except TLR3) and to the IL-1 receptor. Accordingly, MyD88-deficient mice fail to respond to IL-1 or microbial components recognized by TLR2, TLR4, TLR5, TLR7 or TLR9 (Adachi, O. et al, 1998; Akira S et al, 2004). Among the TLRs that use MyD88, TLR7 and TLR9 wholly depend on MyD88. TLR2 can use both MyD88 and TIRAP. On the other hand TLR4 signals through recruitment of MyD88, TIRAP, TRIF and TRAM (Akira S et al, 2004).

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#### **1.4. Immune recognition of nucleic acids: pathogens vs. the host**

After it was discovered that bacterial DNA could stimulate cells of the mammalian immune system through TLR9 (Hemmi H et al, 2000), it was closely followed by the discovery of a group of related TLRs residing in the intracellular endo-lysosomal compartments. These TLRs, TLR3, TLR7, TLR8 and TLR9, have significant sequence homology among them (Wagner H, 2004), and recognize the nucleic acids. TLR9 was reported to detect DNA molecules with unmethylated CpG-containing motifs commonly found in the viral and bacterial genomes (Hemmi H et al, 2000; Bauer S et al, 2001). Synthetic oligonucleotides rich in CpG dinucleotides have been shown to interact directly with the ectodomain of TLR9 inducing conformational changes in the receptor, leading to downstream signaling (Latz E et al, 2007). TLR7 and TLR8 recognize guanosine- or uridine-rich single stranded RNA (ssRNA) molecules from viruses, the synthetic imidazoquinoline compounds (imiquimod and resiquimod) and guanosine analogues (Lund JM et al, 2004; Heil F et al, 2004). TLR3 recognizes double stranded RNA molecules of viral origin and its synthetic mimic polyI:C (Akira S et al, 2004).

Some other independent sensory systems other than the TLRs, that can also detect nucleic acids, not in the endosomes but in the cytosol, have recently been discovered. Three genes identified both in humans and mice encode a group of such proteins known as the RIG-I-like receptors (or RLRs) — RIG-I (or the retinoic-acid-inducible gene I), MDA5 (or the Melanoma differentiation-associated

antigen 5) and LGP2 (or the Laboratory of genetics and physiology 2) (Yoneyama, M et al, 2007; Stetson DB et al, 2006). All of the RLRs are DExD/H box RNA helicases. RIG-I and MDA5 also contain an N-terminal CARD or the Caspase-recruitment domain. They bind to double stranded RNA molecules poly(I:C), poly(A:U) (Yoneyama M et al, 2005) and, 5'-triphosphate RNA in case of RIG-I (Hornung V et al, 2006; Pichlmair A et al, 2006). These molecules are present in viral genomes and viral replication intermediates. All the RLRs recruit a central adaptor molecule called IPS-1 (or the IFNB promoter stimulator 1) resulting in a signaling complex with TRAF3 or the TNF-receptor-associated factor 3, TBK1 or the TRAF-family-member-associated NF- $\kappa$ B activator-binding kinase 1 and IKK $\epsilon$  or the Inhibitor of NF- $\kappa$ B kinase  $\epsilon$ . This complex signals through IRF3 and induce type I IFN expression (Kawai T et al, 2005; Seth RB et al, 2005; Matsui, K. et al, 2006).

More nucleic acids sensing systems are being reported in recent years in mammalian cells. A system of intracellular DNA sensors have been reported in macrophages called the inflammasome, by which cytoplasmic DNA is able to trigger inflammatory responses, through activation of caspases independent of TLRs and IRFs (Muruve DA et al, 2008). Another newly discovered molecule termed DAI (or the DNA-dependent activator of IRFs) is a cytosolic DNA sensor. It binds double stranded DNA molecules, signals through TBK1 and IRF3 and activate NF- $\kappa$ B to induce type I IFN production (Takaoka A et al, 2007). In addition to these nucleic acid recognition systems, RNAs with short stem-loops is

able to activate PKR or the protein kinase R that depends on presence of 5'-triphosphate (Nallagatla SR et al, 2007) and small RNA cleavage products are derived from self RNA through the action of RNase-L activated by 2',5'-linked oligoadenylate. These small RNA fragments can amplify antiviral IFN responses (Malathi K et al, 2007).

The fact that the designated receptors of the innate immune system can differentiate between self nucleic acids from nucleic acids of microbial or other foreign origin has been a long-standing enigma intriguing the immunologists over the years. While the chemical structure of microbial and mammalian nucleic acids is essentially similar, microbial DNA is immunogenic but mammalian DNA is not. Later the heterogeneity of DNA sequence and structure between different species has been revealed. The unmethylated cytidine in CpG motifs of DNA has been shown to be the structural basis of its immune recognition. Thus bacterial DNA rich in unmethylated CpG sequences activate toll-like receptor 9 in the plasmacytoid dendritic cells. But this concept was questioned by a study that showed that artificial relocation of TLR9 on the cell surface of the cells instead of inside the endosomal compartments can enable it to recognize and respond to self-DNA (Barton GM et al, 2006). Thus endosomal seclusion of the DNA-recognizing TLR9 is another way of helping it to discriminate between DNA of self and non-self origin. Viral and bacterial DNA get access to the endosomal compartments in the dendritic cells after being phagocytosed while extracellular self DNA is barred from entering those compartments (Barton GM et al, 2006). Another recent study revealed that the phosphodiester backbone of the DNA

molecule, which is similar in all species, is recognized by the TLR9 (Haas T et al, 2008). Moreover, presence of extracellular nucleases cause fast and efficient degradation of self nucleic acids released into the extracellular spaces in the body and prevent them from accessing the immune receptors. Thus, at least in case of DNA, the discrimination between the nucleic acid molecules of self and pathogenic origin is in effect through other important parameters, beyond the structural differences in the molecules, like seclusion from the immune-receptor containing compartments of the immune cells and efficient extracellular degradation.

Mammalian cells have 5-10 times more RNA than DNA (Kariko K et al, 2005). Also the antigen presenting cells are equipped with toll-like receptors that can recognize viral RNA. TLR7 and TLR8 in plasmacytoid DCs and myeloid DCs are able to recognize single stranded RNA species. On the other hand TLR3 in myeloid DCs recognizes double stranded RNA. Thus the issue of discriminating self-RNA from microbial RNA is similarly, if not more, important just like DNA. A lot of studies have looked into whether the immunogenicity of RNA is also regulated by means of structural modifications like DNA. RNA is known to undergo different nucleoside modifications (Rozenski et al., 1999) that depend on the types of RNA and where the organism in question belongs on the evolutionary ladder. Human ribosomal RNAs (rRNAs) have been shown to contain 10 fold more pseudouridine and 25 fold more 2'-O-methylated nucleosides than ribosomal RNA molecules of bacterial origin. Interestingly, rRNAs from human mitochondria, which is thought to be a remnant of eubacteria

(Margulis and Chapman, 1998), show very few modifications (Bachelierie and Cavaille, 1998). Also mammalian messenger RNAs (mRNAs) have been shown to undergo different modifications, like 5-methylcytidine, N6-methyladenosine, inosine, 2'-O-methylated nucleosides, N7-methylguanosine in contrast to bacterial mRNA that shows no such nucleoside modifications (Bokar and Rottman, 1998). Also a lot of these nucleoside modifications are unique to in either microbial or mammalian RNA. Thus these may constitute an important molecular pattern for the toll-like receptors to distinguish between RNA of self or microbial origin. A recent study, that aimed at elucidating the role of nucleoside modifications on the immunogenicity of RNA by using modified RNA species, has been shown that modifying U, A, and C nucleosides can suppress the RNA induced activation of human dendritic cells (Kariko K et al, 2005). In the same study different TLRs were found to be responsive to RNA differently based on modifications present and unmodified RNA molecules could activate all of them, thus making a strong case for the RNA modifications to be the ultimate determinants for origin discrimination and immunogenic potential. But other studies have shown that modified nucleosides are also present in the many viral RNAs like influenza, adeno, and herpes simplex and content of modified nucleosides were found to be more in viral than in host cell mRNAs (Bokar and Rottman, 1998). Thus nucleoside modification by itself cannot wholly explain the self-nonself discrimination for ribonucleic acid molecules.

Keeping in mind all the experimental data, generated to explain how nature conjures up an efficient system to recognize and respond to nucleic acid

molecules of microbial origin while preventing autoreactivity, perhaps it will be more prudent to think of a multipronged regulatory algorithm, based on mechanisms like barring self nucleic acids from accessing the responsive receptors, differential structural modifications and extracellular clearance of the released self nucleic acids etc., which work together toward achieving a more secure self-nonself discrimination.

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### **1.5. Cell death sensing: ‘danger’-driven immune activation**

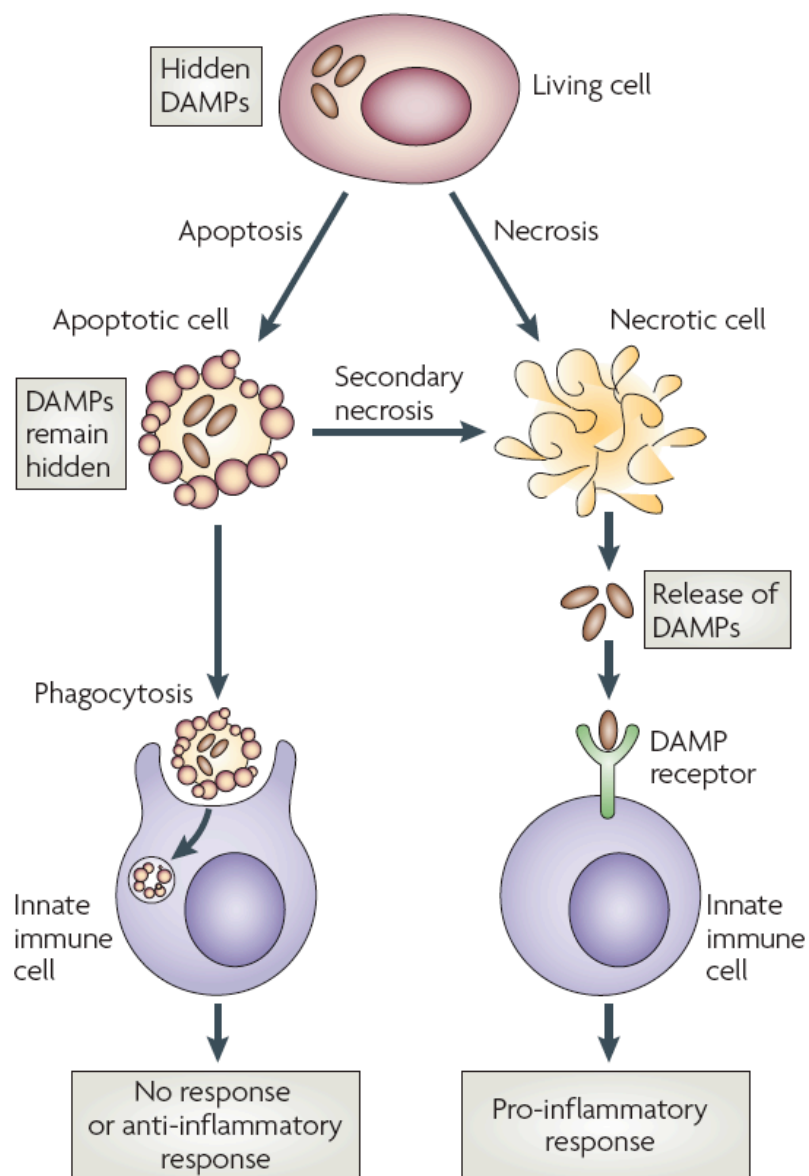
Association of inflammatory response with tissue injury is well appreciated. Mechanistically this association depends on different phenomena encountered in injury. More often tissue injury is complicated by microbial invasion and that leads to the usual innate and adaptive immune activation to the non-self entities associated with microbes and in the process body tries to get rid of those invading microbes. On the other hand, sterile tissue injury in the absence of microbial presence is also found to incite immune activation and inflammation. The inflammatory events associated with sterile tissue injury serves homeostatic purposes. Vasodilatation that follows local tissue injury play a role in increasing blood perfusion at the site of injury, thereby delivering blood-borne immune cells and defense mediators to combat possible pathogen invasion. The increased blood flow also helps to dilute and drain away the potential chemical mediators of injury. Upregulated local expression of chemokines and vascular adhesion molecules attract leukocytes to the site of injury that in addition to combating

potential microbial invasion, play a role in sequestering the injurious agents to prevent spread of injury, and to scavenge the cellular debris generated as a result of cell death at the site of injury. Finally a local inflammation following tissue injury serves to facilitate the repair mechanisms. The inflammatory response in tissue injury results either from release of biochemical mediators from the injured or dying cells or through activation of hemostatic pathways following injury to blood vessels.

Although tissue injury and consequent cell death induces inflammatory responses in the body, they are desired to be short lived. Release of intracellular contents following host cell death poses the danger of presenting certain self-molecular entities to the immune cell-associated pattern recognition receptors, that might result in autoimmune activation. One of the important examples of such self-molecules is the self nucleic acids released from dying cells. To prevent exposure of such dead cell-derived potential immune mediators robust clearance mechanisms are in place. The potential fall-out of the release of these mediators following cell death is established through instances of autoimmune activation in the context of clearance mechanism deficiency disorders. These include phagocytic functions of recruited macrophages and neutrophils as well as the extracellular nucleases and other enzymes.

In addition to injury-induced cell death, normal embryogenesis, development and homeostasis are associated with continuous turnover of host cells though out the life of the organisms. The physiological cell death mostly occurs through apoptosis or the programmed cell death in contrast to the necrotic cell death in

Figure 1.4



**Figure 1.4. Necrotic cell death is immunogenic due to the exposure of the cell associated danger signals.** According to the 'danger' model the immune cells are activated in the context of tissue injury (associated with microbial invasion or other endogenous causes) and this activation is triggered by some danger associated molecular patterns derived from the stressed/damaged host cells. The exposure of these DAMPs occurs in the necrotic form of host cell death (the 'pathological' cell death) due to release of intracellular contents following breach of cell membrane integrity. In contrast to this in apoptosis (the 'physiological' cell death) intracellular contents of the dying cells are released enclosed in membrane-bound vesicles which are efficiently cleared by phagocytic cells thus preventing immune recognition and activation. (Kono H et al, 2008, reproduced with permission from Nature Reviews Immunology)

response to tissue injury. In apoptotic cell death the cell shrinks, chromatin condenses, DNA gets fragmented, and then the cellular components are encased in membrane-bound apoptotic vesicles. These apoptotic vesicles are then rapidly and efficiently cleared by the recruited phagocytes. The process is highly regulated and the plasma membrane of the dying cells remains intact, thereby preventing release of intracellular components with potential immune-activating properties, into the extracellular space (**Figure 1.4**). On the contrary, necrotic cell death in response to infection or bodily injury is associated with release of cytosolic and nuclear materials, including nucleic acids, into the surrounding tissue spaces, resulting in immune cell recruitment and immune activation (**Figure 1.4**). These different categories of cell death seem to be a teleologically viable strategy, whereby a potent immune activation ensues in response to pathological cell death, preventing immune activation to physiological cell death. However, deficient or delayed clearance of apoptotic cells makes the process continue to secondary necrosis characterized by loss of plasma membrane integrity and release of intracellular contents. Secondary necrosis thus poses the danger of triggering pro-inflammatory immune activation.

Recent studies have identified a number of molecules of self-origin that get released in tissue injury and cell death and alert the immune system about the impending or ongoing 'danger'. To appreciate similarity of these molecules with the PAMPs, or the molecular patterns on pathogens that incite immune response in case of a foreign invasion, at times these self-molecules are termed 'DAMPs' or the Danger Associate Molecular Patterns (Kono H et al, 2008). A bona fide

DAMP molecule should fulfill certain criteria like being active in pure form (activity not due to contaminating PAMPs) and active at patho-physiologically relevant concentrations. A number of candidates for such molecules have been proposed and identified over the past several years. Some of the established DAMPs are heat shock proteins (Basu S et al, 2000), uric acid (Shi Y et al, 2003), the high mobility group box 1 protein or HMGB1 (Lotze MT et al, 2005) and double stranded genomic DNA (Lande R et al, 2007).

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### **1.6. Psoriasis: cutaneous autoimmunity**

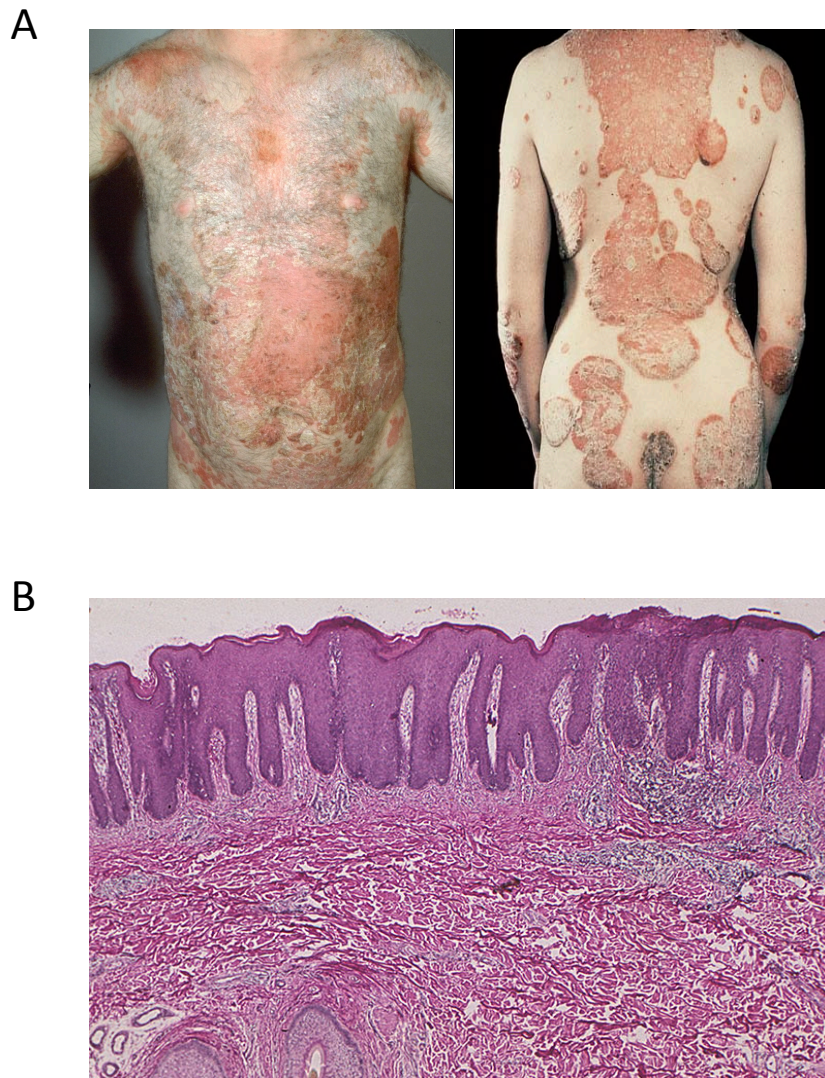
Psoriasis is a chronic, inflammatory disease of the skin characterized by sharply demarcated scaly erythematous plaques. It is one of the longest known illnesses of humans. On the other hand it is one of the most elusive and misinterpreted one. Psoriasis was frequently confused as a type of leprosy due to resemblance in morphology. The Greeks used the term 'lepra' (λεπρα) for scaly skin conditions and the term 'psora' to describe itchy skin conditions. In the late 18th century it came to be called as Willan's lepra after the English dermatologists Robert Willan and Thomas Bateman who perhaps for the first time differentiated it from other skin diseases. Thus it has always been visually, and later semantically, confused with leprosy and it was not until 1841 that the Viennese dermatologist Ferdinand von Hebra gave the name psoriasis for this disease, derived from the Greek word psora which means to itch (Freedberg, et al., 2003). During the 20th century studies identified specific types of psoriasis of which there are five major types:

plaque, guttate, inverse, pustular and erythrodermic. Plaque psoriasis, the most common form of the disease, is characterized by raised, red patches or lesions covered with silvery white scales of dead keratinocytes. Psoriasis can occur on the skin of any part of the body (**Figure 1.5**). Histological features of psoriasis characteristically show epidermal thickening due to hyper-proliferating keratinocytes, elongation of dermal papillae or dermal acanthosis, neoangiogenesis and immune cell infiltration (**Figure 1.5**).

Estimated prevalence of psoriasis is around 2% worldwide, but this data comes from studies from a few populations. A comprehensive study done in the Faroe Islands reported a prevalence of 2.8% (Lomholt G, 1964) while central Europe has a prevalence of approximately 1.5 percent (Farber EM et al, 1998). Ethnic factors have also been implicated in psoriasis. For example, no cases are found in the Samoan population while a prevalence of 12 percent is noted in Arctic Kasach'ye (Farber EM et al, 1998). Also in the United States, the prevalence among blacks (0.45-0.7%) (Kenney JA, 1971) is insignificant compared to the remainder of the U.S. population (1.4-4.6%) (Christophers E, 2001).

Family studies for psoriasis indicates a genetic predisposition to psoriasis with albeit unclear inheritance pattern (Henseler T et al, 1985). Population studies clearly indicate that the incidence of psoriasis is greater among first-degree and second-degree relatives of patients than among the general population (Farber EM et al, 1974). In other studies the concordance rate among monozygotic twins was found to be around 70%, as compared to 20% for dizygotic twins, bringing further supports for genetic predispositions (Farber EM et al, 1974; Brandrup F et

Figure 1.5



**Figure 1.5. Macroscopic and microscopic features of psoriatic skin. (A)** Characteristic chronic psoriatic lesions with scaly plaques. **(B)** Microscopic histology of lesional skin of psoriasis stained with H&E showing characteristic hyperproliferation of epidermal cells and dermal acanthosis. (Courtesy: Michel Gilliet, MD)

al, 1982). In one study almost 71% of children with psoriasis have a positive family history (Morris A et al, 2001).

There is a well-established notion that the disease course is largely influenced by environmental factors like seasonal changes (in some patients the symptoms improve in the summer and worsen in the winter) and physical trauma (local injuries can trigger exacerbations of the lesions, called Koebner's phenomenon) (Eyre RW et al, 1982). Certain drugs have also been found to induce disease exacerbation but the molecular mechanisms are not fully understood for most of the reactive flares except a few like beta-adrenergic blockers (decrease intra-epidermal cyclic AMP and thereby induce epidermal hyperproliferation) and lithium (induces proinflammatory cytokine production thereby stimulating immune cell recruitment to the skin) (Tsankov N et al, 2000). Events of systemic infection have also been found to induce the disease in some patients. Upper respiratory tract infection by streptococcus is well recognized as a trigger of psoriasis (Owen CM et al, 2000). Also exacerbation or even initial manifestation of psoriasis has been observed in HIV infected patients (Mallon E, 2000). On the other hand there is gross lack of any report showing infection of the involved skin in psoriasis, which is conventionally explained by very high expression of endogenous antimicrobial peptides in the psoriatic epidermis (Davidson DJ et al, 2004).

Pathologic hallmarks of psoriatic lesions are hyperproliferation of keratinocytes, infiltration of activated T cells in the dermis *in situ* production of proinflammatory cytokines (Lowes MA et al, 2007). Gene expression profiling with microarrays in psoriatic skin samples, compared with uninvolved skin samples or other

unrelated cutaneous diseases, revealed transcriptome signatures corresponding to proinflammatory cytokines and chemokines in the lesional skin and molecular markers signifying dendritic cell activation in nonlesional skin. Several members of the IL-1 cytokine family as well as IFN-inducible genes are upregulated in psoriatic lesions of patients (Nomura I et al, 2003; Bowcock AM et al, 2001; Zhou X et al, 2003). Gene expression studies on the peripheral blood mononuclear cells collected from psoriatic patients also reveal a differential gene expression signature when compared to healthy individuals or patients with rheumatoid arthritis, another autoimmune disorder with involvement of joints (Pascual V et al, 2010). Cluster analysis of the transcripts that were downregulated in these studies pointed to certain chromosomal regions, that included the psoriasis susceptibility loci PSORS1 and PSORS2 found through genome-wide linkage studies (Nair RP et al, 1997). Genes that have been attributed to regulation or suppression of immune responses in both innate and adaptive axes were found to be among the downregulated genes in these gene expression studies, reaffirming the proinflammatory milieu associated in this disease (Pascual V et al, 2010).

Plasmacytoid dendritic cells have been found to be instrumental in initiating the disease process in psoriasis. PDCs have been found to infiltrate the lesional skin and type I interferons produced by activated pDCs that populate the lesional skin have been reported to be very important for the disease initiation. IFN $\alpha$  initiates the autoimmune-inflammatory a process characterized by the activation of other immune cells like myeloid DCs, which in turn get matured and stimulate

pathogenic autoreactive T cells (Nestle et al., 2005). But the specific details of these mechanisms are not yet fully elucidated.

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### **1.7. Systemic lupus erythematosus: systemic autoimmunity**

Systemic lupus erythematosus is a systemic autoimmune disease that presents with variable symptoms and signs. Diverse combinations of clinical presentations are seen in SLE patients, like the characteristic butterfly rash on cheek leading to global rash, arthritis, anemia, thrombocytopenia, serositis, nephritis, seizures and also psychosis. As a result in the differential diagnosis of patient presenting with one of these clinical problems SLE is considered, especially in female patients of 15 - 50 years of age, as they are more commonly affected (Rahman A et al, 2008).

SLE has a variable prevalence rate that range from 0.04% among North Europeans to 0.2% among blacks (Johnson AE et al, 1995). In the United States, the number of patients with lupus exceeds 250,000. There has been significant improvement in life expectancy of patients powered by greater understanding of the pathogenesis and available treatment modalities. While the 4-year survival rate in the 1950s was reported to be around 50% (Merrell M et al, 1955), at present the 15-year survival rate of lupus patients has reached almost to 80% (Abu-Shakra M et al, 1995).

Monozygotic twins show a concordance rate of 25% and which among dizygotic twins reaches to around 2% (Sullivan KE, 2000), indicating that genetic predisposition operates but is not sufficient for the disease. Genome-wide scans among families with multiple affected members have identified a lot of genes that may take part in lupus pathogenesis (Wakeland EK et al, 2001). Genes for the MHC molecules, specifically HLA-A1, HLA-B8, and HLA-DR3, have been linked to lupus pathogenesis (Walport MJ et al, 1982). Null alleles leading to deficiency of the early complement components C1q, C2, or C4 are taken to be strong risk factors (Walport MJ, 2002). Genetic loci that promote lupus have also been identified in mouse models of the disease (Morel L et al, 2000; Wakeland EK et al, 2001), designated Sle 1, Sle 2, and Sle 3. The genes they contain regulate immunologic tolerance to nuclear autoantigens, B-cell hyperactivity, and T-cell dysregulation, respectively (Wakeland EK et al, 2001). The Sle 1 cluster contains genes homologous to genes in the 1q21–23 and 1q41 regions in human that are also linked to the human disease. As evident from the examples depicted here, a lot of the genes found to play a role in the disease pathogenesis encode components of the immune system, supporting the notion of an autoimmune pathogenesis.

SLE pathogenesis is characterized by hyperreactive B lymphocytes producing autoantibodies against self-DNA, chromatin, and RNA-associated proteins that can be identified in the blood stream. These circulating autoantibodies form insoluble immune complexes with extracellular host-derived self-nucleic acids

and get deposited in different tissue beds like skin, kidney and brain, which in turn results in local inflammation and tissue damage.

There have been reports that suggest a key role for type I interferons in the initiation and progression of SLE pathogenesis (Theofilopoulos AN et al, 2005). Expression of type I IFNs and downstream genes have significant correlation with the pathogenesis. Type I IFNs drive activation of monocytes that differentiate into mature dendritic cells, which in turn induce and stimulate autoreactive B lymphocytes, finally leading to autoantibody production (Blanco P et al, 2001). Type I IFNs can also enhance B cell receptor signaling (Braun D et al, 2002). Plasmacytoid dendritic cells (pDCs) are the most efficient producers of type I IFNs in the body and activation of these cells have been implicated in SLE pathogenesis. pDCs have been shown to accumulate in tissue lesions and sense self-DNA contained in immune complexes, which is accompanied by reduction in their numbers in circulating blood of patients (Cederblad B et al, 1998; Blomberg S et al, 2001).

According to several recent studies circulating neutrophils also play a key role in SLE pathogenesis. SLE patients have been shown to have much higher frequency of apoptotic neutrophils in the circulation. Frequency of apoptotic neutrophils seems to correlate with the disease progression and the circulating anti-DNA antibodies (Courtney PA et al, 1999; McConnell JR et al, 2002). Thus in SLE patients there is a left shift in granulopoiesis as reflected in the circulating blood with higher numbers of immature neutrophils and gene expression

signature of granulopoiesis that strongly correlates with expression of type I IFN genes and disease activity (Bennett L et al, 2003).

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### **1.8. Antimicrobial peptides: old players with new roles**

Innate immunity forms a first line of defense against infection by thousands of potentially pathogenic microorganisms which the multicellular organisms are continuously exposed to. A group of peptides with antimicrobial activity, present in even most primitive multicellular organisms and thus showing outstanding evolutionary conservation, also take part in the innate immune processes. Since the initial reports of such peptides in plants with antibacterial or antifungal functions in early 1970s (Fernandez de Caley R et al, 1972) a lot of similar peptides has been discovered across the species. A growing interest in these peptide antibiotics originated from the ever-growing resistance the targeted pathogens to the conventional antibiotics and the possibilities of therapeutic applications of these peptides or their synthetic analogues in infectious diseases (Reddy KV et al, 2004). Most of these peptides have a broad range of antimicrobial properties, ranging from antibacterial and antifungal activities to antiviral or anticancer properties in some of them. Moreover over the years they have been found to directly influence different inflammatory processes, like

cellular proliferation, wound healing, induction of cytokines and chemotaxis (Bals R, 2000). Although the antimicrobial peptides are very different in terms of amino acid composition, peptide length and secondary structure, most of them exert the antimicrobial effects by interaction with and disruption of the microbial cell membrane leading to cell death. Different mechanisms that these peptides take to kill microbial pathogens have been proposed such as, inhibition of membrane protein synthesis (Engstrom P et al, 1984; Axen A et al, 1997), interference with stress response (Groisman EA et al, 1996), inhibition of DNA synthesis (Boman HG et al, 1993), production of hydrogen peroxide (Leem JY et al, 1996).

Cathelicidin is a group of antimicrobial peptides containing a conserved signal sequence and a pro-region showing homology to cathelin, a cathepsin L inhibitor, while C-terminal domain shows no conservation (Hancock RE et al, 2000). LL37 is the only human cathelicidin characterized till date. LL37 is small peptide composed of 37 amino acids with a linear structure. Final LL37 peptide forms by cleavage of the C-terminal end of the human CAP18 protein (hCAP18) (Cowland JB et al, 1995; Gudmundsson GH et al, 1996). LL37 has been shown to adopt a random coil structure in a hydrophilic milieu and an alpha-helical conformation in a hydrophobic environment (Turner J et al, 1998).

LL37 is expressed in monocytes, neutrophils, T and B lymphocytes, NK cells and epithelial cells in different parts of the body like skin, testis, gastrointestinal and respiratory tracts (Cowland JB et al, 1995; Gudmundsson GH et al, 1996; Frohm M et al, 1997; Bals R et al, 1998).

Both Gram positive and Gram negative bacteria has been shown to be susceptible to the antimicrobial activity of LL37 (Turner J et al, 1998). LL37 has also been shown to have chemotactic activity for neutrophils, monocytes, mast cells and T cells.

The concentration of LL37 in the body differs among different physiological condition and pathological contexts. For example according to some reports concentration of LL37 in adult sweat is around 1  $\mu\text{M}$ . In the bronchoalveolar lavage fluid of infants also LL37 reaches similar concentrations (Davidson DJ et al, 2004). On the other hand in the context of infections or inflammation expression of this peptide in neutrophils, keratinocytes and in other epithelial cells is enhanced by several folds (Dorschner RA et al, 2001). An example very appropriate for our studies is the cutaneous autoimmune disease psoriasis, where LL-37 concentration in the lesions have been reported to reach a median value of 304  $\mu\text{M}$  (Davidson DJ et al, 2004).

A shared property of most of the antimicrobial peptides described so far is found to be a tendency to adopt an amphipathic conformation with clusters of hydrophobic and cationic amino acids positioned in different spaces of the molecules. The fact that bacterial cell wall is sensitive to these peptides is attributed to a very high content of anionic phospholipid moieties in the outer membrane leaflet of the bacterial cell wall. This is in stark contrast with the outer membrane of cells rich in electro-neutral lipids in higher organisms conferring a relative specificity for the antimicrobial action of these peptides (Ganz T, 2002). A study on bacterial infections with *Shigella* reported an interesting observation of

an inhibition of AMP expression, which was mediated by the plasmid released by the bacteria (Islam D et, 2001). Another study reported that LL-37 can bind to plasmids and target them to the nuclear compartment of mammalian cells followed by the expression of those plasmids (Sandgren S et al, 2004). These studies, along with the cationic amphipathic domains of LL37 molecule and the observation that LL37 is overexpressed in the psoriatic skin lesions led to the previous study of our group where Lande R et al found that LL37 can bind to human DNA and target it to the endosomal compartment in the plasmacytoid dendritic cells leading to their activation through toll-like receptor 9 (Lande R et al, 2007). Thus apart from contributing to innate immune response by direct killing of microorganisms other facets of innate immune function of the antimicrobial peptides has become an interesting area of study.

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## **2. Description of the research study**

## **2.1. Chapter 1: Antimicrobial peptide LL37 binds self ribonucleic acids and forms particulate complexes**

### **2.1.a. Background and Rationale:**

**R**ole of plasmacytoid dendritic cells (pDCs) in the pathogenesis of autoimmune inflammation has been studied in different clinical contexts. The characteristic endosomal toll-like receptors (see section 1.4) in the pDCs that can recognize nucleic acid molecules and a very efficient intracellular signaling machinery for induction of type I interferon gene expression and secretion of these cytokines, equip this subset of dendritic cells with the ability to respond to microbial challenge by producing very high amounts of type I interferons, thus earning them the name professional type I interferon producing cells (see section 1.2). Type I interferon plays a great role in driving antiviral immunity making pDCs the key immune cells in combating such infectious challenges. The discrimination of microbial nucleic acids from self nucleic acids by these TLRs in pDCs (see section 1.4) is conventionally explained by recognition of structural changes in the nucleic acid molecule that differentiate between the molecules in lower organisms from higher organisms. But it has also been shown that endosomal seclusion of the receptors play a vital role in the discrimination process as self nucleic acid can not normally get access to these intracellular compartments (discussed in section 1.4). It has been shown by previous studies in systemic lupus erythematosus (SLE) that self nucleic acids in complex with respective autoantibodies do access the endosomal compartments of pDC via FcγRII-

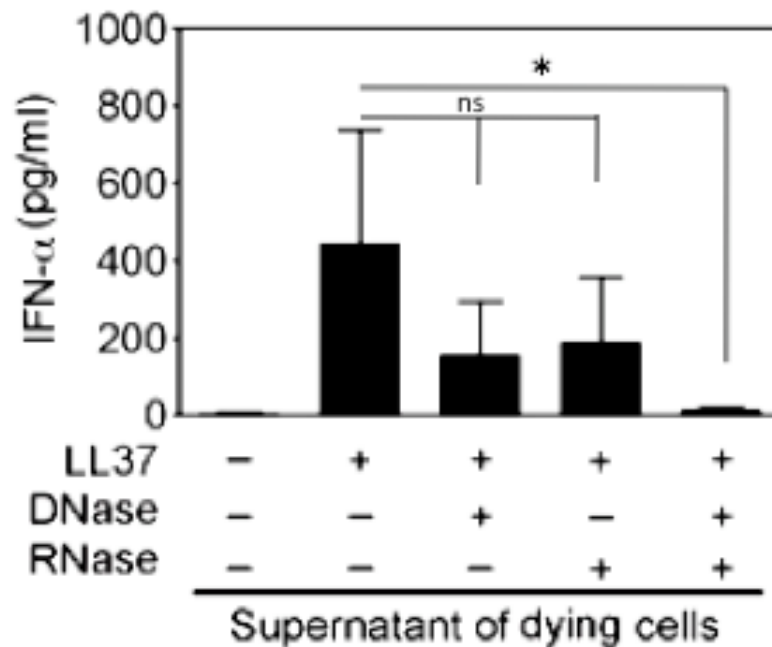
mediated endocytosis (Ronnblom L et al, 2003; Means TK et al, 2005; Barrat FJ et al, 2002). Ensuing pDC activation and production of type I IFNs drives the autoimmune pathogenesis in SLE. This notion has been supported by studies on murine models of SLE in TLR7 or TLR9 deficient mice (Christensen SR et al, 2006; Leadbetter EA et al, 2002), and also by a study that showed TLR7 gene duplication induces a lupus-like disease in mouse (Deane JA et al, 2007).

**P**revious studies done in our lab by other investigators had revealed an instrumental role of pDC-derived IFN $\alpha$  in the initiation of the disease process in the cutaneous autoimmune disease psoriasis (Nestle FO et al, 2005). Assessment of fractionated skin extracts from psoriatic skin to activate pDC cultures revealed a fraction containing the antimicrobial cathelicidin LL37 to be able to achieve that (Lande R et al, 2007). This study found that LL37 peptide can bind to self DNA molecules through an electrostatic interaction between the anionic nucleic acid molecules and the cationic peptides forming complexes. These complexes then are taken up by pDCs into the endosomal compartments where they trigger TLR9 activation resulting in type I IFN production (Lande R et al, 2007). As discussed in the section 1.6, psoriatic skin is known to have a very high expression of endogenous antimicrobial peptides and this fact was cited to explain lack of infections involving psoriatic skin. But Lande R et al showed a new role of the antimicrobial peptide in breaking the innate tolerance of self DNA and activating innate immune cells. The hypothesis implied from these findings was that self nucleic acids extracellularly released by host cell death can bind this antimicrobial peptide when it is around and then get access to the

endosomal compartments in pDCs to trigger TLR activation. Very high cell turn-over in the epidermis and resulting release of self nucleic acids and associated high expression of antimicrobial peptides in psoriasis thus predisposes this clinical condition to such innate immune activation. DNA molecules are relatively less susceptible to fast extracellular degradation than RNA molecules and thus seem to fit into the hypothesis convincingly. Thus access of the host-derived nucleic acids to the relevant TLRs in the host cell can lead to the break of innate tolerance to them and autoreactivity.

To confirm that the antimicrobial peptide LL37 can really enable pDCs to sense native self nucleic acids released by dying cells, we tried to simulate the condition *in vitro*. U937 cells, a human cell-line, were subjected to secondary necrotic process by UV-irradiating the cells and then incubating them overnight at 37°C. PDCs were stimulated with supernatants of these UV-irradiated cells, which undergo apoptosis and secondary necrosis and release self-nucleic acids (Lovgren T et al, 2004). These supernatants induced significant amount of IFN- $\alpha$  in pDCs when mixed with LL37 but not when given alone (**Figure 2.1**), thus confirming ability of LL37 to bind nucleic acids released by cell death and taking them inside pDC endosomes. But interestingly, depletion of DNA from the supernatants by treating the supernatant with DNase could inhibit IFN- $\alpha$  induction only partially but not completely (**Figure 2.1**). That created the possibility that other elements released by the dying cells (possible other nucleic acid species) can also bind to the peptide and work in the same way. Depletion of RNA from the supernatants by treating them with RNase also leads to partial inhibition,

Figure 2.1



**Figure 2.1. Both DNA and RNA released during cell death can activate pDCs in the presence of LL37.** IFN- $\alpha$  produced by pDCs after stimulation with supernatants from dying (UV-irradiated) U937 cells, either alone or premixed with LL37. In some experiments, DNA and/or RNA was depleted from supernatants of dying cells by pretreatment with DNase I (2,000 U/ml) and/or RNase A (50  $\mu$ g/ml). Data are the mean  $\pm$  SEM of four independent experiments. \*p value <0.05, paired Student's T test. ns = not significant.

while the combined depletion of RNA and DNA completely abolished IFN- $\alpha$  induction (**Figure 2.1**). These results indicated that not only self-DNA released by host cell death, but also self-RNA released simultaneously, despite its extreme susceptibility to nuclease degradation, can contribute to the pDC activation in presence of LL37 peptide. These findings led to the present study investigating whether these cationic antimicrobial peptides can bind to ribonucleic acid molecules in the same manner as they bind to DNA and what are the implications of this interaction.

### **2.1.b. Materials and Methods:**

#### **Reagents.**

The synthetic human antimicrobial peptide LL37 (amino acid sequence: LLGDDFRKSKEKIGKEFKRIVQRIKDFLRNLVPRTES), the corresponding scrambled peptide GL37 having same amino acids in different sequence (amino acid sequence: GLKLRFESKIKGEFLKTPEVRFRDIKLDNRISVQR) and Tamra-labeled LL37 were purchased from Innovagen, Lund, Sweden. LyoVec-free single stranded 40 base long phosphothioate linked RNA sequences named ssRNA40 (5'-GsCsCsCsGsUsCsUsGsUsUsGsUsGsUsGsAsCsUsC-3', 's' denotes phosphothioate linkage) was from Invivogen, CA and used at a final concentration of 5  $\mu$ g/ml. Ribogreen RNA quantitation kit was purchased from Molecular Probes, CA. The Ulysis Nucleic Acid Labeling Kit for Alexa 488 fluorophore labeling of nucleic acids was from Molecular probes, CA.

**Isolation of nucleic acids.**

Total human RNA (self-RNA) and genomic DNA (self-DNA) were extracted from cell lines or inflammatory skin samples using standard procedures. In brief, cells were homogenized in Trizol reagent (Invitrogen), followed by phase separation with chloroform. RNA was precipitated from the aqueous phase by isopropanolol (Sigma Aldrich) and was washed in 75% ethanol. DNA was precipitated from the interphase and the organic phase with ethanol followed by wash in 0.1M sodium citrate (Sigma Aldrich) in 10% ethanol.

**Nucleic acid condensation assay**

Ability of the LL37 peptide to bind to ribonucleic acid was assessed by a spectrophotometric assay using the RNA-binding dye Ribogreen. 10  $\mu$ M LL37 peptide was added with 5ug/ml U937 RNA in a volume of 20  $\mu$ l (volume made up by nuclease free water) and was incubated at room temperature for 20 min. The dye Ribogreen for a final dilution of 1:20 (5  $\mu$ l in 80  $\mu$ l) was added in TE buffer. 80 $\mu$ l of the TE buffer with the dye was added to the 20  $\mu$ l of the nucleic acid-peptide suspension, mixed well and put in a cuvette to measure fluorescence in 500nm to 600nm emission wavelength range in a Jasco FP-6300 spectrofluorometer (Jasco Inc., Easton, MD). The data was analyzed on a Jasco Spectra Manager software.

**Generation of nucleic acid-LL37 complexes.**

10 µg of the antimicrobial peptide LL37 or the scrambled peptide GL37 were added with 1 µg of self-RNA or self-DNA in 20 µl of PBS. To look for nucleic acid complexes in the culture supernatant of necrotic cells, U937 cells were UV-irradiated (480 mJ/cm<sup>2</sup> for 30 min) in a UVP TL-2000 ultraviolet crosslinker (UVP, LLC, Upland, CA), washed and then resuspended in complete medium to be cultured overnight (50×10<sup>6</sup>/ml) for inducing secondary necrosis. After harvesting the supernatant by spinning down the necrotic cells at 3000 rpm for 5 min, 100 µl of the supernatant was added with 10 µg of LL37. The supernatant added with LL37 was then incubated at room temperature for 30 min and then spun down to collect the pellet. The pellet was resuspended in 20 µl PBS. Ribogreen (Molecular Probes, CA), a nucleic acid-binding dye sensitive to low concentrations of RNA, was diluted with PBS. To visualize the RNA and DNA complexes the suspensions were added with 1:10000 Ribogreen and 0.1 ng/ml DAPI (Sigma-Aldrich, MO) respectively and incubated at room temperature for 10 min. 5 µl of the suspension was put on a glass slide, covered with a cover slip and visualized by confocal microscopy. In some experiments the supernatant of the necrotic cells after harvesting were first treated with DNase I (200U/ml), RNase A (10 µg/ml) or both together for 1 h at 37° C, and then were added with LL37, to differentiate between and quantitate the RNA and DNA particles being formed in presence of the LL37 peptide.

### **Fluorescence resonance energy transfer.**

Self-RNA was labeled with Alexa 488 fluorophore using the Ulysis Nucleic Acid Labeling Kit according to manufacturer's instruction. Labeled self-RNA (1 ug) was added with Tamra-labeled LL37 (10 ug, procured from Innovagen, Sweden) in a volume 20 ul made with PBS. 5 ul of this nucleic acid-AMP suspension was put on slide, covered with a coverslip and sealed. Image was acquired on a Leica TCS SP2 confocal microscope using 488 nm and 543 nm lasers working in a sequential scanning scheme. Fluorescence resonance energy transfer (FRET) was analyzed based on donor recovery after acceptor photobleaching. Fluorescence intensity of Alexa 488 in selected complexes was imaged at the 488 nm excitation and 496-535 nm emission. TAMRA was photobleached by illuminating a rectangular area (shown in the first panel) with the 543 laser at the maximum power (160 scans), and the complexes were imaged again for Alexa 488 using the same settings. The extent of TAMRA photobleaching was about 80%. FRET analysis was done using the SlideBook 4.2 software (3i, Denver). The calculated FRET efficiency was 96.5% based on the equation  $E = (D_{\text{pre}} - D_{\text{post}}) / D_{\text{pre}}$ , where  $D_{\text{pre}}$  and  $D_{\text{post}}$  are average donor intensities in the areas before and after acceptor photobleaching.

**Scanning electron microscopy.**

50 µg of the peptides LL37 was mixed with 5 µg of self-RNA or ssRNA40 (used as uniformly sized control RNA molecule) in 100 µl of PBS. The self-RNA-LL37 and ssRNA40-LL37 complex suspensions were then taken to High Resolution Electron Microscopy Core Facility at the MD Anderson Cancer Center for scanning electron microscopy. At the core facility the suspensions of the nucleic acid-peptide complexes were air-dried overnight on poly-L-lysine-coated coverslips and then the samples were mounted on to double-stick carbon tabs (Ted Pella Inc., CA), which have been previously mounted on to aluminium specimen mounts (Electron Microscopy Sciences, PA). The samples were then coated under vacuum using a Balzer MED 010 evaporator (Technotrade International, NH) with platinum alloy for a thickness of 25 nm. It was followed by a flash carbon coating. Samples were examined in a JSM-5910 scanning electron microscope (JEOL USA Inc., MA) at an accelerating voltage of 5kV.

**Particle size estimation:**

To assess the size distribution of the nano-scale particles formed by the self-RNA-LL37 complexes we used a particle-sizing instrument Zetasizer Nano (courtesy Malvern Instruments Ltd, Worcestershire, UK) that works based on dynamic light scattering from the particles in suspension. RNA-LL37 complexes were prepared with both self-RNA isolated from U937 cell line and a uniformly sized 40 nt long short sequence of RNA called ssRNA40, which is a TLR7/8

agonist. 100  $\mu$ l of the suspensions in PBS were made with adding 5  $\mu$ g of RNA with 50  $\mu$ g of LL37. Then volume was made to 1 ml in these suspensions with additional PBS and after vortexing the particle size distribution was quantitated in the Zetasizer Nano instrument. The data was analyzed in Zetasizer software provided by Malvern Instruments.

### **Nuclease protection assays.**

10  $\mu$ g of the peptides LL37 or GL37 were mixed with 1  $\mu$ g of self-RNA or self-DNA in 20  $\mu$ l of double distilled water. This suspension containing the RNA-LL37 complexes was then left at room temperature for 30min and then incubated for different time-points with 50  $\mu$ g/ml RNase A at 37°C. Then RNA in the suspensions was quantitated using the fluorometric Ribogreen RNA Assay Kit (Molecular probes, CA). In short, the dye Ribogreen for a final dilution of 1:20 (5  $\mu$ l in 80  $\mu$ l) was added in TE buffer. 80 $\mu$ l of the TE buffer with the dye was added to the 20  $\mu$ l of the nucleic acid-peptide suspension, mixed well and put in a cuvette to measure fluorescence in 500nm to 600nm emission wavelength range. To visualize the RNA and the RNA-peptide suspension and effect of RNase on them in an agarose gel, the naked RNA and the RNA complexes were run on a 1% agarose gel (with 0.005% ethidium bromide). The gel was imaged in a UV transilluminator gel imager (Biorad Laboratories, CA).

### **2.1.c. Results & Discussion:**

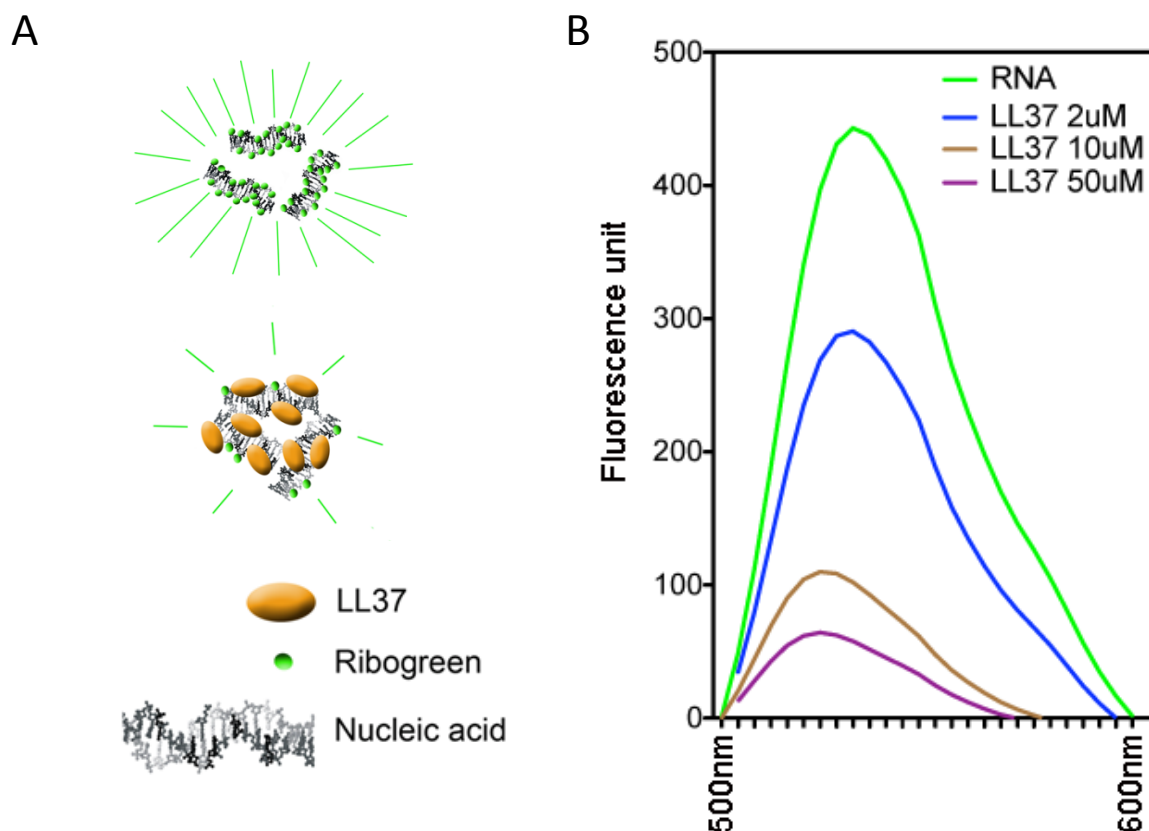
#### **Antimicrobial peptide LL37 binds and condenses self-RNA molecules.**

To investigate whether host-derived RNA can interact with the antimicrobial peptide LL37 to form characteristic complexes similar to DNA we used a fluorometric assay using the nucleic acid binding fluorophore Ribogreen (Jones LJ et al, 1998). Self-RNA alone or added with LL37 was quantitated using this assay. Ribogreen reagent binds naked RNA molecules and the bound fluorophore when illuminated with 488 nm light source gives fluorescence in the range 525nm to 530nm. But if the LL37 peptides bind to and condenses RNA the fluorophore is not able to access the as many binding sites as it would have done if given to naked RNA molecules (**Figure 2.2.A**). Thus the fluorescence detected is diminished. We found that presence of LL37 with self-RNA greatly diminished the Ribogreen fluorescence (**Figure 2.2.B**). With increasing concentration of the peptide there was greater reduction in fluorescence revealing 10 uM peptide concentration to be optimal for the condensation and binding.

#### **Self-RNA-LL37 complexes are visible under microscope.**

Interestingly, when self-RNA purified was added with LL37 to make the complexes we observed formation of particulate precipitates at the bottom of the

Figure 2.2



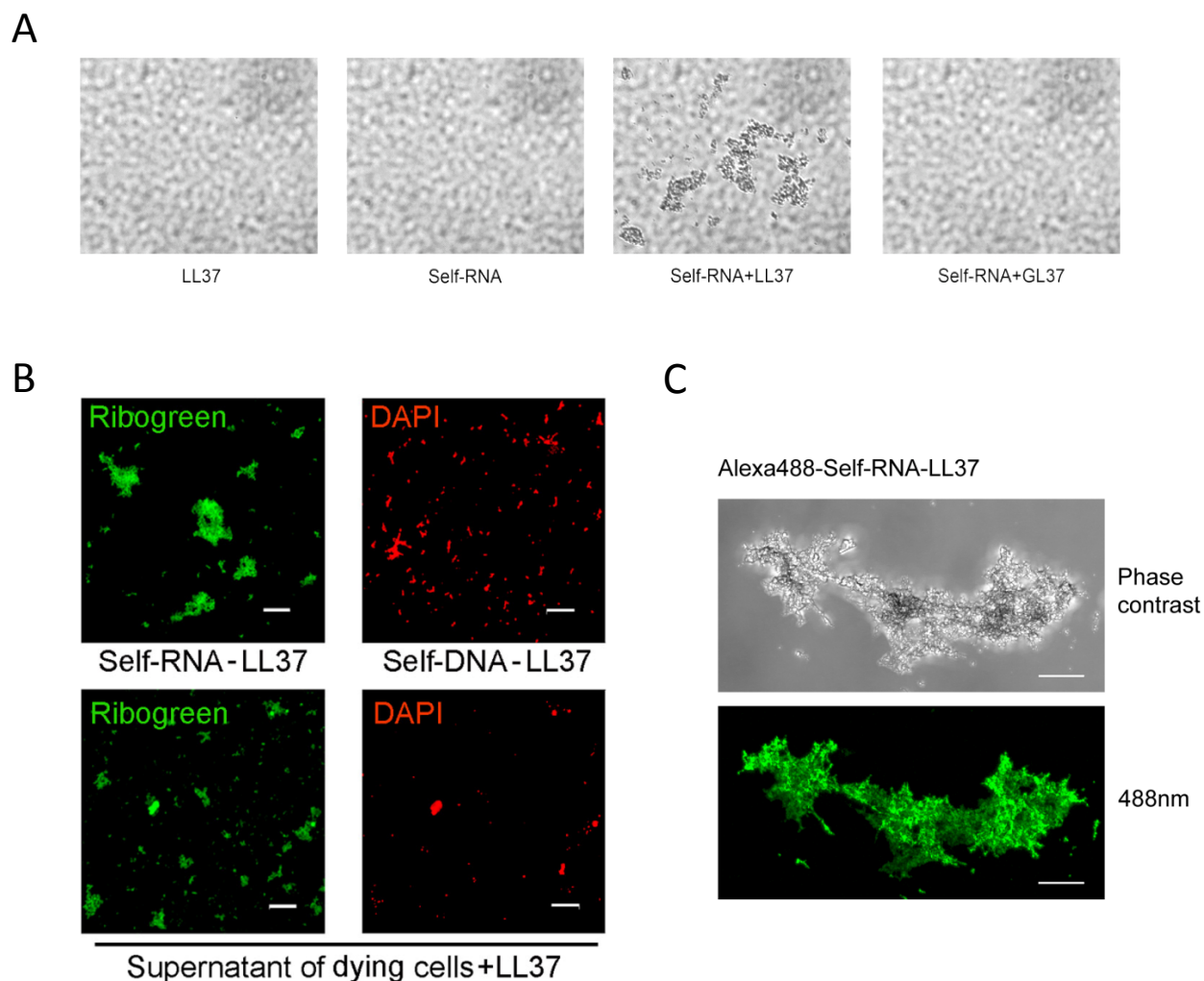
**Figure 2.2. LL37 can bind to self ribonucleic acid molecules and condense them. (A)** Schematic representation of the principle of the fluorometric assay with the RNA-binding dye Ribogreen to assess peptide binding to RNA molecules and resulting condensation leading decrease in Ribogreen binding and fluorescence. **(B)** Indicated amounts of LL37 peptide was incubated with self-RNA (10  $\mu\text{g/ml}$ ) for 30 min and then a fluorometric assay was done with the RNA-binding dye Ribogreen. Data representative of multiple experiments.

culture wells ranging in size from 1–30  $\mu\text{m}$  (**Figure 2.3.A**). But addition of GL37, a scrambled form of the peptide with the same amino acid composition but with a different sequence, did not form these particulate complexes. On fluorescence microscopy the visible particulate complexes fluoresced when stained with Ribogreen (stains RNA preferentially, but also DNA) but not with DAPI (stains DNA exclusively) showing they contained RNA (**Figure 2.3.B**). Self-DNA when complexed with LL37 also formed these particles and they could be stained with DAPI (**Figure 2.3.B**).

We wanted to see whether similar particles also form in the supernatants collected from the dying cells undergoing secondary necrosis when added with the LL37 peptide. When added with LL37 but not GL37, the supernatants of dying U937 cells also formed similar particulate complexes. A large number of these complexes contained RNA as they stained as Ribogreen<sup>+</sup> DAPI<sup>-</sup> (**Figure 2.3.B**) and because these particles were not seen with RNase pretreatment of the supernatants to specifically deplete free RNA (not shown). Number of visible RNA particles was more than DNA particles, identified as DAPI<sup>+</sup> particles (**Figure 2.3.B**). Predominance of RNA particles in the supernatants from necrotic cells is supported by the fact that mammalian cells contain much more RNA than DNA (Kariko K et al, 2005). When imaged in high magnification these RNA-LL37 complexes had a characteristic morphology with aggregation and branching of bead-like structures (**Figure 2.3.C**).

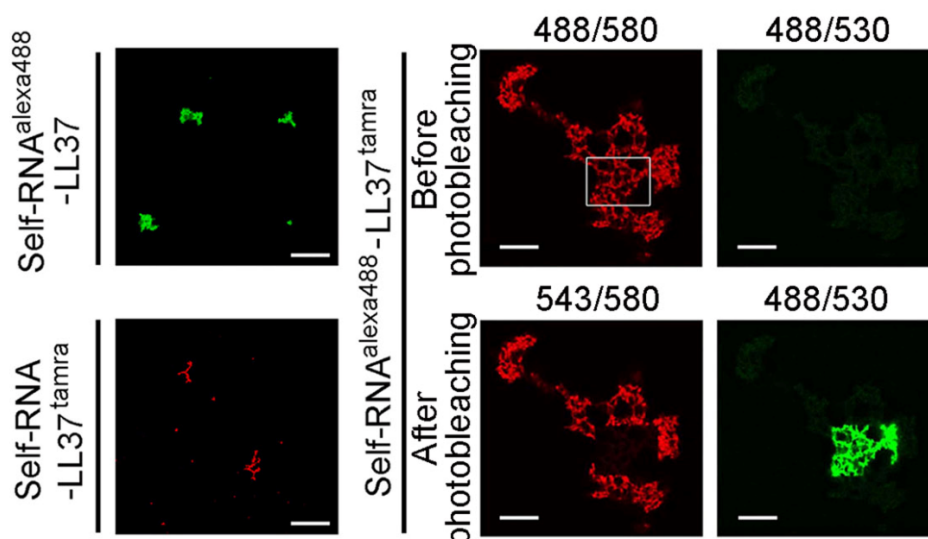
To prove that these microscopically visible particulate complexes indeed form by molecular interaction between RNA and LL37 we used a fluorescence resonance

Figure 2.3



**Figure 2.3. LL37 binds self-RNA and forms aggregated particles.** (A) Characteristic particle formation in culture plates by self-RNA and LL37 as seen under a light microscope at 40X. The scrambled peptide GL37 fails form such complexes. (B) Confocal microscopy images of self-RNA–LL37 and self-DNA–LL37 complexes generated in vitro (top panels) or formed by mixing supernatant of dying U937 cells with LL37 (bottom panels) and stained with Ribogreen (which stains both RNA and DNA) and DAPI (which stains DNA exclusively). RNA and DNA complexes were detected as Ribogreen<sup>+</sup>/DAPI<sup>-</sup> and Ribogreen<sup>+</sup>/DAPI<sup>+</sup> complexes, respectively. Bar, 20  $\mu$ m. (C) Complexes formed by Alexa 488-labeled self-RNA and the LL37 peptide showing characteristic branched aggregation of bead-like structures. Imaged in confocal microscope, bar=5 $\mu$ m.

Figure 2.4



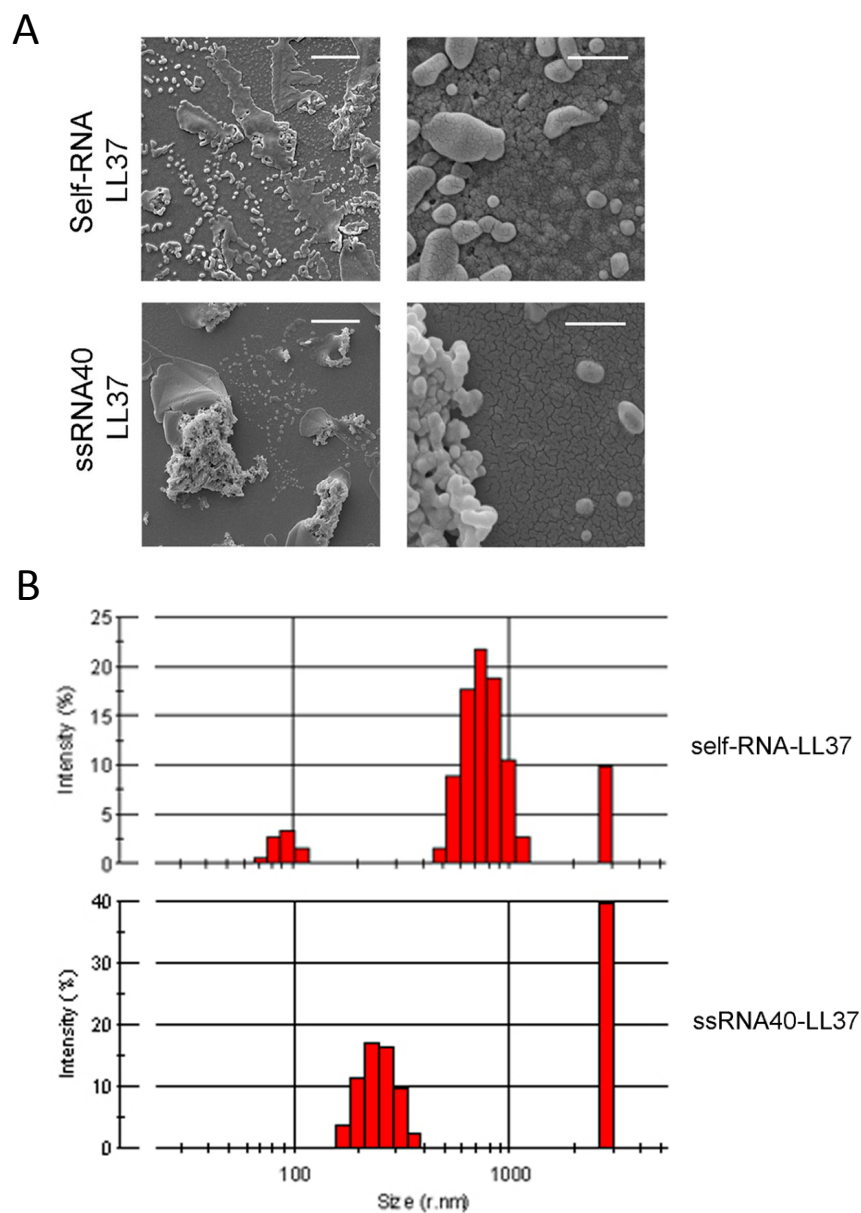
**Figure 2.4. The microscopically visible complexes form by molecular interaction between self-RNA and LL37.** Confocal microscopy of self-RNA<sub>Alexa488</sub>-LL37 (top left panel) and self-RNA-LL37<sub>TAMRA</sub> (bottom left panel), or self-RNA<sub>Alexa488</sub>-LL37<sub>TAMRA</sub> complexes (right panels). Self-RNA<sub>Alexa488</sub>-LL37<sub>TAMRA</sub> complexes did not appear in the green channel after excitation at 488 nm (top right quadrant) but did appear in the red channel (top left quadrant), suggesting FRET. Photobleaching of the red fluorophore TAMRA with the 543 laser (bottom left quadrant) resulted in recovery of green fluorescence of the Alexa Fluor 488 fluorophore in response to excitation at 488 nm (bottom right quadrant). Numbers on the top of the right panels indicate excitation/emission wavelength. Size-bars: (left panels) 20  $\mu\text{m}$ ; (right panels) 10  $\mu\text{m}$ .

energy transfer (FRET) experiment using the efficient donor-acceptor pair of Alexa 488 and Tamra fluorophores to do the FRET imaging. Human RNA labeled with fluorescent dye Alexa 488 (self-RNA<sub>Alexa488</sub>) and Tamra-labeled LL37 (LL37<sub>Tamra</sub>) were added together to generate the complexes. Excitation of these self-RNA<sub>Alexa488</sub>/LL37<sub>Tamra</sub> complexes at 488 nm failed to elicit green fluorescence but gave a red fluorescence due to efficient transfer of the energy imparted on the Alexa 488 fluorophores to Tamra, as the emission spectrum of Alexa 488 (500-620nm) overlap considerably with the excitation spectrum of Tamra (450-600nm) (**Figure 2.4**). On the other hand, after photobleaching of the Tamra fluorophore at 543 nm in a selected area of a complex, excitation at 488 nm could induce the green fluorescence from that area of the complex, demonstrating a FRET efficiency of over 90%. A FRET efficiency of this magnitude points to a close molecular proximity between RNA and LL37 or in other words these two entities bind to each other to form these particulate complexes.

**The particles formed by self-RNA-LL37 complexes are micro-aggregates of nano-scale particles.**

Further analysis of the RNA complexes by scanning electron microscopy, revealed that they consisted of aggregates of smaller electron-dense nano-scale particles. With total human RNA (self-RNA) the particles were more pleomorphic that may result from different sizes of the individual RNA molecules in that

Figure 2.5



**Figure 2.5. Self-RNA-LL37 complexes are composed of micro-aggregates of nano-scale particles. (A)** Scanning electron microscopy of RNA-LL37 complexes formed by self-RNA fragments (top panels) or the short ssRNA sequence ssRNA40 (bottom panels). Bars: (left panels) 10  $\mu\text{m}$ ; (right panels) 1  $\mu\text{m}$ . **(B)** Particle size distribution of self-RNA-LL37 (top) and ssRNA40-LL37 complexes.

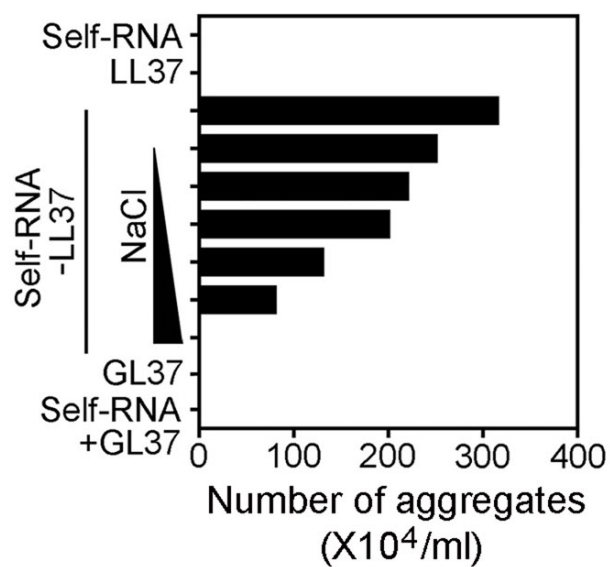
sample. With the uniformly sized short RNA sequence ssRNA40 the complexes showed more uniform particle sizes. By visual estimation from the SEM images we found the sizes of the particles ranging from 100 nm to 700 nm when total human RNA was used and ranging from 100 to 300 nm when short single-stranded RNA sequences were used (**Figure 2.5.A**).

Particle sizing experiments on a Zetasizer instrument also revealed a similar size range for the particles. The sizes ranged from 100–900 nm with self-RNA and 100–300 nm when short single-stranded RNA sequences were used (**Figure 2.5.B**).

### **The electrostatic interaction between LL37 and RNA results in the particulate complexes.**

The complex formation between the anionic ribonucleic acid molecules and the cationic peptide LL37 suggests an electrostatic interaction between the two molecular entities. LL37 has previously been shown to interact with human deoxyribonucleic acid in the same manner and the resulting complexes were found to be dissolved on increasing the ionic strength of the suspension by adding sodium chloride solution. In our experiments also the self-RNA-LL37 particles were rapidly dissolved by addition of sodium chloride, indicating that ionic interactions between the cationic peptide and the negatively charged phosphate groups of the nucleic acid are involved in the complex formation (**Figure 2.6**). While lower strengths of the salt solution could not achieve this

Figure 2.6



**Figure 2.6. Self-RNA-LL37 complexes form by electrostatic interaction between the anionic nucleic acid molecule and the cationic peptide.** Number of self-RNA–LL37 complexes counted as visible precipitates by phase-contrast microscopy. Increasing concentrations of NaCl (10, 50, 100, 200, 500, and 1,000 mM) were used to dissolve the complexes. Data are representative of three independent experiments.

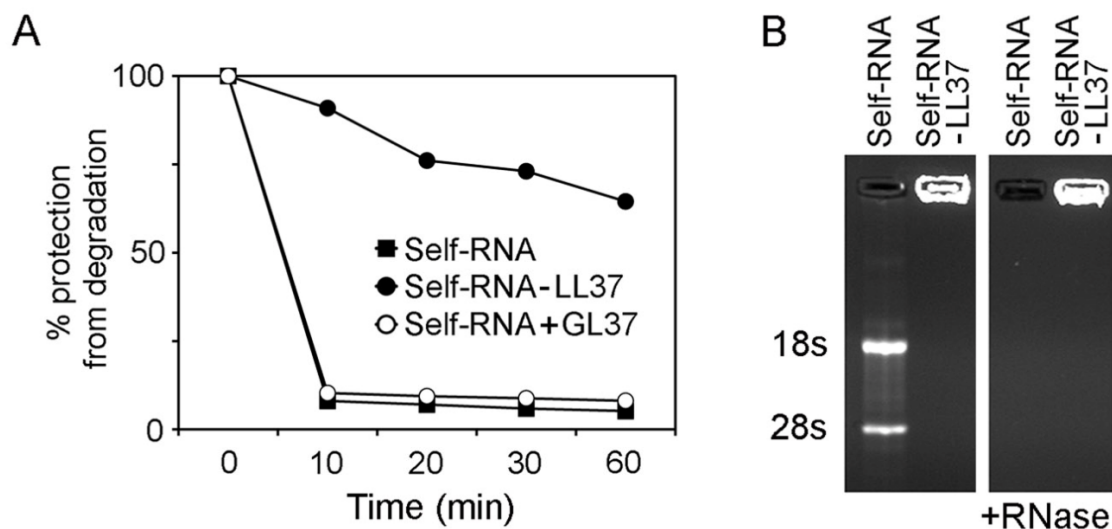
dissolution, at higher concentrations (>500mM) could reverse the electrostatic interaction between nucleic acids and the peptide and the particles dissolved.

### **Self-RNA-LL37 particles are protected from enzymatic degradation.**

Free RNA is exquisitely sensitive to degradation by extracellular RNases. Thus self-RNA, even if released extracellularly by the dying cells may not be available for a long time for immune cells to take them up. Efficient extracellular nucleases work toward clearing the extracellularly released nucleic acids. But the electrostatic interaction of RNA with the LL37 peptide and resulting condensation of the RNA molecules creates a possibility of protection of these condensed particles from the extracellular enzymatic degradation. To test whether LL37 can protect self-RNA from nuclease activity, self-RNA-LL37 complexes, self-RNA alone or self-RNA mixed with the control peptide GL37 were treated with RNase A and RNA was quantitated by a fluorometric assay using the RNA-sensitive dye Ribogreen at different time-points. As expected RNA alone and RNA mixed with GL37 were rapidly degraded after treatment with RNase A. But the self-RNA-LL37 complexes were found to be resistant to this degradation (**Figure 2.7.A**).

This ability of LL37 to protect RNA from RNase degradation was also confirmed in an agarose gel electrophoresis-based nuclease protection assay. Self-RNA alone or in complex with LL37, with or without a treatment with RNase A, were run on a gel and we found that self-RNA, when in complex with LL37 was retained in the loading wells and was protected from digestion by RNase (**Figure**

Figure 2.7



**Figure 2.7. Self-RNA-LL37 complexes are protected from enzymatic degradation. (A)** Self-RNA alone, self-RNA-LL37 complexes, or self-RNA mixed with the control peptide GL37 were treated with RNase A and quantified over 60 min by a fluorometric assay. The percentage of protection was calculated as the ratio of remaining RNA over the initial RNA input. Data are representative of at least three experiments. **(B)** Agarose gel electrophoresis of self-RNA alone and self-RNA-LL37 complexes before and after RNase A treatment visualized by ethidium bromide staining.

**2.7.B).** Retention of the complexes in the wells may result from neutralization of the anionic charge on the RNA molecules due to the electrostatic interaction with the cationic peptide and also due to greater mass of the RNA complexes compared to RNA alone. The condensed structure of the self-RNA-LL37 complexes may prevent the enzymes to access the cleavage sites of the RNA molecules resulting in the protection. Thus, LL37 condenses RNA to form compacted particles, which are highly protected from extracellular degradation.

## **2.2. Chapter 2: Self-RNA-LL37 complexes activate dendritic cells through endosomal toll-like receptors**

### **2.2.a. Background and Rationale**

In our studies, as described in Chapter 2.1, we found that the endogenous antimicrobial peptide LL37 can interact with human ribonucleic acid molecules based on electrostatic interaction of the cationic domains on the peptide and anionic nucleic acid molecule. Previous studies done in our lab had found LL37 as a key molecule expressed in the psoriatic skin that can bring about activation of the plasmacytoid dendritic cells resulting in type I IFN production (Lande R et al, 2007). LL37 was found to make complexes with human DNA and these DNA-LL37 complexes were then internalized into the pDCs to trigger activation of TLR9, the DNA-recognizing TLR present in pDC endosomes (see Sections 1.6 & 1.8; Lande R et al, 2007). Similar involvement of host-derived RNA in the activation of pDCs was plausible given the endosomal expression of TLR7 in pDCs that can recognize RNA molecules. But access of extracellularly released self-RNA to the pDC endosomes was physiologically somewhat improbable given the exquisite susceptibility of RNA molecules to extracellular enzymatic degradation. But we found that the condensation of RNA molecules resulting from electrostatic interaction with LL37 rendered them significantly resistant to enzymatic digestion and thus fuelled the probability of self-RNA molecules being able to access the endosomal TLRs in pDCs and contributing to the autoreactive innate immune activation.

**P**soriatic skin shows early pDC infiltration and the chemokine Chemerin is overexpressed in early psoriatic lesions and has been implicated in the recruitment of pDCs (Albanesi C et al, 2009). PDC-derived type I interferons have been shown to be instrumental for the initiation of the disease (Nestle FO et al, 2005). But it has also been shown that psoriasis is essentially a T-cell mediated disease and there is involvement of myeloid DCs. The pDC activation in response to DNA-LL37 complexes induce very potent type I IFN production but fails to drive maturation of these dendritic cells to be able to express the costimulatory molecules, produce other proinflammatory cytokines and finally to induce T cell activation by autoreactive priming (Lande R et al, 2007). MDCs have also been shown to infiltrate the lesional skin in psoriasis and there are obvious reasons to believe that these mDCs drive the T cell activation in psoriasis. MDCs do have endosomal TLRs that can recognize nucleic acids but they are specific for RNA molecules (TLR3 for dsRNA and TLR8 for ssRNA). DNA recognizing TLRs are not present in mDC endosomes. Ability of LL37 to bind self-RNA and protect them from extracellular enzymatic degradation may enable the RNA molecules to access the endosomal compartments in both pDCs and mDCs, thus providing a potential mechanistic explanation for mDC activation in psoriasis.

**I**n light of these possibilities we did experiments to find out whether the self-RNA-LL37 complexes can access the endosomal compartments of the dendritic cells and if they can whether they are able to activate the DCs by triggering toll-like receptors.

## 2.2.b. Materials and Methods

### Reagents.

LyoVec-free single stranded RNA sequences ssRNA40 (5'-GsCsCsCsGsUsCsUsGsUsUsGsUsGsUsGsAsCsUsC-3', 's' denotes phosphothioate linkage) and ssPolyU (single-stranded poly-uridine) were from Invivogen, CA and used at a final concentration of 5 µg/ml. TLR9 agonist oligonucleotide CpG-2006 (5'-tcgtcgttttgcgttttgcgtt-3') was produced by Trilink (San Diego, CA) and used at a final concentration of 1 µM. Synthetic TLR7 agonist R837 (final concentration used 1 µM) and TLR8 agonist R848 (final concentration used 100 ng/ml) were from Invivogen, CA. The specific human TLR7 oligonucleotide antagonist C661 and the corresponding control oligonucleotide sequence (ctrl-ODN, 5'-tcctgcagggttaagt-3') were kindly provided by Dr. Franck Barrat from Dynavax Corporations, CA and they were used at a final concentration of 1 µM to pre-treat pDC for 30min at 37°C before stimulation. The endosomal acidification inhibitor Bafilomycin A1 from *Streptomyces griseus* was purchased from Sigma-Aldrich (St. Louis, MO).

### Isolation of nucleic acids.

Total human RNA (self-RNA) and genomic DNA (self-DNA) were extracted from cell lines or inflammatory skin samples using standard procedures as described in the section 2.1.b. Skin samples from patients with Psoriasis vulgaris, Atopic dermatitis and healthy individuals were taken by 6-mm punch biopsies as

previously described (27), under approved protocols at the M. D. Anderson Cancer Center in Houston and the Heinrich-Heine University in Dusseldorf.

### **Isolation and generation of pDCs and mDCs.**

Use of human blood cells for this study was approved by the Institutional Review Board for Human Research at the M.D. Anderson Cancer Center. PDCs were isolated from buffy coats of healthy volunteers. In short peripheral blood mononuclear cells (PBMCs) were isolated from the buffy coats by centrifugation on Ficoll-Paque (GE Healthcare, Uppsala, Sweden). PDCs were enriched from the PBMCs by magnetic immuno-selection with anti-BDCA-4 microbeads (Miltenyi Biotec, CA) and were then sorted by FACS Aria (BD Bioscience, CA) according to their lineage<sup>-</sup> CD11c<sup>-</sup> CD4<sup>+</sup> CD123<sup>+</sup> phenotype. Immature myeloid DCs were generated from blood monocytes. Monocytes were first obtained from PBMC by magnetic immuno-selection using anti-CD14 microbeads (Miltenyi Biotec) and cultured for 5 days with GM-CSF and IL-4 (both at 100ng/ml, from R&D Systems, CA). Circulating conventional DCs were isolated from PBMC by magnetic immuno-depletion of B cells with anti CD19 microbeads, followed by magnetic immuno-selection using anti-CD1c microbeads (Miltenyi Biotec). All the primary cell cultures were done in RPMI 1640 medium added with 10% fetal calf serum, 10mM HEPES buffer, 1mM sodium pyruvate and penicillin-streptomycin antibiotic cocktail. Primary pDCs were cultured and stimulated in round bottom 96 well plates while myeloid DCs were cultured in flat bottom plates.

**Stimulation and analysis of pDC and mDC.**

Suspensions containing RNA-LL37 or DNA-LL37 complexes made in 20  $\mu$ l volume of PBS or 100  $\mu$ l of supernatants of dying cells were added to pDC and mDC cultures in a final volume of 200  $\mu$ l in RPMI 1640 medium with 10% fetal calf serum. Stimulations with the positive controls were done using CpG-2006 for TLR9, R837 for TLR7, R848 for TLR8 and Poly I:C for TLR3. We used Bafilomycin (Sigma-Aldrich, MO), a vesicular proton pump inhibitor, to inhibit endosomal acidification and TLR activation. To inhibit TLR7 we used C661, a specific oligonucleotide provided kindly by Dr. F.J. Barrat from Dynavax Technologies, CA. After overnight stimulation with indicated stimuli, supernatants of pDCs and mDC cultures were harvested. Production of IFN- $\alpha$  (PBL Biomedical Laboratories), IL-6 and TNF- $\alpha$  (R&D Systems) in the supernatants were measured by ELISA. The stimulated pDCs and mDCs were also stained with fluorochrome-labeled anti-CD80, anti-CD86 and anti-CD83 antibodies (BD Pharmingen, CA) for analysis by flow cytometry. In some experiments myeloid DCs were primed with supernatant from pDCs cultures activated by self-DNA-LL37 before stimulating the mDCs with self-RNA-LL37 complexes. The pDC supernatants used in these experiments were sometimes added with a combination of anti-human IFN- $\alpha$  ( $10^4$  U/ml), anti-human IFN- $\beta$  ( $2 \times 10^3$  U/ml) antibodies and anti-human IFN- $\alpha\beta$  receptor antibody (clone: MMHAR-2, 10  $\mu$ g/ml) before adding to the mDC cultures (all these antibodies were from PBL Biomedical Laboratories, NJ).

### **Uptake of LL37–human-RNA complexes in pDCs and mDCs.**

To assess the internalization of the self-RNA-LL37 complexes we used RNA labeled with Alexa 488 fluorophore. PDC or mDC cultures were added with self-RNA<sub>Alexa488</sub> alone or Self-RNA<sub>Alexa488</sub>-LL37 complexes. After 4 h of incubation at 37°C, the cells were harvested, and after washing with FACS buffer (PBS with 2% human serum and 0.5 mM EDTA) were analyzed by flow cytometry. To confirm entry of the complexes inside the cell we did confocal microscopy of the DCs after adding Self-RNA<sub>Alexa488</sub>-LL37 complexes in culture. After 4 h of incubation, cells were stained with Alexa 647-conjugated anti-HLA-DR (BioLegend, CA) and the slides were mounted in Prolong Gold antifade mounting media (Molecular Probes, CA). A Leica TCS SP2 confocal microscope was used to acquire the images, which was then analyzed with the Leica confocal software.

### **Luciferase reporter assay.**

Transient transfection was done in HEK293T cells with 500 ng of TLR3-GFP or TLR8-HA expression vectors (both from Invivogen, CA) and 100 ng of NFκB-luciferase reporter plasmid. Transfection was done with lipofection using Fugene 6 (Roche Applied Science, IN). The plasmids (indicated amounts) were added with Fugene 6 (3 µl) in final volume of 50 µl serum-free Opti-Mem I medium (Invitrogen, CA) and 250 X 10<sup>3</sup> cells/well in 6-well plates were transfected. Simultaneous transfection of 5 ng Renilla luciferase reporter gene (pRL-TK) was

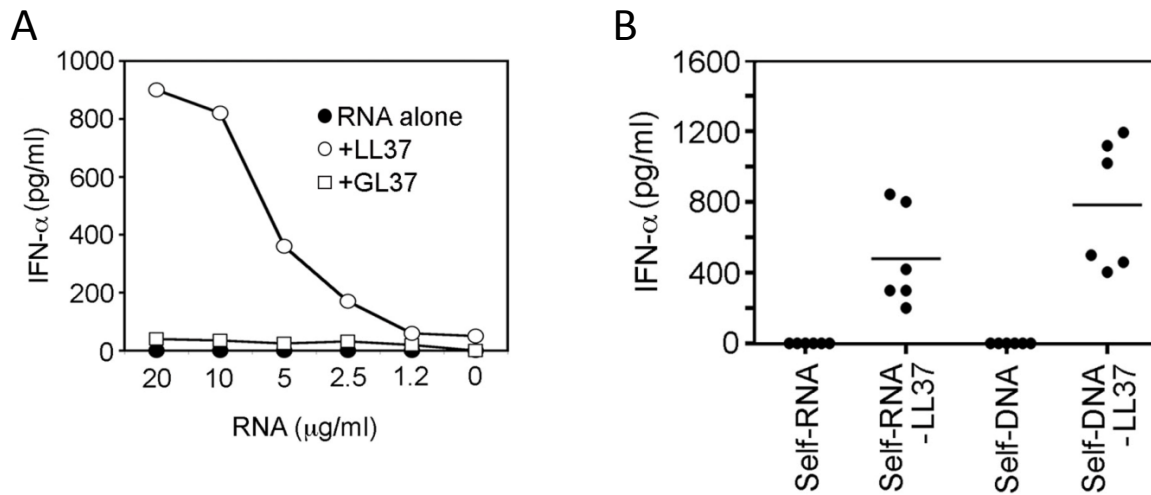
used as an internal control for transfection efficiency. After overnight incubation the transfected cells were collected, plated  $50 \times 10^3$ /well in a 24 well plate and stimulated as indicated for another 24 h. Poly I:C and ssPolyU complexed with cationic lipids (DOTAP, from Roche Applied Science, IN) were used as positive controls for activation of TLR3 and TLR8 respectively. Luciferase activity was measured with a dual-luciferase reporter assay system (Promega Corp., WI).

### **2.2.c. Results & Discussion:**

#### **Self-RNA can induce IFN- $\alpha$ production by plasmacytoid dendritic cells in complex with LL37.**

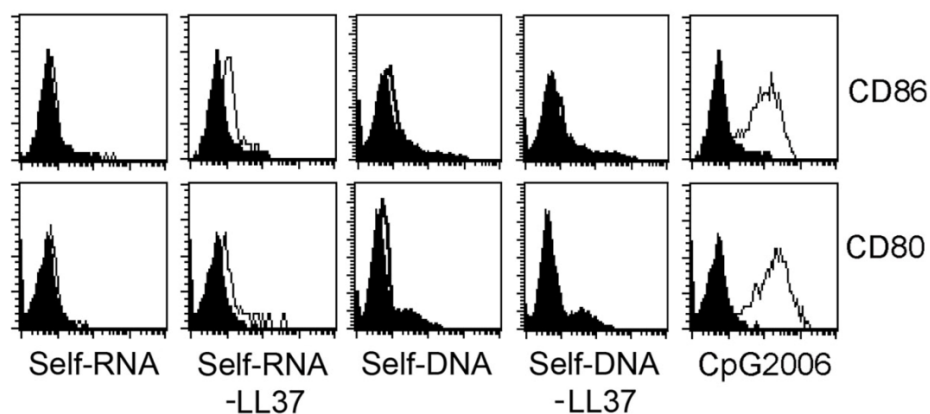
To investigate whether the complexes formed by self-RNA with LL37 can activate plasmacytoid dendritic cells similar to self-DNA as described in previous studies, pDCs were stimulated with total human RNA (self-RNA) extracted from U937 cells in complex with LL37. Self-RNA induced IFN- $\alpha$  production in a dose-dependent manner when in complex with LL37 but it failed to do the same when given to the pDC cultures alone or added with the scrambled peptide GL37 (**Figure 2.8.A**). PDCs when activated by self-RNA-LL37 complexes produced high levels of IFN- $\alpha$ , but this activation was not accompanied by production of the proinflammatory cytokines TNF- $\alpha$  or IL-6. There was no significant qualitative difference between pDC activations induced by self-DNA and self-RNA thus implicating both the species of self nucleic acids in this

Figure 2.8



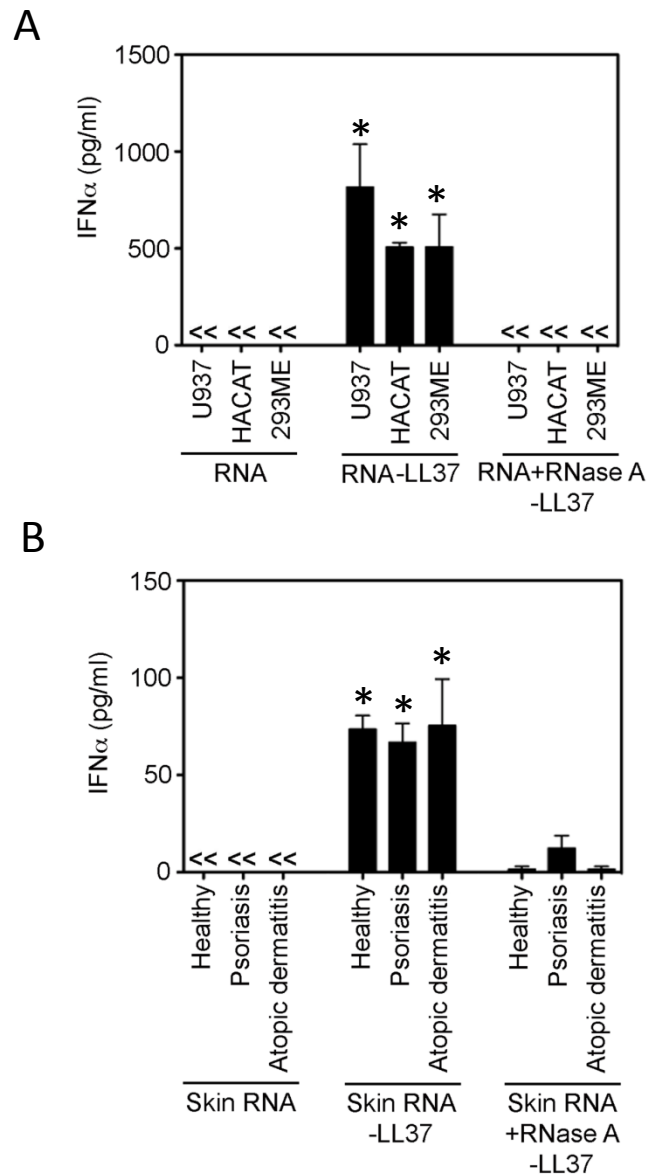
**Figure 2.8. LL37 converts self-RNA into a trigger for pDCs to produce IFN- $\alpha$ .** (A) IFN- $\alpha$  produced by pDCs after stimulation with increasing concentrations of total human RNA purified from U937 cells (self- RNA), either alone (closed circles) or premixed with LL37 (self-RNA-LL37; open circles) or the scrambled peptide GL37 (self-RNA+GL37; open squares). (B) IFN- $\alpha$  produced by pDCs after stimulation with self-RNA or self-DNA (both at 5  $\mu$ g/ml) alone or in complex with LL37 (self-RNA-LL37 and self-DNA-LL37). Each symbol represents an independent experiment; horizontal bars represent the mean.

Figure 2.9



**Figure 2.9. Self-RNA-LL37 complexes fail to induce maturation of pDCs.** Flow cytometric analysis of stimulated pDCs for CD80 and CD86 surface expression. CpG-2006 (1  $\mu$ M) was used as a control to induce pDC maturation. Data are representative of at least three independent experiments.

Figure 2.10



**Figure 2.10. Self-RNA from different sources induce similar levels of IFN- $\alpha$  in pDCs.** **(A)** IFN- $\alpha$  produced by pDCs after stimulation with total RNA (5 $\mu$ g/ml) from U937 (myelomonocytic leukemia), HACAT (keratinocytes) and 293ME (melanoma) cells with or without premixing with LL37 (50  $\mu$ g/ml). Data are presented as mean + s.e.m. of 3 independent experiments. **(B)** IFN- $\alpha$  produced by pDCs after stimulation with total RNA (5 $\mu$ g/ml) isolated from healthy skin (n=4), plaque psoriasis (n=5), or atopic dermatitis (n=3) with or without premixing with LL37 (50 $\mu$ g/ml). Mean + s.e.m of a representative experiment is given. Experiments in (A) and (B) were also performed by pretreatment of purified RNA with RNase A (50 $\mu$ g/ml) to ensure that responses were RNA-mediated. \*p value <0.05, paired Student's T test.

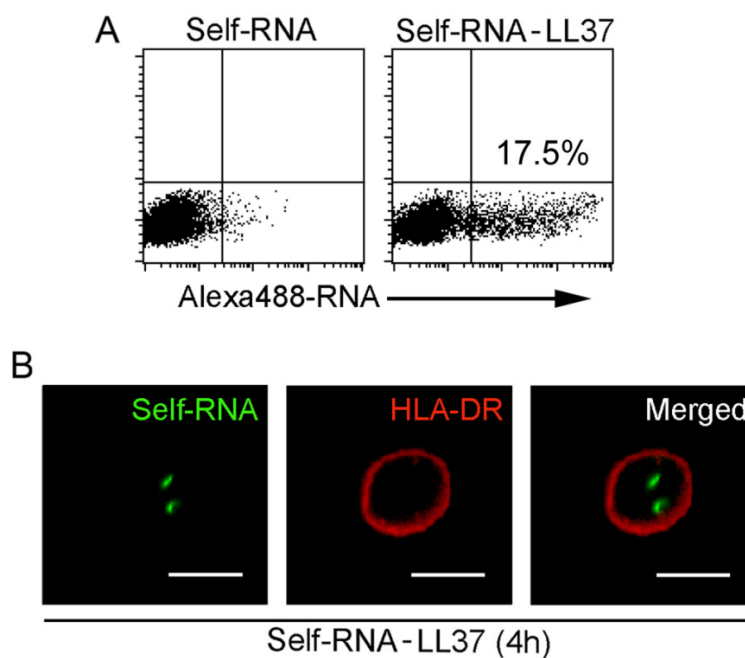
response. Also the pDCs failed to undergo maturation as assessed by surface expression of the costimulatory molecules CD80 and CD86 (**Figure 2.9**).

To investigate whether the source of the total human RNA had something to do with this activation event we used self-RNA isolated from a variety of cell types and biopsies from various types of skin pathologies to do the stimulation of the pDCs and we found RNA from all these sources induced similar levels of IFN- $\alpha$  when in complex with LL37. This indicated that different cellular sources or disease-dependent variations in total RNA composition do not have any differential role to play in this activity (**Figure 2.10.A&B**). These experiments proved that LL37 when interact with self-RNA can convert otherwise non-stimulatory self-RNA into a trigger for activation of the plasmacytoid dendritic cells leading to production of IFN- $\alpha$ . Thus self-RNA released during host cell death is able to induce innate immune activation.

### **Self-RNA-LL37 complexes are internalized into the endosomal compartments of pDCs.**

A potent interferon production response of the pDCs to the self-RNA-LL37 complexes indicated that the pDCs are able to take up the particulate complexes intracellularly. Indeed using Alexa 488 fluorophore labeled self-RNA (self-RNA<sub>Alexa488</sub>) in the RNA-peptide complexes after incubating the complexes with the pDCs for 4 h 10-15% cells were found to be positive for Alexa 488 fluorescence on flow cytometry, indicating uptake of the fluorescently labeled

Figure 2.11

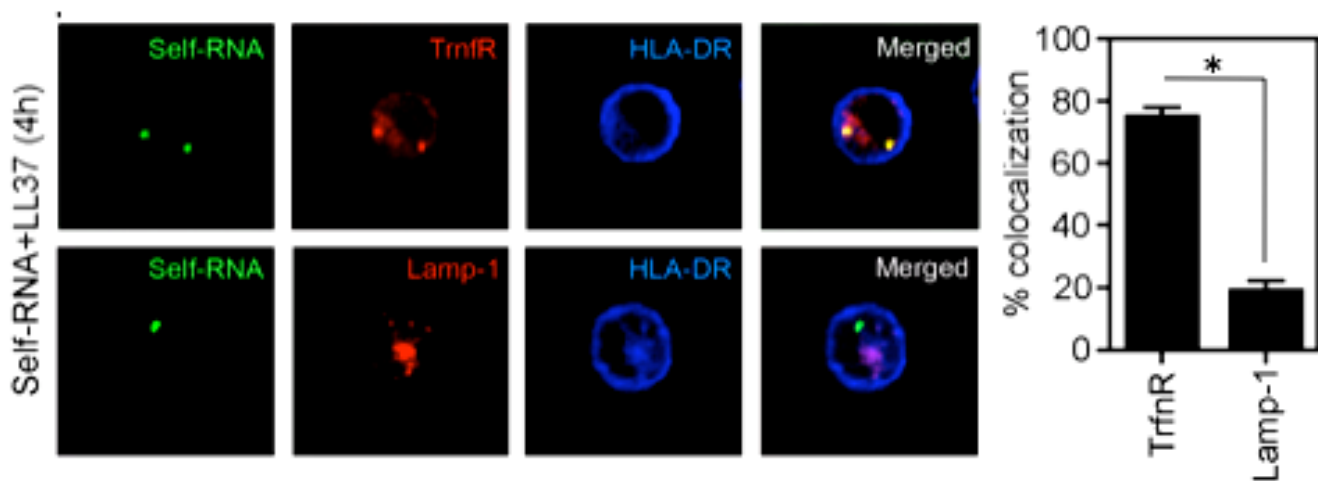


**Figure 2.11. LL37 transports self-RNA intracellularly into pDCs.** (A) pDCs were stimulated for 4 h with self-RNA<sub>Alexa488</sub> alone or self-RNA<sub>Alexa488</sub>-LL37 and analyzed by flow cytometry for self-RNA<sub>Alexa488</sub>-containing pDCs. (B) Confocal microscopy of pDCs stimulated for 4 h with self-RNA<sub>Alexa488</sub>-LL37 complexes and stained with Alexa 647-labeled anti-HLA-DR antibody to visualize the contour of the cell.

complexes by the pDCs. When given alone self-RNA<sub>Alexa488</sub> could not get internalized (**Figure 2.11.A**) showing importance of the complex formation in this internalization process. To confirm the localization of the self-RNA-LL37 complexes inside the cell we performed confocal microscopy using the labeled complexes incubated with the pDCs for 4 h and then staining the pDCs with anti-HLA-DR antibodies to delineate the cell boundary. On acquiring the images on a confocal microscope the complexes were again found to be located inside the cells (**Figure 2.11.B**).

Previous study on DNA-LL37 complexes done in our lab reported retention of the DNA complexes in the early endosomes of pDCs (Lande R et al, 2007). To check for the endosomal localization of the self-RNA-LL37 complexes in the pDCs the cells after incubation with the labeled complexes for 4 h, were stained with either the early endosomal marker Transferrin receptor or the late endosomal marker LAMP-1. On confocal microscopy of these cells we found a lot more labeled complexes to co-localize with the Transferrin receptor containing early endosomes compared to the LAMP-1-positive late endosomes (**Figure 2.12**). Thus the RNA-LL37 complexes after getting internalized into the pDCs were retained in the early endosomal compartments for a long period of time and possibly triggered the cognate receptors at this compartment. This phenomenon could explain the absence of costimulatory molecule expression and proinflammatory cytokine production in the pDCs in addition to induction of IFN- $\alpha$  in line with previous reports, which showed early endosomal retention of TLR-ligands in pDCs lead to more robust type I IFN induction in the absence of

Figure 2.12



**Figure 2.12. Self-RNA-LL37 complexes are retained in early endosomes in pDCs.** pDCs were stimulated for 4 h with self-RNA<sub>Alexa488</sub>-LL37 (green) and then stained for HLA-DR (blue) along with either Transferring receptor or Lamp-1 (red). Confocal microscopy of the stained pDCs were counted for relative abundance of complexes co-localizing with either the Transferring receptor or LAMP-1 were assessed by counting 50 cells in multiple fields. \*p value <0.05, Student's T test.

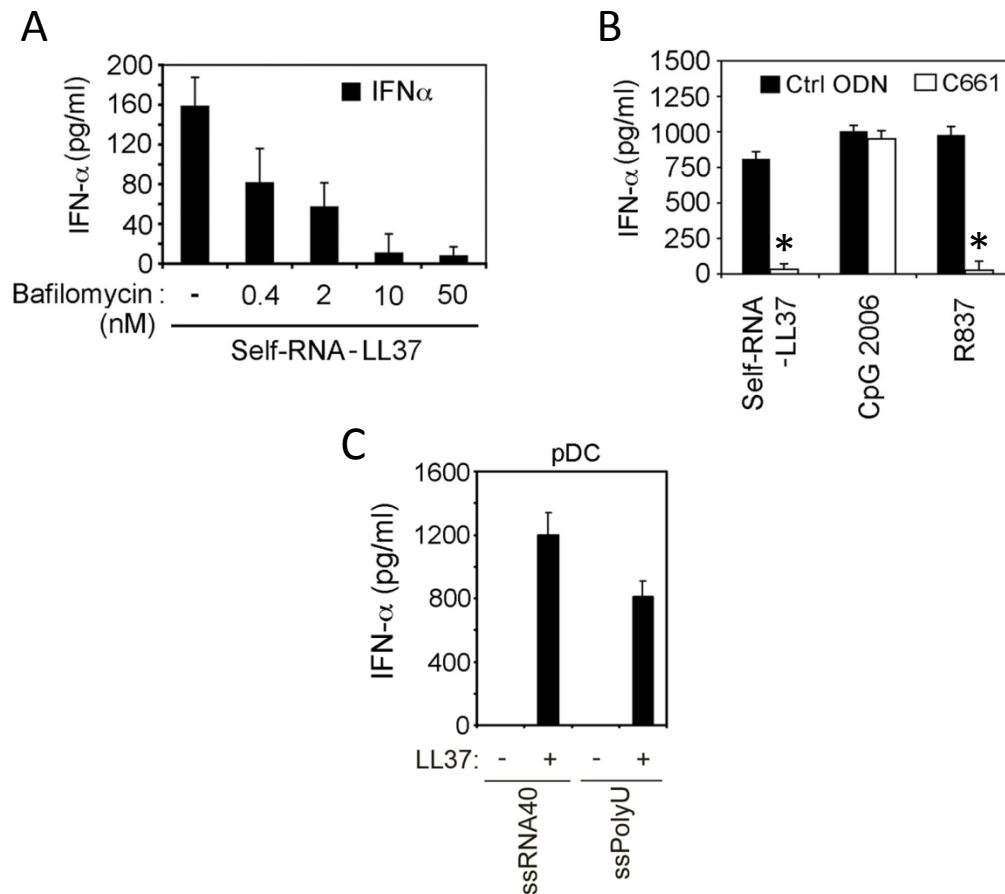
maturation of pDCs and proinflammatory cytokine production (Honda K et al, 2005). On the contrary a late endosomal localization of the TLR-ligands leads to an opposite response showing pDC maturation and proinflammatory cytokine production while having less type I IFN induction (Honda K et al, 2005).

### **Activation of endosomal TLR7 in pDCs by the self-RNA-LL37 complexes.**

To investigate whether the self-RNA-LL37 complexes, after being internalized by pDCs and being located in the endosomal compartments, are able to trigger endosomal TLR activation and whether this TLR activation is the mechanism of induction of IFN- $\alpha$  by the pDCs, we used an inhibitor for vesicular H<sup>+</sup>K<sup>+</sup>-ATPase Bafilomycin A1. Acidic pH is crucial for the toll-like receptor triggering in the endosomes (Rutz M et al, 2004) and the vesicular proton pumps bring about this acidification. Thus inhibiting the proton pumps leads to inhibition of TLR triggering in the endosomes. When pDCs were stimulated with self-RNA-LL37 complexes in the presence of Bafilomycin, the IFN- $\alpha$  induction was inhibited in a dose dependent-manner by this proton pump inhibitor, which blocks endosomal acidification and TLR signaling (**Figure 2.13.A**).

PDCs have endosomal toll-like receptors for recognizing both DNA (TLR9) and RNA (TLR7). The endosomal localization of the self-RNA-LL37 complexes and the dependence of the activation response on endosomal acidic pH pointed to possible involvement of TLR7 in the recognition of this complexed self-RNA. To confirm this possibility we used a specific oligonucleotide inhibitor for TLR7

Figure 2.13



**Figure 2.13. Self-RNA-LL37 complexes trigger endosomal TLR7 activation in pDCs.** (A) IFN- $\alpha$  produced by pDCs stimulated with self-RNA-LL37 complexes after pretreatment with increasing concentrations of bafilomycin. (B) IFN- $\alpha$  produced by pDCs after stimulation with self-RNA-LL37, CpG-2006 (1  $\mu$ M), or R837 (10  $\mu$ g/ml) after pretreatment with 1  $\mu$ M TLR7 inhibitor C661 or a control oligonucleotide (ctrl-ODN). Data in A–D are representative of at least three independent experiments; error bars in C and D represent the SD of triplicate wells. (C) IFN- $\alpha$  produced by pDCs after stimulation with short single-stranded RNA sequences ssRNA40 and ssPolyU alone (both 5 $\mu$ g/ml) or in complex with LL37.

named C661 (Barrat FJ et al, 2005). There was inhibition of IFN- $\alpha$  induction by the synthetic TLR7 agonist R837 but not the TLR9 agonist CpG2006 in presence of when pDCs were pre-treated with C661 (**Figure 2.13.B**). Also the pretreatment of pDC with C661 prior to stimulation with the self-RNA-LL37 complexes abolished the IFN- $\alpha$  induction (**Figure 2.13.B**). This experiment confirmed the involvement of TLR7 triggering by the self-RNA-LL37 complexes in the activation of pDCs by these complexes.

To further support these data, we investigated whether the short synthetic single-stranded RNA sequences that can activate TLR7 in pDC upon liposomal transfection (Diebold SS et al, 2004; Heil, F et al, 2004) were also internalized in presence of LL37 and were able to trigger IFN- $\alpha$  induction. These short sequence RNA molecules also formed particulate complexes with LL37 (data not shown) and these complexes were able to induce IFN- $\alpha$  production by pDCs (**Figure 2.13.C**).

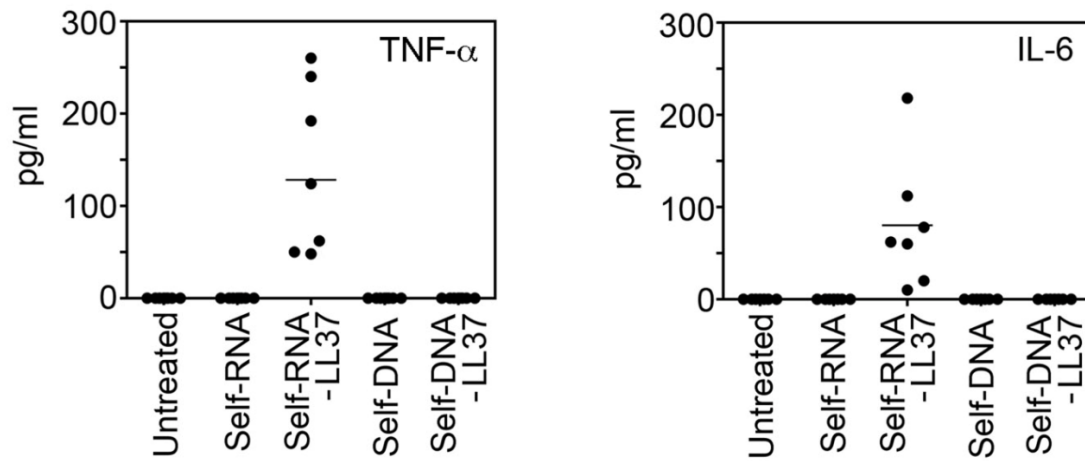
### **Self-RNA-LL37 complexes activate myeloid DCs leading to their maturation and production of proinflammatory cytokines.**

Ability of the self-RNA-LL37 complexes to access the endosomal compartments in the plasmacytoid dendritic cells opened up the possibility of activation of myeloid dendritic cells by these nucleic acid-endogenous peptide complexes and thus leading to broader autoimmune activation. Although DNA-LL37 complexes also are able to access pDC endosomes and trigger TLR9 to induce type I IFN

production in pDCs, they failed to show any activating response on mDCs. That could be explained by the absence of TLR9 or any other DNA-responsive receptor in the endosomal compartments of human mDCs (discussed in Section 1.3 & 1.4). But mDCs in human do express multiple toll-like receptors in the endosomal compartments that recognize RNA (Kadowaki N et al, 2001; Jarrossay D et al, 2001; Hornung V et al, 2002). TLR3 and TLR8, expressed in mDC endosomes, recognize double-stranded and single-stranded RNA molecules respectively.

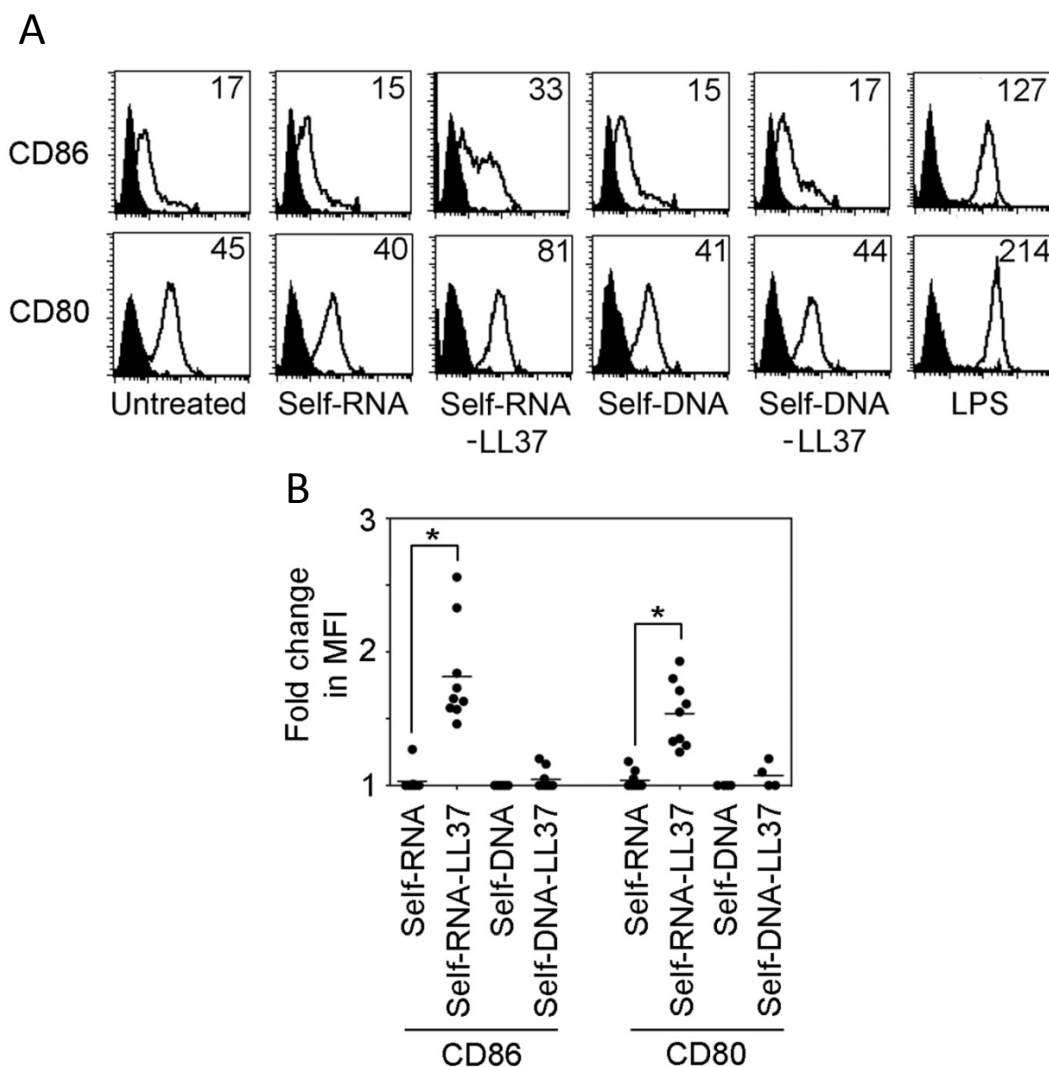
To investigate whether mDCs can respond to self-RNA when complexed with LL37 leading to their activation, we stimulated immature monocyte-derived mDCs or mDCs isolated directly from human peripheral blood with self-RNA alone or in complex with LL37. Self-RNA, when in complex with LL37 but not when given alone, induced activation of mDCs. The mDCs thus stimulated produced proinflammatory cytokines TNF- $\alpha$  and IL-6 (**Figure 2.14 and 2.16.B**). The mDCs were not able to produce any IFN- $\alpha$  unlike the pDCs stimulated in the same manner. Self-RNA-LL37 complexes also led to maturation of the mDCs as evident by upregulation of the B7 costimulatory molecules CD80 and CD86 expression on the cell surface (**Figure 2.15.A&B**). This activation of mDCs in response to self-RNA-LL37 complexes was entirely dependent on presence of self-RNA in the complex, found by abrogation of these responses by decreasing the amount of self-RNA in the complexes (data not shown). As expected self-DNA-LL37 complexes, as opposed to self-RNA-LL37 complexes, were not able to bring about similar activation of the mDCs (**Figure 2.14 and Figure 2.15**).

Figure 2.14



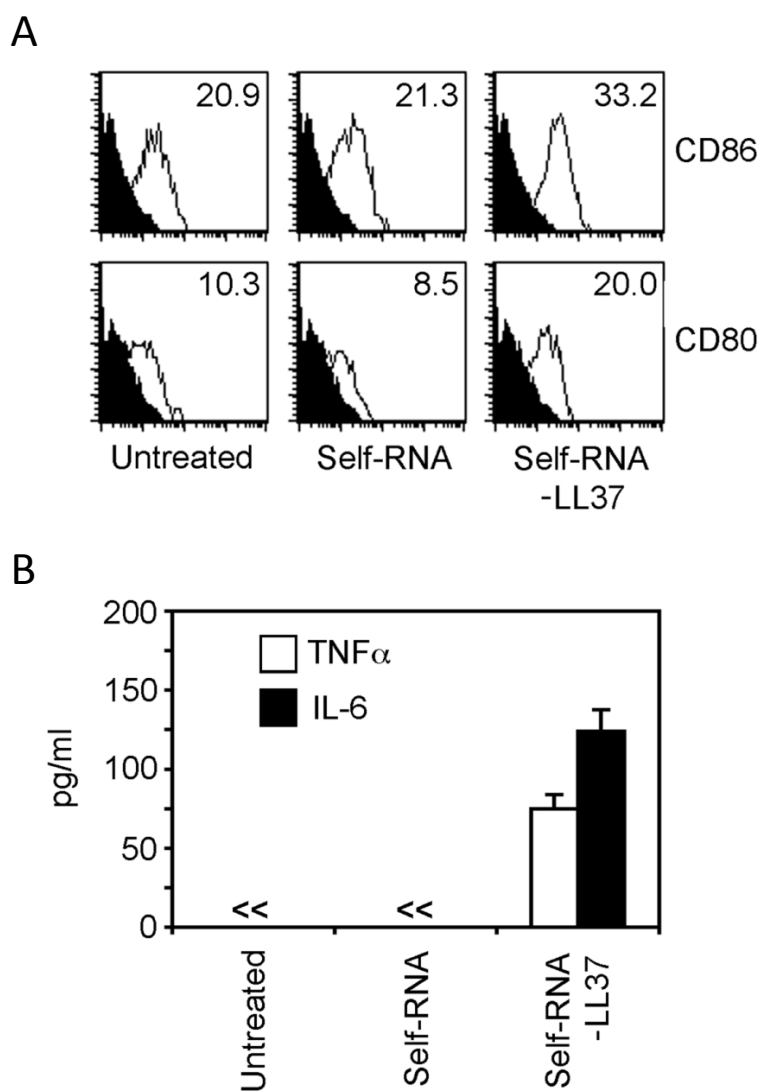
**Figure 2.14. Self-RNA but not self-DNA in complex with LL37 activates monocyte-derived mDCs to secrete TNF- $\alpha$  and IL-6.** Immature monocyte-derived mDCs were stimulated with self-RNA, self-DNA, self-RNA-LL37, or self-DNA-LL37. TNF- $\alpha$  and IL-6 secretion was measured after overnight culture. Each symbol represents an independent experiment, and horizontal bars represent the mean.

Figure 2.15



**Figure 2.15. Self-RNA-LL37 complexes induce maturation in monocyte-derived mDCs.** **(A)** Flow cytometry for surface expression of CD86 and CD80 on stimulated mDCs. LPS was used as a control to induce mDC maturation. **(B)** Mean fluorescence intensity (MFI) changes for CD86 and CD80 expression on mDCs in response to stimulation with self-RNA-LL37 complexes. Each symbol represents an independent experiment, and horizontal bars represent the mean. \*,  $P < 0.0005$ ; paired Student's T test.

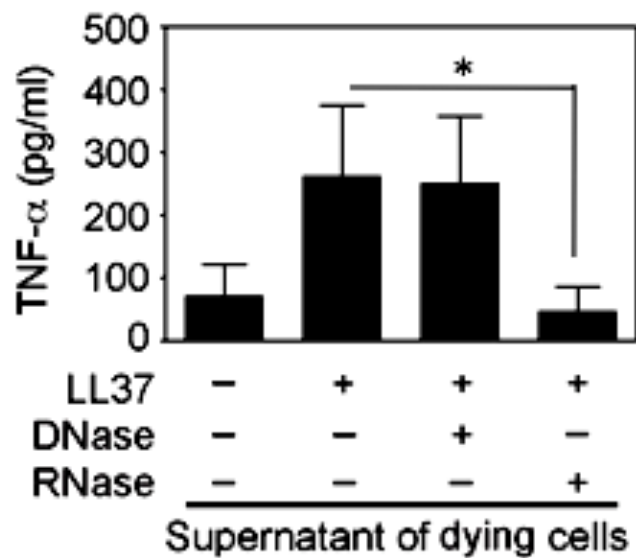
Figure 2.16



**Figure 2.16. Self-RNA-LL37 complexes induce proinflammatory cytokine production and maturation in circulating CD1c<sup>+</sup> mDCs.** (A) Flow cytometry for surface expression of CD86 and CD80 on stimulated CD1c<sup>+</sup> mDCs isolated from peripheral blood. (B) TNF- $\alpha$  and IL-6 production by circulating CD1c<sup>+</sup> mDCs stimulated with self-RNA either alone or mixed with LL37. Data are presented as mean + s.e.m. of 3 independent experiments.

These phenomenon of the ribonucleic acids of self origin being able to access the endosomal compartments of the mDCs when in complex with the endogenous peptide LL37 leading to activation and maturation again pointed to the possibility of innate immune activation of the mDCs in response to nucleic acids released during host cell death. These experiments were done with pure RNA molecules isolated from human cells and so it remained to be seen whether RNA derived from a more physiological source like the same released by dying human cells were also able to show similar activity. Toward this we used supernatants of UV-irradiated human cells as done with pDCs before (described in Section 2.1.a). As described before UV-irradiation of the cells followed by overnight incubation leads to apoptosis of the cells followed by secondary necrosis and as a result nucleic acids are released into the supernatant. Stimulation of the myeloid DCs with these supernatants added with LL37 also induced activation of the mDCs as evident by the production of the proinflammatory cytokine TNF- $\alpha$  (**Figure 2.17**). In case of mDCs, as opposed to pDCs, this activity was completely abolished when self-RNA was depleted from those supernatants by pre-treating the supernatants with RNase before adding LL37 showing that this activity was entirely dependent on the self-RNA released during cell death (**Figure 2.17**). DNA present in the supernatants was not taking part in this activation as shown by lack of abolition of activity on pre-treatment of the supernatants with DNase (**Figure 2.17**). This was in line with our finding that self-DNA-LL37 complexes were unable to induce mDC activation.

Figure 2.17



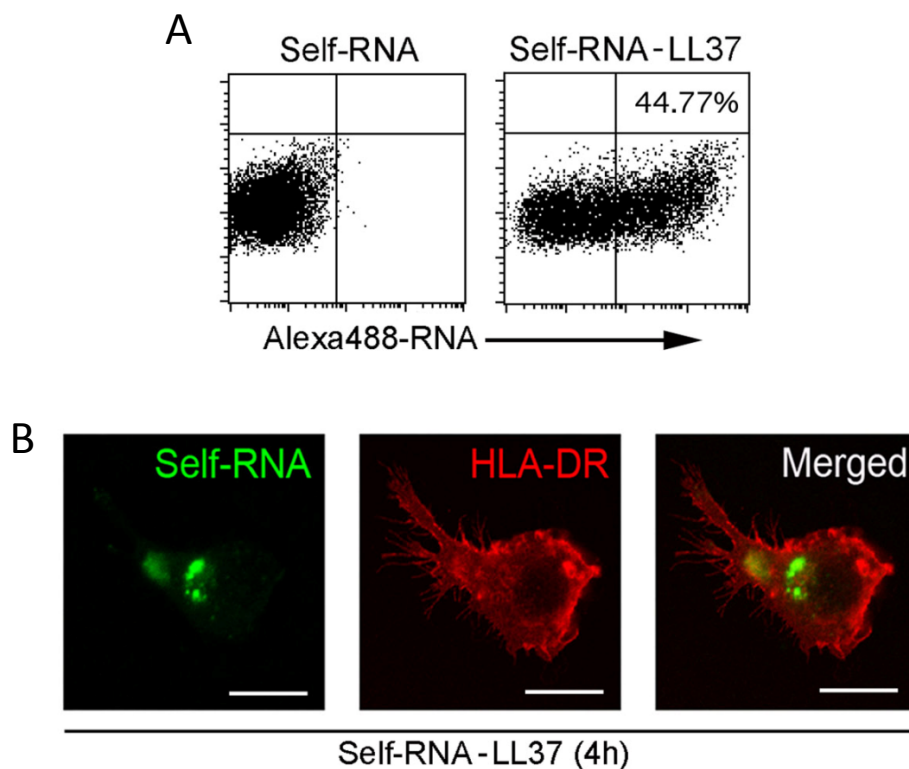
**Figure 2.17. RNA, but not DNA, released during cell death can activate mDCs in presence of LL37.** TNF- $\alpha$  production by mDCs stimulated with supernatants of dying U937 cells, either alone or premixed with LL37. In some experiments, DNA and/or RNA was depleted from supernatants of dying cells by pretreatment with DNase I and/or RNase A. Data indicate the mean  $\pm$  SEM of three independent experiments. \*p value <0.05, paired Student's T test.

### **Self-RNA-LL37 complexes trigger TLR8 activation in the endosomal compartment in mDCs.**

To investigate the mechanism of this activation of the myeloid DCs in response to the self-RNA-LL37 complexes we wanted to confirm access of these complexes to the endosomal compartments of the mDCs and TLR triggering therein. Self-RNA<sub>Alexa488</sub> was added to mDC cultures alone or in complex with LL37 to look for uptake of the complexes by the mDCs. On doing both flow cytometry and confocal microscopy, we found the labeled RNA to be internalized into the mDCs when in complex with LL37 but not when given alone after 4h (**Figure 2.18**). To investigate the endosomal localization of the self-RNA-LL37 complexes inside the mDCs the cells were also stained with early and late endosomal markers (Transferrin receptor and LAMP-1 respectively) and the complexes were seen co-localizing with these markers, thus confirming their endosomal location. But, in contrary to pDCs, there was no relative retention of these complexes in the early endosomes.

The production of the proinflammatory cytokines TNF- $\alpha$  and IL-6 by the mDCs in response to the self-RNA-LL37 complexes but not by TLR4 agonist LPS was found to be completely abrogated when Bafilomycin was added to the cultures to block the vesicular proton pumps in the DCs to reverse the acidic pH conducive for endosomal TLR triggering (**Figure 2.19A**). This demonstrated that this activation of the mDC by self-RNA-LL37 complexes involved activation of the TLRs in the endosomes.

Figure 2.18



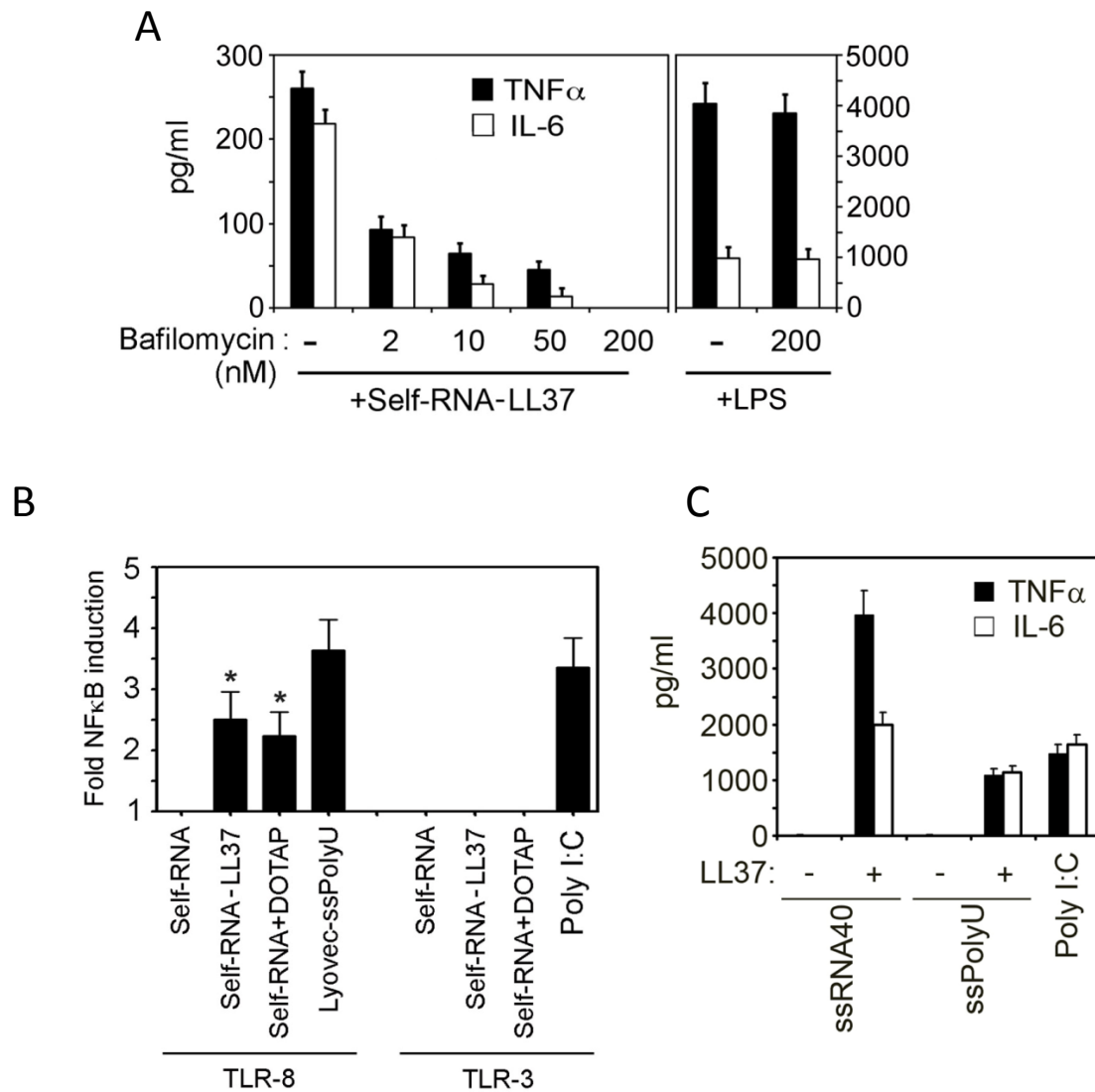
**Figure 2.18. LL37 transports self-RNA into mDCs. (A)** mDCs were stimulated for 4 h with self-RNA<sub>Alexa488</sub> alone or self-RNA<sub>Alexa488</sub>-LL37 and analyzed by flow cytometry for self-RNA<sub>Alexa488</sub>-containing mDCs. **(B)** Confocal microscopy of mDCs stimulated for 4 h with self-RNA<sub>Alexa488</sub>-LL37 complexes and stained with Alexa 647-labeled anti-HLA-DR antibody to visualize the contour of the cell. Data in A and B are representative of at least five independent experiments.

MDCs express multiple endosomal TLRs (TLR3 and TLR8) that can recognize RNA. Total human RNA consists of RNA molecules that can potentially be recognized by both TLR3 and TLR8, recognizing respectively double-stranded and single-stranded RNA molecules. To investigate whether both of these TLRs are involved in sensing self-RNA-LL37 complexes or one of them is responsible for this recognition, we performed gain-of-function reporter assay using 293T cells. In this experiments using 293T cells transfected with either TLR8 or TLR3 expression vectors along with an NF $\kappa$ B-luciferase reporter plasmid, it was revealed that self-RNA-LL37 complexes could induce NF $\kappa$ B activation only in the TLR8-transfected 293T cells, but not in the TLR-expressing cells. Thus we found that these RNA-peptide complexes were sensed by TLR8 but not by TLR3 (**Figure 2.19.B**). Bringing further support to this finding, we used the synthetic short RNA sequences that activate TLR8 in human mDCs (Diebold SS et al, 2004; Heil, F et al, 2004), added them in mDC cultures alone or in complex with LL37 and found that they could also activate the mDCs when complexed with LL37 but not when given alone (**Figure 2.19.C**).

#### **PDC-derived IFN- $\alpha$ enhances self-RNA-LL37 induced maturation of mDCs.**

Type I IFNs have been shown to directly induce myeloid DC activation and maturation (Luft T et al, 1998; Santini SM et al, 2000) and plasmacytoid DCs are the most potent producers of type I IFN in the body. Thus through type I IFNs pDCs and mDCs can cooperate and synergize during an immune response. In

Figure 2.19

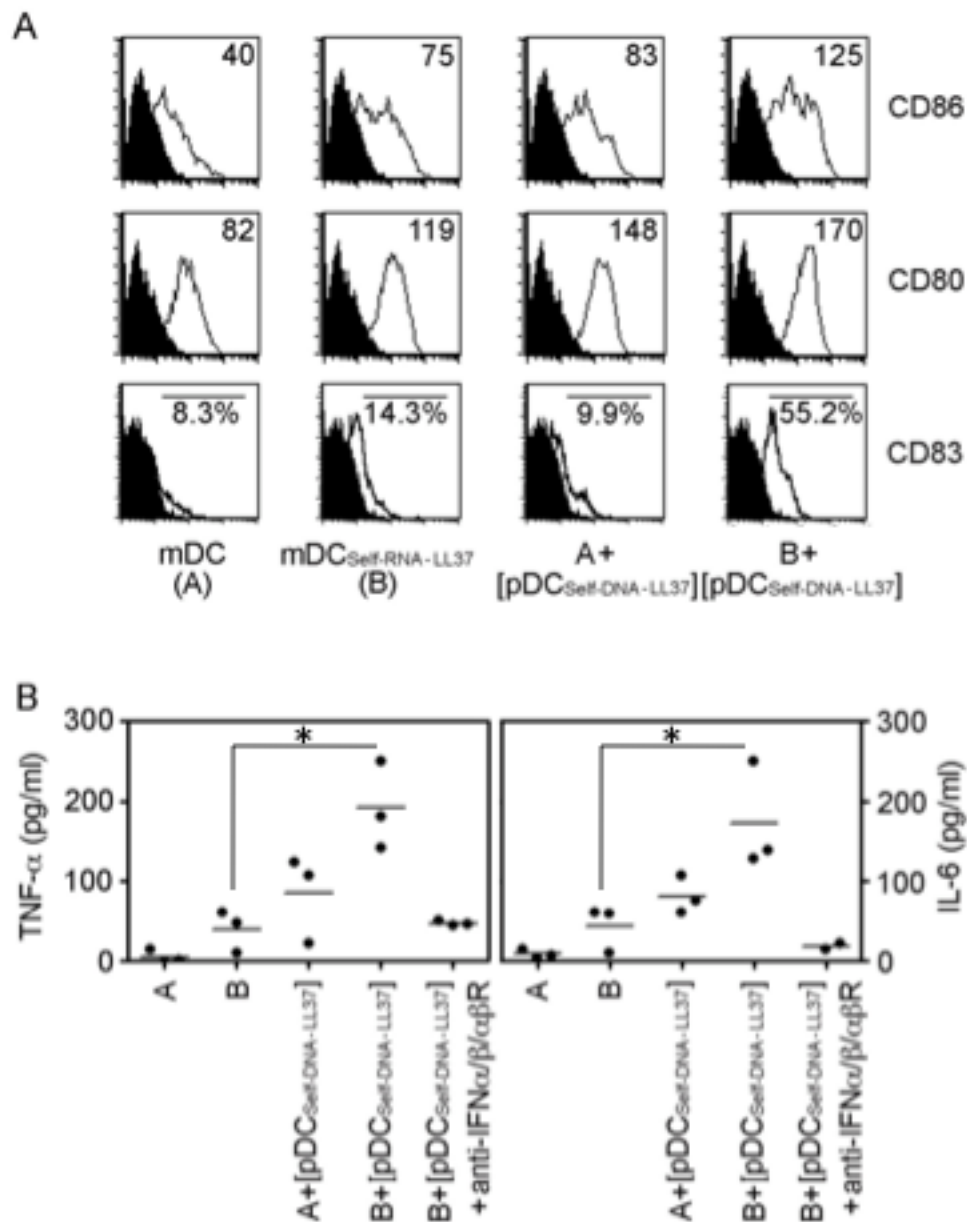


**Figure 2.19. Self-RNA-LL37 complexes trigger endosomal TLR8 activation in mDCs.** (A) TNF- $\alpha$  and IL-6 secretion by mDCs stimulated with self-RNA-LL37 complexes after pretreatment with increasing concentrations of bafilomycin. Stimulation of the mDCs with LPS was used as negative control. Data are representative of three independent experiments. (B) NF- $\kappa$ B promoter activity of TLR8- or TLR3-transfected HEK293 cells measured by luciferase reporter assay after stimulation at the indicated conditions. Data in D are the mean  $\pm$  SEM of five independent experiments. \*,  $P < 0.05$ ; paired Student's T test. (C) TNF- $\alpha$  (black) and IL-6 (white) secretion by mDCs after stimulation with short single-stranded RNA sequences ssRNA40 and ssPolyU alone or in complex with LL37.

our studies we found that both these subsets of human dendritic cells are able to sense self nucleic acids, in the context of the endogenous antimicrobial peptide LL37, through triggering of the endosomal TLRs. While pDCs can respond to both DNA and RNA, mDCs are able to respond only to RNA in this context. But the type I IFN produced by pDCs in response to the self nucleic acid-AMP complexes is potentially able to affect also the mDCs. As the responsible AMP is expressed in a clinical context involving host cell death associated with extracellular release of both DNA and RNA molecules and both pDCs and mDCs have been shown to be recruited in such inflammatory processes, there is possibility of a cooperative synergy between these two DC subsets mediated through type I IFNs.

To investigate this possibility we wanted to see whether supernatants, derived from the pDC cultures stimulated with self-DNA-LL37 complexes, when added in mDC cultures before being stimulated with the self-RNA-LL37 complexes, could enhance the activation and maturation response. When we compared the effects of stimulation with either the supernatant of activated pDCs or self-RNA-LL37 alone on mDCs with the response in presence of a combination of both, we found significant enhancement of production IL-6 and TNF- $\alpha$  by the mDCs (**Figure 2.20.A**) and their differentiation into mature CD83<sup>+</sup> DCs by the combined stimulation (**Figure 2.20.B**). When we added an antibody cocktail containing neutralizing antibodies against IFN- $\alpha$ , IFN- $\beta$  and the IFN- $\alpha\beta$  receptor in the supernatant from the activated pDC culture this cooperative enhancement response in mDCs was completely blocked (**Figure 2.20.B**). This demonstrated

Figure 2.20



**Figure 2.20. IFN- $\alpha$  induced by concomitant pDC activation enhances maturation and cytokine production by mDCs. (A)** Flow cytometry for CD86, CD80, and CD83 surface expression. Data are representative of at least three experiments. **(B)** TNF- $\alpha$  and IL-6 secretion measured by ELISA. Each symbol represents an independent experiment, and horizontal bars represent the mean. \*p value <0.05, paired Student's T test.

that the enhancement of mDC activation, in presence of the supernatant from activated pDC culture, was mediated by the type I IFNs present in that supernatant. Thus, the activation and maturation of mDCs in response to the self-RNA-LL37 complexes can be enhanced by the concomitant activation of pDCs to produce IFN- $\alpha$ .

### **2.3. Chapter 3: Pathologic relevance of immune recognition of self nucleic acids in the context of autoimmune diseases**

In the studies done in our lab we found that the endogenous cationic antimicrobial peptide LL37 can go into an electrostatic interaction with anionic nucleic acid molecules of self origin and transport them into the endosomal compartments of both pDCs and mDCs to induce TLR activation (Lande R et al, 2007; Ganguly D et al, 2009). These findings identify a novel pathway for autoimmune activation to self nucleic acids in human diseases. To confirm the relevance of these biological phenomena in clinical settings we wanted to look at human autoimmune diseases where this pathway can play a key role hypothetically.

**F**irst of them is naturally Psoriasis, as LL37 is over-expressed in psoriatic epidermis and it was identified to be the active principle present in psoriatic skin extract that was shown to trigger pDC activation and type I IFN induction (Lander R et al, 2007) and this pDC-derived type I IFNs have been shown to be instrumental for disease initiation (Nestle FO et al, 2005). Ability of LL37 to interact with self-RNA opened the possibility of TLR activation concomitantly in pDCs and mDCs through the same pathway that leads to self-DNA-mediated pDC activation and thus could explain the presence of activated mDC in the psoriatic skin as reported earlier (Lowes MA et al, 2005).

**A**nother autoimmune condition, where this autoimmune activation to self nucleic acids can play a key role, is systemic lupus erythematosus or SLE. In SLE

autoreactive B cells producing autoantibodies against self-DNA, chromatin, and RNA-associated proteins are circulating in blood and insoluble immune complexes formed by these circulating autoantibodies with extracellular self-nucleic acids get deposited in different tissues resulting in local inflammation and tissue injury. Moreover, type I IFNs and pDCs have been reported to play an important role in SLE pathogenesis (Theofilopoulos AN et al, 2005; Blomberg S et al, 2001).

### **2.3.A. Psoriasis**

#### **2.3.A.a. Background and Rationale**

As depicted above psoriatic skin lesions show a very high level of expression of the cationic antimicrobial peptide LL37 and we found that LL37 can interact with both DNA and RNA molecules of self-origin to make particulate complexes that are then internalized into both the plasmacytoid and myeloid dendritic cells. This enables the self nucleic acids to access the endosomal toll-like receptors and trigger innate immune activation. On the other hand local tissue injury has been shown to trigger aggravation of psoriatic disease progression, termed as Koebner's phenomenon (discussed in Section 1.6). To link these findings and to establish our hypothesis of innate immune activation in Psoriasis in response to Nucleic acid-AMP complexes, we wanted to look for the particulate complexes formed by self-nucleic acid and the AMP molecules *in situ* taking cryosections from involved skin of psoriatic patients. Also we wanted to investigate whether

the activation of infiltrating mDCs in the lesions can be linked to the presence of the ribonucleic acid particles being formed *in situ*.

### **2.3.A.b. Materials and Methods**

#### **Collection of human samples.**

The study with clinical samples from patients was approved by the Institutional Review Board for human research at the M. D. Anderson Cancer Center in Houston and the Heinrich-Heine University in Düsseldorf. Skin samples were collected by 6-mm punch biopsies from normal skin of healthy subject, from lesional skin of patients with chronic plaque psoriasis and atopic dermatitis. The clinical conditions were defined according to standard clinical and histopathological criteria. Cryosections were prepared from these biopsy specimens.

#### **Immunofluorescence and immunohistochemistry.**

Immunofluorescence staining for nucleic acids was done on cryosections with the nucleic acid dyes DAPI (Sigma-Aldrich, MO) and Ribogreen (Molecular Probes, CA). The cryosections after thawing were fixed in acetone for 10 min at 4°C. Acetone-fixed sections were then washed in PBS and incubated with the blocking buffer (PBS with 2% goat serum, 0.1% fish gelatin and 0.05% Tween20) for 30 min at room temperature. Then the sections were incubated with mouse

anti-LL37 (mAb 3d11, Hycult Technologies) or anti-DC-LAMP antibody (mAb 104.G4, Coulter Immunotech) or isotype control overnight, followed by further washes and incubation with Alexa 647-conjugated anti-mouse IgG secondary antibody for 30 min at room temperature. After washes the sections were incubated with DAPI (0.1ng/ml) and Ribogreen (1:100000) for 5 min to stain the nucleic acids. Then the slides were washed, mounted with prolong gold mounting medium (Molecular Probes, CA) and stored at 4°C till the images were acquired. Ribogreen and DAPI were added following the antibody incubations for best staining efficiency. The images were acquired in a Leica TCS SP2 confocal microscope and analyzed with Leica confocal software.

Immunohistochemical staining for DC-LAMP on these cryosections from psoriatic skin biopsies were done using mouse anti-DC-LAMP antibody (mAb 104.G4, Coulter Immunotech, Marseille, France). Peroxidase-conjugated secondary antibody was used with DAB detector reagent. Images were acquired with an Olympus DP70 microscope.

The extracellular RNA complexes and the DC-LAMP<sup>+</sup> DCs in the tissue sections were quantitated by two independent investigators who counted three best fields in each section.

### **Statistical analysis.**

Comparison between samples from different clinical conditions for the number of extracellular RNA particles was statistically validated by unpaired Students' T

test. Correlation studies were analyzed by two-tailed Pearson's correlation ( $p < 0.05$  was considered statistically significant). Statistical evaluations were done on GraphPad Prism software (La Jolla, CA).

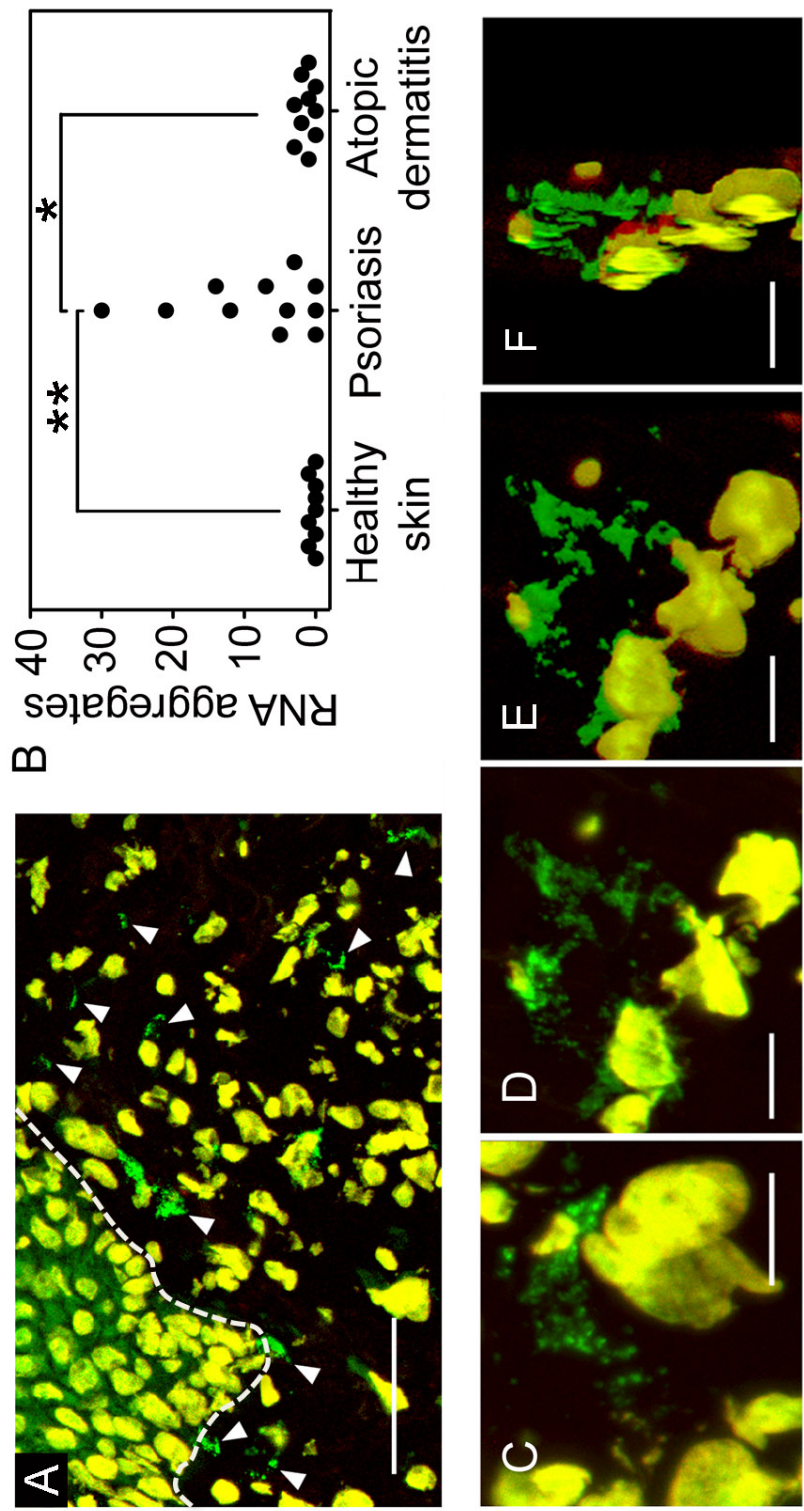
### **2.3.A.c. Results & Discussion:**

#### **Nucleic acid complexes are present in psoriatic skin.**

Our lab has previously demonstrated that the antimicrobial peptide LL37 is highly expressed in the epidermis of the inflamed skin in psoriatic patients, while they are undetectable in the skin affected with atopic dermatitis or healthy skin (Lande R et al, 2007). The *in vitro* studies, revealing the ability of the LL37 peptide to interact with self-origin DNA and RNA molecules and form particulate complexes, led to the possibility of these complexes to be formed also *in vivo*. Moreover, as these nucleic acid-peptide particulate complexes were microscopically visible we set on to find these complexes *in situ* in the cutaneous cryosections from psoriatic patients.

The initial goal was to find extracellular nucleic acids in the tissue sections and for these we stained the cryosections with two nucleic acid dyes DAPI and Ribogreen to differentiate between DNA and RNA complexes. DAPI binds preferentially to DNA, while Ribogreen binds both DNA and RNA and is very sensitive to even low amounts of RNA (Jones LJ et al, 1998). Thus we defined extracellular RNA complexes to be extracellular particulate structures stained only with Ribogreen but not with DAPI, and DNA complexes as similar structures

Figure 2.21



**Figure 2.21. Extracellular RNA complexes are present in psoriatic skin.** (A) Confocal microscopy image of a representative psoriatic skin lesion stained with DAPI (red) and Ribogreen (green). DNA in cell nuclei appears yellow (Ribogreen<sup>+</sup>/DAPI<sup>+</sup>), and arrowheads indicate extracellular RNA aggregates appearing green (Ribogreen<sup>+</sup>/DAPI<sup>-</sup>). Bar, 50  $\mu$ m. (B) Numbers of visible extracellular RNA aggregates in the dermal compartment of healthy skin (n = 9), psoriatic skin (n = 11), and atopic dermatitis (n = 10). \*\*, P = 0.0295; \*, P = 0.042; unpaired Student's T test (both two-tailed). (C-F) High magnification images of extracellular RNA aggregates in the dermal compartment of psoriatic skin lesions. Bar, 10  $\mu$ m. (E and F) SFP projection of panel D.

doubly stained by the both dyes. Under a laser-scanning confocal microscope we could find a lot of extracellular Ribogreen<sup>+</sup>DAPI<sup>-</sup> complexes in the dermal compartment of psoriatic skin lesions (**Figure 2.21.A**).

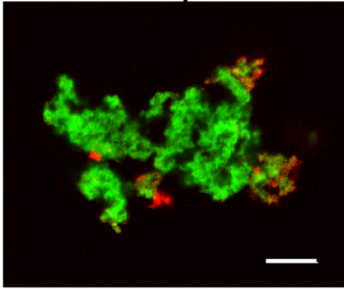
We also looked for similar complexes in cryosections from the skin affected with atopic dermatitis or in skin samples from healthy donors and the abundance of such particulate RNA complexes were significantly low in these cryosections (**Figure 2.21.B**). This was compatible with our hypothesis as healthy skin as well as skin affected with atopic dermatitis do not express the cationic antimicrobial peptides in contrast to their high expression in psoriatic skin. The self-RNA-LL37 complexes showed a characteristic branched and aggregated beads structure as found *in vitro* (**Figure 2.21.C-F**). As expected a lot of Ribogreen<sup>+</sup>DAPI<sup>+</sup> DNA complexes were also found in the psoriatic skin sections.

### **Nucleic acid complexes in psoriatic skin contain LL37.**

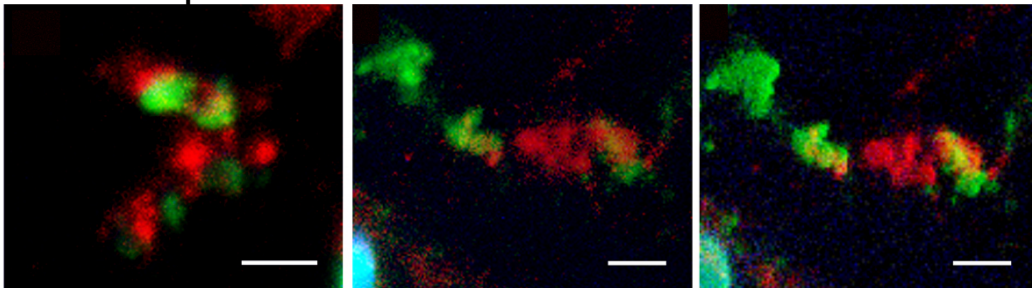
The extracellular RNA complexes *in situ* shared several morphological features with the RNA-LL37 complexes generated *in vitro*. The similarities in the size of the particles, the characteristic morphology of branched aggregates of bead-like structures resulting from the aggregation of smaller particles and the very fact of their sustenance in frozen tissue perhaps due to relative resistance to extracellular enzymatic degradation raised the possibility of similar interaction between cationic peptides and nucleic acids to be behind their formation as we found *in vitro*.

Figure 2.22

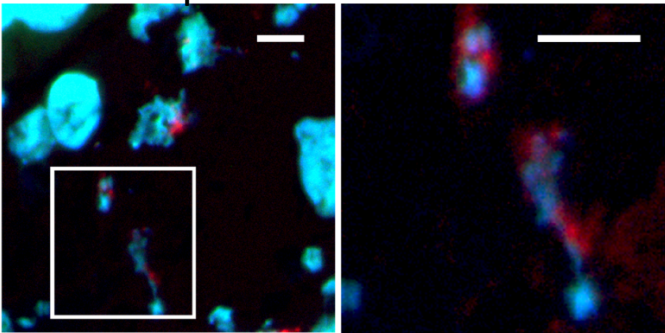
## A RNA complexes in vitro



## B RNA complexes in situ



## C DNA complexes in situ



**Figure 2.22. Extracellular RNA complexes in psoriatic skin show presence of LL37.** (A) Representative confocal microscopy images of in vitro-generated self-RNA-LL37 complexes stained with Ribogreen (green) and an anti-LL37 antibody (red). (B) Representative confocal microscopy images of RNA-LL37 aggregates in psoriatic skin cryosection stained with Ribogreen (green) and an anti-LL37 antibody (red). Extreme right: SFP projection of middle panel. (C) Representative confocal microscopy images of DNA-LL37 complexes in psoriatic skin stained with DAPI (blue), Ribogreen (green) and anti-LL37 antibody (red). Bars, (A) 10  $\mu$ m; (B) 2  $\mu$ m.

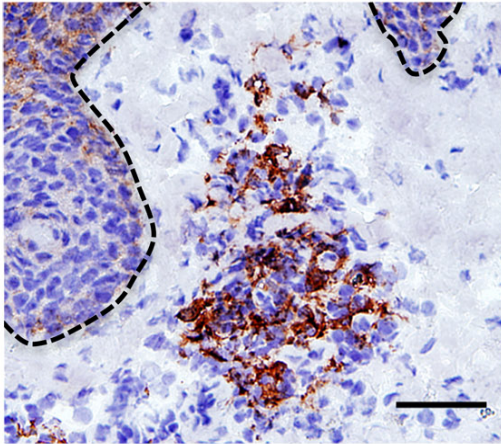
To confirm this speculation we investigated whether the extracellular RNA complexes found in the tissues showed concomitant presence of LL37. First we wanted to establish the detection of this peptide on the *in vitro* formed RNA particles with an antibody. We could stain LL37 on the *in vitro* generated complexes by staining the self-RNA-LL37 particles with anti-LL37 and Ribogreen (**Figure 2.22.A**). Interestingly, we found that the antibody was not bound all over perhaps due to a mutual exclusion of sites for antibody binding to the complex and Ribogreen staining. By doing similar staining on the cryosections from psoriatic skin biopsies for the complexes with an anti-LL37 antibody we found that on a lot of these extracellular RNA complexes presence of LL37 could be detected (**Figure 2.22.B**), similar to the RNA-LL37 complexes *in vitro*. A lot of the extracellular DNA complexes, identified as Ribogreen<sup>+</sup>DAPI<sup>+</sup> structures, were also found to show similar presence of LL37 on them (**Figure 2.22.C**).

**Abundance of self-RNA complexes in psoriatic skin can be linked to mDC activation.**

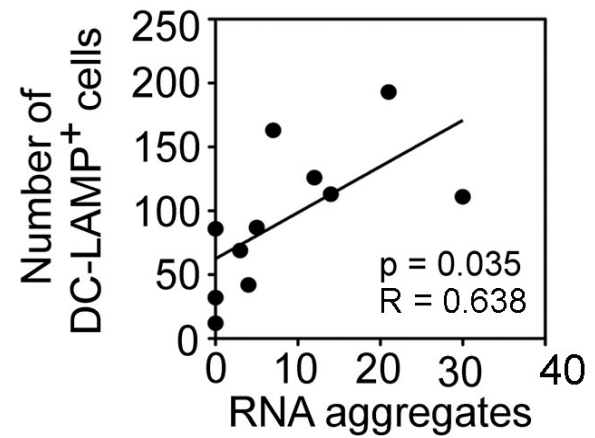
Finding the RNA-LL37 complexes *in situ* in the lesional skin of psoriatic patients led to further investigations to look for whether the presence of these RNA-complexes is linked to the activation of mDCs that infiltrate the psoriatic skin lesions. We looked in serial sections of lesional skin for the activated mDCs by immunohistochemical staining of the sections for the expression of DC-LAMP, a lysosomal marker that is specifically expressed in activated and mature mDCs.

Figure 2.23

A



B



**Figure 2.23. Extracellular RNA complexes in psoriatic skin is associated with mDC activation *in situ*.** (A) Immunohistochemical results for DC-LAMP in a representative psoriatic skin lesion. Bar, 50  $\mu$ m. (B) Correlation between the numbers of DC-LAMP<sup>+</sup> mDCs and the numbers of visible extracellular RNA aggregates in multiple psoriatic skin lesions (n = 11). Pearson R = 0.615, two-tailed p = 0.044. SFP, simulated fluorescence Projection.

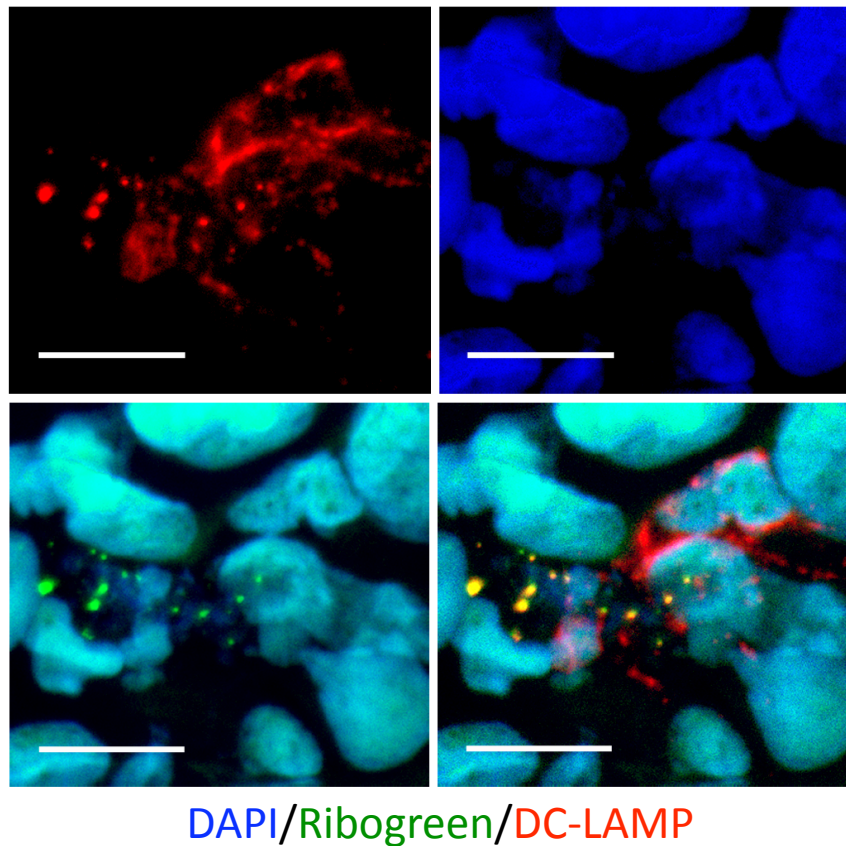
We found clusters of DC-LAMP positive mature mDCs at the epidermal-dermal junctions and in the dermis of the lesional skin (**Figure 2.23.A**), which was consistent with earlier reports (Lowes MA et al, 2005).

To investigate whether relative abundance of the RNA-complexes in the lesions could be linked to the frequency of activated mDCs found in the same lesions we compared lesional tissue sections from 11 psoriatic patients. We found a very significant correlation between the numbers of RNA-complexes found with the number of activated mDCs seen (**Figure 2.23.B**). This quantitative correlation pointed to a mechanistic link between the *in situ* RNA-complexes and mDC activation.

### **Self-RNA complexes can be seen associated with endolysosomal compartments in mDCs**

In our experiments where we stained cryosections from lesional skin from psoriatic patients we found numerous RNA-complexes near or within the clusters of activated mDCs. The activation of mDC in these was indicated by the expression of DC-LAMP. DC-LAMP is a membrane-bound glycoprotein expressed in the endolysosomal compartments of myeloid dendritic cells induced upon maturation of the DCs (de Saint-Vis B et al, 1998). We found (described in Section 2.2) that the RNA-LL37 complexes are taken up into the endosomal compartments in mDCs where they trigger TLR8 activation. In some of the stained cryosections of lesional skin from psoriatic patients we could even find

Figure 2.24



**Figure 2.24. Extracellular RNA complexes can be found within endolysosomal network of activated mDCs *in situ*.** Confocal microscopy images of DC-LAMP<sup>+</sup> mature mDCs (red) in the dermal compartment of a representative psoriatic skin lesion co-stained with DAPI (blue) and Ribogreen (green). The images depict DC-LAMP (top left), DAPI (top right), and the merged image of DAPI and Ribogreen (bottom left). The merged image (bottom right) shows the colocalization of RNA complexes (Ribogreen<sup>+</sup>/DAPI<sup>-</sup>; green) with DC-LAMP-positive organelles (red) appearing yellow. Bar, 10  $\mu$ m.

RNA-complexes inside the endo-lysosomes of mDCs. The Ribogreen<sup>+</sup>DAPI<sup>-</sup> complexes were found to co-localize with DC-LAMP positive vesicles on co-stained sections (**Figure 2.24**).

Taken together our findings strongly supported the hypothesis that extracellular RNA complexes present in lesional skin can drive mDC activation *in vivo*. Also these studies confirmed involvement of this antimicrobial peptide-driven immune recognition of self nucleic acids leading to innate immune activation in this cutaneous autoimmune disease.

## **2.3.B. Systemic lupus erythematosus**

### **2.3.B.a. Background and Rationale**

We have found in our studies with tissue samples from lesional skin of psoriatic patients that the antimicrobial peptide-driven innate immune recognition of self nucleic acid plays a major role in the initiation of the inflammatory cascade in this cutaneous autoimmune disease. On the other hand other studies done in the lab interestingly found that the antimicrobial peptide LL37 is present in the immune complexes isolated from the serum of patients affected with systemic lupus erythematosus or SLE (Lande R a et al, 2010). Moreover these immune complexes could activate pDCs to induce type I IFN production and this activity could be abolished when the immune complexes were depleted of the antimicrobial peptides (Lande R et al, 2010). These findings, along with the established knowledge of autoimmunity to self nucleic acids and nucleoproteins

and circulating autoantibodies to these molecules in SLE, intrigued us to investigate whether this presence of antimicrobial peptides in the SLE immune complexes is also due to the electrostatic interaction with the self nucleic acid molecules and whether they bear the characteristic morphology as seen with nucleic acid-AMP complexes *in vitro* and in psoriatic lesions (Ganguly D et al, 2009). This was interesting as it may be that the existence of these complexes in the circulating blood and in different tissue spaces is due to the relative resistance of the complexes to extracellular enzymatic degradation conferred by the interaction with antimicrobial peptides. It was also interesting to explore the potential source of the nucleic acids in the SLE immune complexes.

### **2.3.B.b. Materials and Methods**

#### **Reagents.**

Synthetic LL37 peptide (LLGDFFRKSKEKIGKEFKRIVQRIKDFLRNLPRTES) was from Innovagen (Lund, Sweden). Natural human neutrophil peptides [HNP<sub>(1-3)</sub>] were from Hycult Biotechnology (Canton, MA). Human genomic DNA (3 µg/ml) was procured from BioChain (Hayward, CA). DNase I (200 U/ml) was from Roche (Nutley, NJ). Monoclonal antibody against LL37 (clone 8A8, IgG2b, OK) was generated by immunizing 6- to 8-wk-old BALB/c mice with synthetic LL37 peptide and was used at 10 µg/ml. Monoclonal antibody against HNPs (clone 3G9, IgG2a) was from Novus Biological (Littleton, CO) and was used at 10 µg

ml<sup>-1</sup>. Monoclonal antibody against dsDNA (clone H241, IgG2b, OK) was a kind gift from Dr. Stollar, Tufts University, and was used at 10 µg/ml.

### **Collection of human samples.**

Studies with human samples were approved by the Institutional Review Board for human research at The University of Texas M. D. Anderson Cancer Center in Houston and the Kansai Medical University in Osaka. The diagnosis of SLE was based on the American College of Rheumatism criteria for SLE. For purification of immune complexes, only sera from SLE patients with anti-DNA titers >60 IU/ml were selected (provided by The Binding Site Inc., San Diego, CA). Total IgGs from the sera of SLE patients and healthy controls were isolated with use of a HiTran™ Protein-G HPLC (GE Healthcare, Piscataway, NJ). Immune complexes (SLE-IC) were separated from monomeric IgGs by using an Amicon centrifugal filter device with a 300,000 nominal molecular weight limit cutoff.

### **Microscopic visualization of immune complexes.**

Total human genomic DNA (self-DNA) was purchased from BioChain (Hayward, CA) or extracted from U937 cells by using standard procedures. Artificial immune complexes containing Self-DNA were generated by adding self-DNA (3 µg/ml) with LL37 (10 µM), HNP<sub>(1-3)</sub> (10 µM), and anti-dsDNA antibodies (1 µg/ml) in 20 µl of phosphate-buffered saline (PBS) and incubated for 30 min at room

temperature. For visualization of the complexes, suspensions containing *in vitro*-generated artificial immune complexes or purified SLE immune complexes were spun down and stained with DAPI (0.1 ng/ml, Sigma-Aldrich) and analyzed by confocal microscopy. In some experiments, the artificial immune complexes and the purified SLE immune complexes, in a volume of 20ul, were treated for 1 h with DNase I (Roche, 600 U/ml) or the anionic polypeptide poly aspartic acids (5,000-15,000 molecular weight), from Sigma-Aldrich and used at 10  $\mu$ M, to provide an excess of negative charge.

#### **Nuclease protection assay.**

To investigate the susceptibility of different DNA complexes self-DNA alone, DNA-LL37 complex, DNA added with anti-DNA antibody and DNA-LL37 added with anti-DNA antibody were run on a 1% agarose gel. The same samples were also treated with DNase 200U/ml before running on the gel to look for sensitivity of the samples to DNase degradation.

#### **Induction of NETosis in neutrophils.**

For isolation of human neutrophils from the circulating blood of healthy donors, we first treated whole peripheral blood with a ACK lysing buffer (Lonza, Walkersville, MD) to lyse erythrocytes and then isolated neutrophils by using anti-CD15-conjugated microbeads (from Miltenyi Biotec). 93-94% of CD15<sup>+</sup> cells were

neutrophils that express high levels of CD15 (eosinophils and some monocytes also express low levels of CD15). Purified neutrophils were seeded at  $50 \times 10^6$  cells/ml in complete RPMI medium and either left untreated or stimulated with PMA (Sigma-Aldrich, 20 ng/ml), anti-LL37 (10  $\mu$ g/ml), anti-HNP (10  $\mu$ g/ml), anti-dsDNA, or IgG control antibodies (10  $\mu$ g/ml) in a 96 well plate followed by transfer of the cells on poly-L-lysine coated coverslips and incubated for 3 h at 37°C.

### **Microscopic visualization of NETs.**

For microscopic visualization and quantitation of NETosis, stimulated neutrophils (as described above) were seeded on coverslips pretreated with poly-lysine. Coverslips were washed gently after 3 hours, put on glass slide and visualized under an immuno-fluorescence microscope (Olympus BX61). We did not use any mounting medium to prevent loss of extracellular DNA fibrils. The quantitation involved scoring of the NET density by two independent observers. The 1-10 scoring system was arbitrated taking less than 10% percent cover-up area of extracellular DNA fibrils as score 0 and more than 90% of the same as score 10.

### **Statistical analysis**

Statistical significance of differences was determined by Student's t test and ANOVA and Bonferroni's t test ( $p < 0.05$  was considered statistically significant).

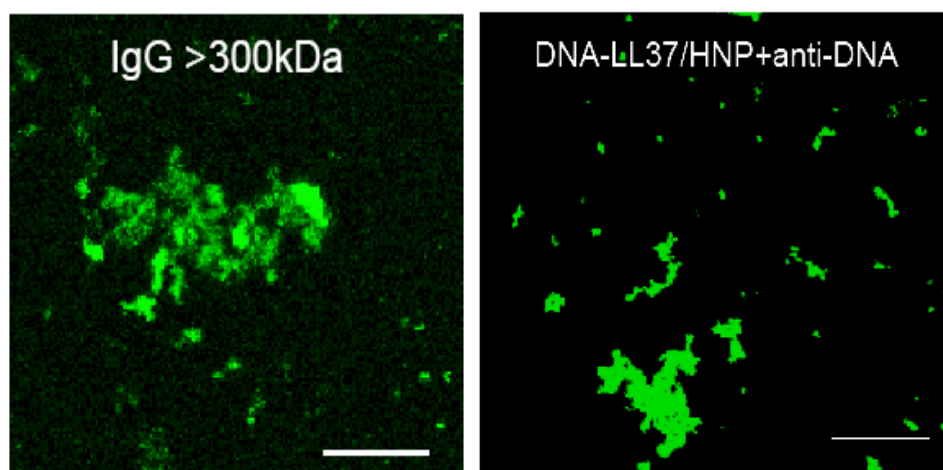
### 2.3.B.c. Results & Discussion:

#### SLE immune complexes consist of DNA micro-particles

As found in other studies done in our lab (Lande R et al, 2010), immune complexes isolated from sera of SLE patients contain neutrophil-derived endogenous antimicrobial peptides LL37 and alpha defensins (HNPs, Human Neutrophil-derived peptides). In our previous studies we found (as described in Section 2.1) that LL37 can bind to both human DNA and RNA and form characteristic micro-particles that are visible under microscope. Lupus immune complexes are conventionally thought to be composed of self nucleic acids, nucleoproteins and antibodies to these self molecules. But the presence of the cationic AMP LL37 in those complexes by Lande R et al led us to investigate whether there is any interaction between the nucleic acid molecules and LL37 contained in the SLE immune complexes.

When the immune complex suspension (>300kD molecular weight) isolated from Lupus sera was spun down we found a precipitate in the centrifugation tube. On re-suspending the precipitate in PBS and staining with DAPI, the DNA-binding dye, we found characteristic particulate aggregates with similar morphology as the DNA-LL37 or RNA-LL37 particles described before (in Section 2.1) (**Figure 2.25**). To confirm this observation we made artificial immune complexes by adding all the identified constituents *in vitro* like human DNA, anti-DNA antibody, LL37 and HNP in indicated amounts. This also gave a precipitate on centrifugation and on staining the precipitate with DAPI after re-suspension in

Figure 2.25



**Figure 2.25. SLE Immune complexes show the characteristic aggregated-particle morphology of nucleic acid-AMP complexes.** Confocal microscopy image of DNA-containing immune complexes (IgGs >300 kDa) (left) and artificial immune complexes (formed by DNA, LL37, HNP and anti-DNA antibody) detected as insoluble particles that stained with DAPI. Bar = 10  $\mu$ M.

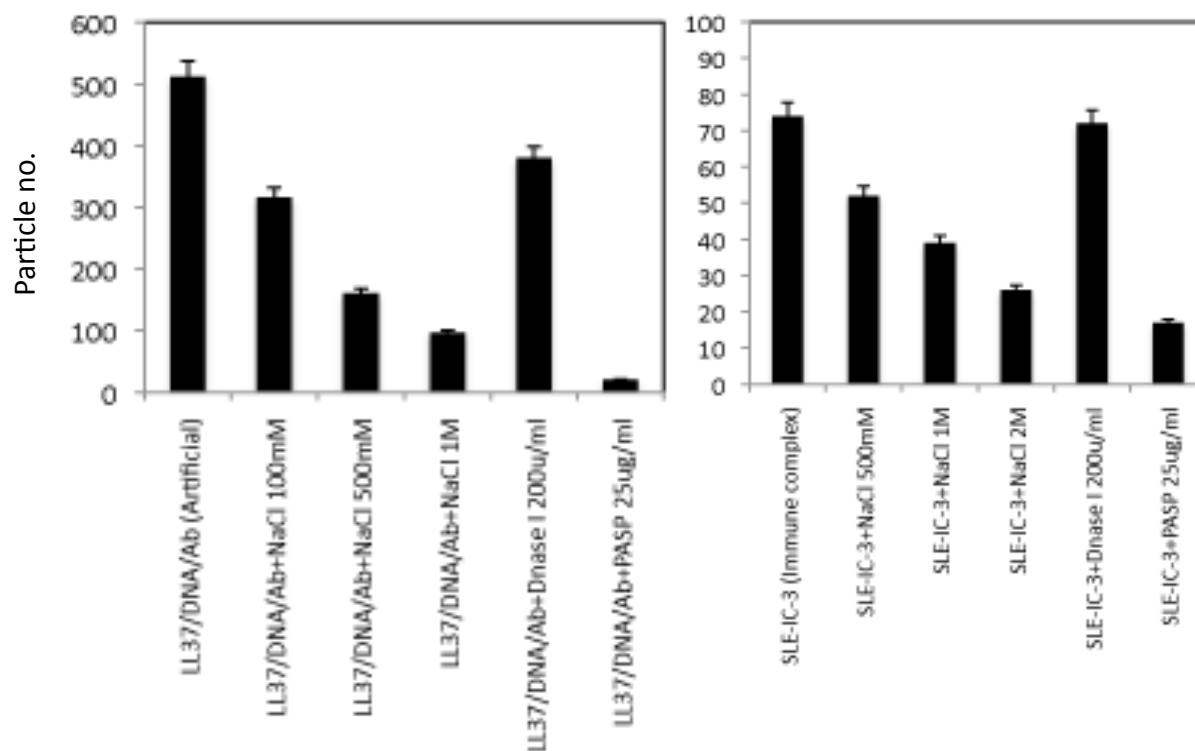
PBS we found similar particulate aggregates just as seen in immune complexes from Lupus sera (**Figure**). These observations indicated that the nucleic acid molecules and the cationic AMPs contained in Lupus immune complexes might interact to form particulate complexes.

### **Immune complex micro-particles form by electrostatic interaction between antimicrobial peptides and nucleic acids**

When we found the characteristic micro-particles formed with the Lupus immune complexes, it led us to speculate that a similar electrostatic interaction as we found *in vitro* between self-DNA or self-RNA and LL37 is also operating in this case (discussed in Section 2.1; Ganguly D et al, 2009). To prove that these complexes are indeed formed by electrostatic interaction between the cationic peptide and anionic nucleic acid molecules, we added poly-aspartic acid, an anionic polymer, to competitively inhibit this interaction. These particulate complexes dissolved rapidly when added with poly-aspartic acid pointing to an electrostatic interaction in forming these complexes (**Figure 2.26**).

We have previously shown that the particulate complexes formed by DNA and RNA with LL37 are protected from enzymatic degradation (discussed in Section 2.1; Ganguly D et al, 2009) due to condensation of the nucleic acids in presence of the cationic peptides. We speculated that similar protection from enzyme digestion could also play a role in sustenance of these nucleic acid containing immune complexes. Both the complexes isolated from Lupus sera and the

Figure 2.26



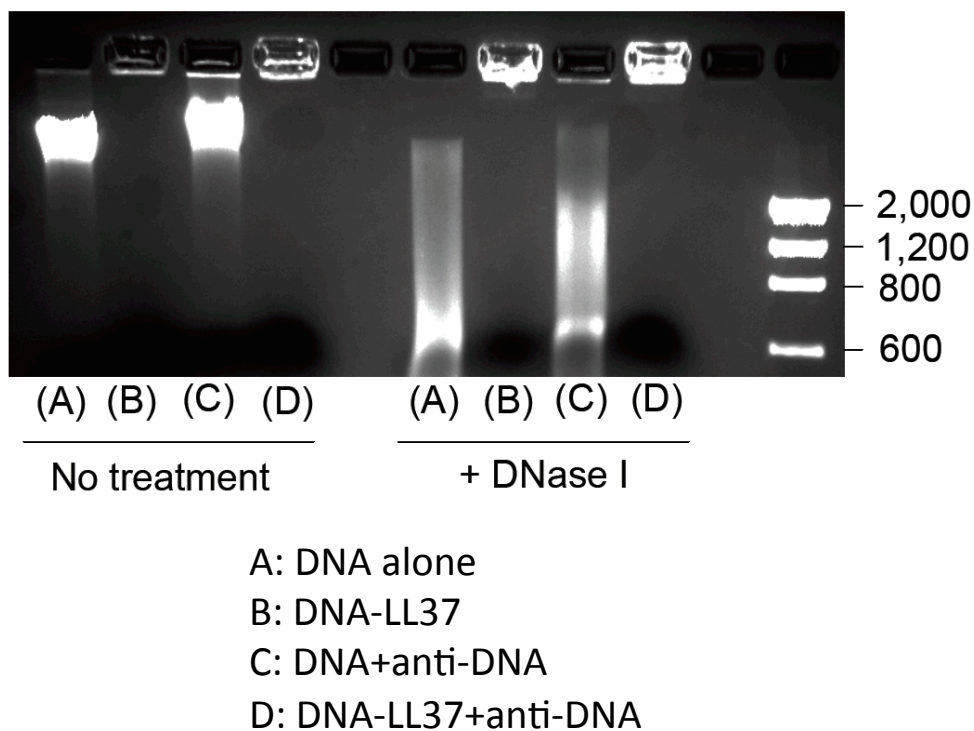
**Figure 2.26. The artificial and natural Immune complexes form by electrostatic interaction and are protected from enzymatic degradation.** Number of DNA-LL37/HNP +anti-DNA artificial complexes (left) and natural DNA/anti-DNA complexes (right) counted as insoluble DAPI+ particles with or without treatment with DNase I or poly-ASP and in the presence of increasing concentration of NaCl. Particles counted under a confocal microscopy and represented as number in a confocal microscopic field with a 63.5X objective.

artificial immune complexes made *in vitro* were protected from DNase degradation (**Figure**). To confirm that the antimicrobial peptides present in the immune complexes are responsible for this protection of DNA and not the anti-DNA antibodies present, we did one agarose gel electrophoresis based nuclease protection assay with DNA alone, DNA-LL37 complex, DNA with anti-DNA antibody and DNA-LL37 complex with anti-DNA antibody. We found that only in presence of LL37 in the complexes the DNA contained in the complex is protected from DNase activity, but not with anti-DNA antibody (**Figure 2.27**). This indicated that the antimicrobial peptides contained in Lupus immune complexes protect the nucleic acids in immune complex micro-particles from enzymatic degradation and this protection cannot be achieved by the antibodies to nucleic acids present in the immune complexes.

### **NETosis in neutrophils is a potential source of the immune complex micro-particles**

In our studies we found that nucleic acid-endogenous antimicrobial peptide complexes, similar to what we found in case of the cutaneous autoimmune disease Psoriasis, is present and they play an important role in immune complex mediated systemic disease in Lupus. But a very important question was what is the source of these complexes in SLE sera. A relevant knowledge derived by previous reports in SLE pathogenesis is the presence of circulating apoptotic neutrophils in SLE patients, abundance of which has been correlated with

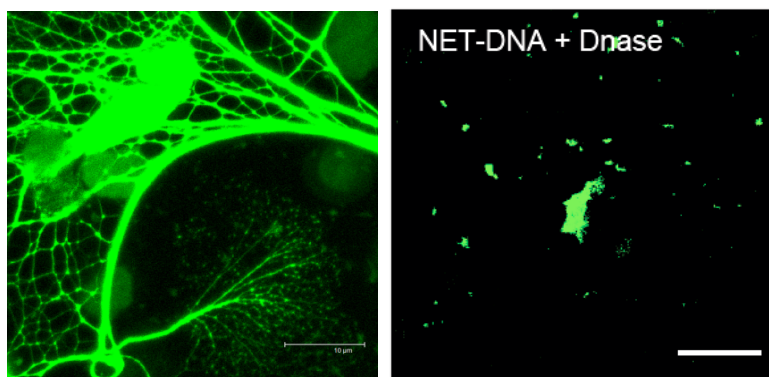
Figure 2.27



**Figure 2.27. Protection of DNA from enzymatic degradation is mediated by the presence of LL37 in the complexes.** Agarose gel electrophoresis of human DNA alone (A), human DNA in complex with LL37 (B), anti-DNA antibodies (C), or LL37 plus anti-DNA antibodies (D) before and after treatment with DNase I. The DNA was visualized by ethidium bromide staining.

disease progression and autoantibody production (discussed in Section 1.7; Courtney PA et al, 1999; McConnell JR et al, 2002). Apoptotic neutrophils can potentially be a rich source of extracellular DNA in the sera. Moreover, very high production of the AMPs in question, LL37 and HNP, by the neutrophils creates the possibility of them being the source of the nucleic acid-AMP complexes we found in the immune complexes. LL37 and HNP are known to be stored in the secretory granules in neutrophils and they are released on degranulation (Lehrer RI, 2004). Recent work has discovered that dying neutrophils release long filament of genomic DNA, termed Neutrophil Extracellular Traps (NETs), in response to stress and bacterial infection (Brinkmann V et al, 2004; Fuchs T et al, 2007). This interesting type of cell death induced in neutrophils has been given the name NETosis. Interestingly these NETs contain the granule-derived antimicrobial peptides LL37 and HNP and they are believed to play a role in killing bacteria trapped on these NET (Brinkmann V et al, 2007). Thus presence of both the antimicrobial peptides and genomic DNA in the NETs and circulating apoptotic neutrophils in Lupus, together point to strong possibility of these NETs to be the source of the DNA-LL37 particulate complexes contained in the Lupus immune complexes. We investigated the possibility of finding nuclease-insensitive domains in the NETs produced by neutrophils in response to nonspecific activation with phorbol myristate acetate. We found the long filaments of DNA on the NET structures disappeared on DNase treatment leaving behind protected DNA particles quite similar in morphology to the small micron-range particulate complexes found in the immune complexes and with *in vitro*

Figure 2.28



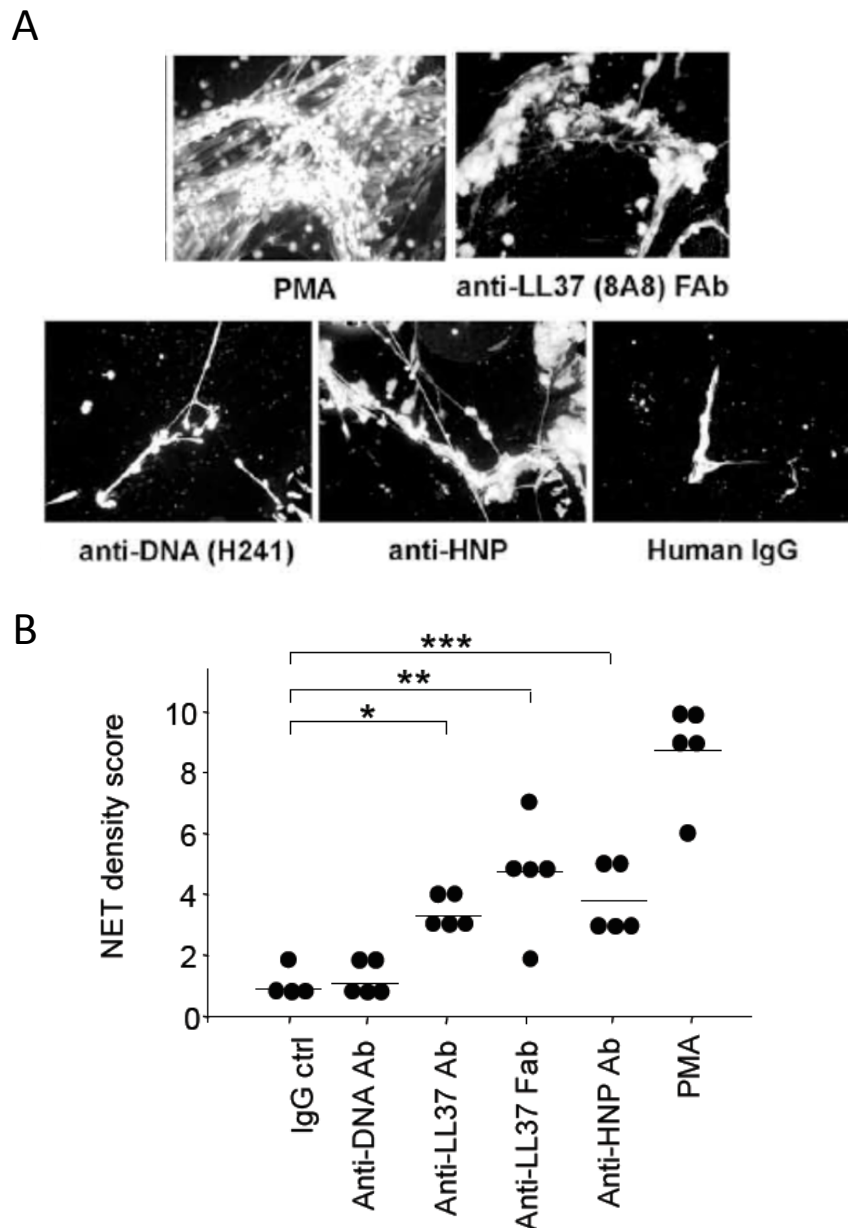
**Figure 2.28. Nuclease-resistant domains in neutrophil-derived NET-DNA.** Neutrophils were stimulated with PMA to induce NETosis on coverslips. NET-DNA was stained with DAPI and imaged under a confocal microscope with (right panel) or without (left panel) a 30-min pretreatment with DNase I. Bar = 10 µm.

formed complexes (**Figure 2.28**). The size and the characteristic morphology of NET-derived DNA particles and their resistance to DNase degradation and presence of antimicrobial peptide LL37 on the NETs as shown earlier, suggests that NETs can be the potential source of self-DNA in immune complexes in SLE patients.

### **Autoantibodies to the antimicrobial peptides can drive neutrophil NETosis**

Although our experiments gave suggestive evidence for the NETs being potential source of the nucleic acid-AMP particles in the Lupus immune complexes, question remained about the cause of the NETosis process in SLE. Neutrophil cell death associated with the original description of NETosis was triggered by microbial infections (Brinkmann V et al, 2007). However, a very recent study reported that auto-antibodies against the enzymes myeloperoxidase and elastase, which are also present in the secretory granules in neutrophils, are able to trigger the release of NETs and inducing cell death (Kessenbrock K et al, 2009). Interestingly, studies done by others in our lab (Lande R et al, 2010) had revealed presence of auto-antibodies to the endogenous antimicrobial peptides LL37 and HNP in SLE sera. This led us to investigate whether auto-antibodies against these neutrophil-derived antimicrobial peptides can also trigger the process of NETosis. We found that antibodies against LL37 and HNP can induce NETosis in neutrophils (**Figure 2.29.A&B**), while anti-DNA antibodies and relevant isotype antibodies were not able to do the same. To exclude the role of

Figure 2.29



**Figure 2.29. The release of NETs by neutrophils is triggered by autoantibodies to antimicrobial peptides.** Purified neutrophils from healthy donors were incubated for 3 h with anti-LL37 (8A8), anti-LL37 (8A8) Fab, anti-HNP, anti-DNA (H241), control IgG antibodies, or phorbol myristate acetate (PMA) and analyzed for NET formation by confocal microscopy. **(A)** Representative confocal images of NET induction in stimulated neutrophils. **(B)** NET density values, scored from 1 to 10 in multiple independent experiments, are given; horizontal line represents the mean. \*,  $p = 0.024$ ; \*\*,  $p < 0.001$ ; \*\*\*,  $p = 0.006$ ; ANOVA (adjusted for Dunnett's test).

cell surface Fc receptor in this antibody-induced cell death we also used F(ab)<sub>2</sub> fragment of the anti-LL37 antibody and found that it was also able to induce similar NETosis (**Figure 2.29.A&B**).

Taken together we found that auto-antibodies against neutrophil derived antimicrobial peptides produced in SLE lead to neutrophil cell death and release of extracellular NETs. These NETs are known to contain the same antimicrobial peptides on them and they are potentially digested by extracellular nucleases leaving behind smaller nucleic acid-AMP particulate domains that are relatively resistant to nuclease degradation. These particulate complexes along with the anti-DNA antibodies constitute the Lupus immune complexes.

These data brings further support in favor of our general hypothesis that the electrostatic interaction between endogenous antimicrobial peptides with self nucleic acid molecules occur in physiological conditions and leads to the formation of nucleic acid-peptide complexes *in vivo*. These complexes can then access the endosomal compartments in the dendritic cell subsets and trigger toll-like receptor activation, and thus incite activation of innate immune response to self nucleic acids initiating an autoimmune inflammatory cascade.

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### **3. General Discussion**

**and**

**Future Directions**

**H**ost cell death is associated with release of the nucleic acid content of these cells into the extracellular space. While innate immune system is able to recognize the nucleic acid molecules and initiate inflammation, more often this results from recognition of nucleic acid molecules of foreign origin derived from microbial invaders. The reasons for this fact of extracellularly released nucleic acid molecules of self-origin usually failing to initiate similar inflammatory cascade, are thought to be structural differences between the nucleic acid molecules from higher organisms compared to those from microbial pathogens and very efficient degradation of the extracellularly released self-nucleic acids by the extracellular nucleases (discussed in Section 1.4). Moreover the toll-like receptors that are able to recognize different species of nucleic acids are located inside the endosomal compartments of the innate immune cells. Nucleic acid molecules of self-origin cannot normally access these endosomal compartments and thus fail to trigger TLR activation.

**I**n our studies we found that an endogenous peptide can circumvent these barriers and bring about innate immune recognition of self-nucleic acids by binding to and transporting the nucleic acid molecules into the endosomal compartments of dendritic cells. First a study by Lande R et al in our lab identified this endogenous peptide LL37, an antimicrobial peptide of Cathelicidin family, in the context of self-DNA recognition in plasmacytoid dendritic cells (Lande R et al, 2007). Then we found in our studies with nucleic acids released by dying human cells, that this pathway is operative in the recognition of these host cell-derived nucleic acids and both DNA and RNA molecules released in this

situation can activate pDCs. As expected we found that the LL37 peptide is also able to interact with and transport RNA molecules into the endosomal compartments of dendritic cells (Ganguly D et al, 2009). Extracellular self-RNA usually cannot lead to innate immune activation because of its exquisite susceptibility to extracellular RNases and as it cannot to enter the endosomal compartments of the dendritic cells to access the RNA-recognizing TLRs. But in our studies we found that the cationic antimicrobial peptide LL37 can also go into a charge-based interaction with self-RNA, like it had been shown with DNA molecules previously (Lande R et al, 2007).

**M**ore importantly we found that this interaction between the cationic peptide and anionic nucleic acid molecules form microscopically visible aggregates of particulate complexes by condensing the nucleic acid molecules and these complexes are protected from enzymatic degradation (described in Section 2.1; Ganguly D et al, 2009). Thus inherent susceptibility of RNA molecules to nucleases is circumvented, thus enabling the self-RNA molecules in these complexes to be available for transport into the endosomal compartments of dendritic cells and access the TLRs. Ability of LL37 to transport self-RNA molecules into the innate immune cells broadens the implications of this pathway, as in addition to pDCs, mDCs can also recognize RNA molecules in their endosomal compartments due to expression of multiple RNA-responsive TLRs. We found that the self-RNA-LL37 complexes trigger TLR7 activation in pDCs to induce production of type I IFNs, on the other hand these complexes induce mDC activation through TLR8 leading to maturation of those mDCs and

production of the proinflammatory cytokines TNF- $\alpha$  and IL-6 (depicted in Section 2.2; Ganguly D et al, 2009). Possibility of mDC activation, in addition to activation of the pDCs, implies a wider propagation of the inflammatory cascade.

**T**he formation of the nucleic acid-AMP complexes is mediated by electrostatic interaction between the cationic residues on the peptide and the anionic phosphate backbone of the nucleic acid (Lande R et al, 2007; Ganguly D et al, 2009). It has been proposed that the alpha-helical domains of LL37 stabilize the interactions with the double-stranded DNA molecules (Sandgren S et al, 2004). Human RNA is single-stranded but LL37 may preferentially bind the regions where the RNA molecule folds into complex structures like double helical regions with hairpin or internal loops. The conversion of the non-stimulatory self-RNA molecules into a trigger of TLR7 and TLR8 in the dendritic cells seem to result from two distinct events — the electrostatic interaction with LL37 condenses the RNA molecules into compacted and aggregated structures that are relatively resistant to enzymatic degradation, which in turn is critical for its availability for the next event of internalization of the nucleic acid molecules into endosomal compartments. A recent paper shows that pDCs are particularly efficient in phagocytosing a particulate antigen load or TLR ligands, although their macropinocytosis efficiency in case of soluble antigens is not significant (Tel J et al, 2010). Thus the particulate complex formation from the interaction of self nucleic acid molecules with the cationic peptides lead to efficient internalization of these self-ligands for triggering endosomal TLRs. Moreover, the nano-scale size distribution of the unit constituent particles in the particulate micro-

aggregates formed by self-RNA and LL37, as resolved by the electron microscopic studies and particle sizing experiments (discussed in Section 2.1; Ganguly D et al, 2009), also may play an important role in their immunogenicity and dendritic cell activation due to higher efficiency in the internalization of the constituent TLR ligands. A recent study revealed that RNA molecules presented in nano-scale particles are selectively phagocytosed by pDCs, triggering TLR7 and inducing IFN- $\alpha$  production in them (Rettig L et al, 2010). These studies reaffirmed the physio-pathological significance of the nano-scale particle formation in the interaction between self nucleic acids and the cationic peptide.

Inside the endosomes self-RNA binds and activates TLR7 and TLR8 that recognize uridine residues present in both viral RNA and RNA of self-origin (Diebold SS et al, 2006). Conventional views about distinction of nucleic acid molecules of self and non-self origin is that self-RNA is highly enriched with several nucleoside modifications like methylation or presence of pseudouridines that make it unable to activate TLR7 and TLR8 (Kariko KM et al, 2005; Robbins MA et al, 2007). The same is proposed to explain the distinction of DNA molecules of self-origin, which have methylated CpG motifs. But our findings, that total cellular RNA isolated from human cells or released during host cell death is able to trigger TLR activation, indicates that even in the presence of these nucleoside modifications, non-modified sequences that are retained in mammalian RNA can induce innate immune activation. But the protection of these RNA sequences from extracellular degradation and their transport into the endosomal compartments, so that they can access the TLRs, are important

prerequisites. This view is supported by previous studies, which showed the ability of self-RNA to trigger TLR7 activation when they were transfected into DCs through lipofection (Kariko KM et al, 2005).

**A**s an indication in support of our hypothesis, that this immune recognition of self-nucleic acids driven by this endogenous antimicrobial peptide can be pathophysiologically relevant and may drive the autoimmune inflammation in human clinical conditions, we could find nucleic acid-LL37 complexes as small extracellular particles in cryosections of the lesional skin from psoriatic patients. We could find both DNA and RNA particles and on a lot of these particles we could detect the presence of LL37 (depicted in Section 2.3.A; Ganguly D et al, 2009). This demonstrated that this interaction between the cationic peptide and the anionic nucleic acid molecules can occur and the particulate complexes can form *in vivo* in the context of an autoimmune inflammation. Moreover, we found a lot of these particulate complexes to be around clusters of mature dendritic cells in the lesions. Occasionally we could even find some activated mDCs with multiple RNA particles within their endolysosomal vesicular network. The relative abundance of the RNA particles also correlated with the number of activated mDCs in the lesional cellular infiltration. These findings provided strong evidence that self-RNA-LL37 particles can form *in vivo* and can activate mDCs in psoriatic lesions.

**P**soriatic lesions are characterized by infiltration of pDCs early during the course of disease progression and type I IFNs produced by these pDCs have been

shown to be instrumental for the initiation of the disease process (Nestle FO et al, 2005). However, at later stages of pathogenesis the cellular infiltrate in the psoriatic lesions is characterized by relative absence of pDCs and presence of large numbers of activated and mature myeloid DCs, which are able to stimulate pathogenic T cells thus driving the inflammation (Nestle FO et al, 2005; Albanesi C et al, 2009). PDC infiltration early in the disease process has been attributed to the expression of the chemokine Chemerin by dermal fibroblasts and presence of the ChemR23 receptor on the infiltrating pDCs early in pathogenesis, which is absent in the chronic plaque form of the disease (Albanesi C et al, 2009). Based on these reports and the findings from our studies, we propose that RNA-AMP and DNA-AMP complexes may drive a concerted activation of pDC and mDC initiating the early inflammatory process in psoriasis. While at the later stages of the more chronic form of the disease, inflammation may be exclusively driven by the mature mDCs activated by RNA-AMP complexes.

**F**inding similar electrostatic interaction between the cationic AMPs and self nucleic acids in our studies with immune complexes isolated from sera of SLE patients brought further support to our notion, that this phenomenon of nucleic acid complexes being formed and driving autoimmune inflammation operates *in vivo* (discussed in Section 2.3.B; Lande R et al, 2010). We found that Lupus immune complexes form particulate complexes, similar to what we found *in vitro* or *in situ* Psoriatic lesions, and the nucleic acid molecules contained in these complexes are protected from enzymatic degradation due to the presence of the cationic AMPs (Lande R et al, 2010). When this electrostatic interaction is

inhibited, by using an anionic polypeptide, the visible particulate complexes disappear, thus demonstrating that the structural integrity of the immune complexes is due to this interaction with cationic AMPs and resulting condensation of the nucleic acid molecules. Also the susceptibility from enzymatic degradation, which is a very important issue for extracellular existence of the immune complexes, were found to reduce in presence of the cationic peptides, while the anti-nucleic acid antibodies contained in the complexes seem unable to revert this susceptibility (described in Section 2.3.B).

**A**ctivation-induced neutrophil death and associated release of genomic DNA in a filamentous network structure called NETs is a recently described antimicrobial effector mechanism (Brinkmann V et al, 2004; Brinkmann V et al, 2007). Original description depicted that on bacterial infection the NETs are generated from the apoptosing neutrophils (by a process termed NETosis). The released NETs are studded with antimicrobial peptide stored in neutrophil granules and the bacteria, after getting trapped on the sticky DNA filaments, get killed by those antimicrobial peptides (Brinkmann V et al, 2004). This report of extracellular release of DNA filaments studded with antimicrobial peptides, along with the reports showing high numbers of apoptotic neutrophils in SLE patients, that correlated with disease progression, led us to hypothesize that NETosis process of the apoptotic neutrophils in SLE may be a potential source of the nucleic acid-AMP complexes found in lupus immune complexes. Particulate domains of NET DNA remaining after DNase treatment, while longer stretches of DNA filaments disappear, provided suggestive evidence in support of this hypothesis (discussed in Section

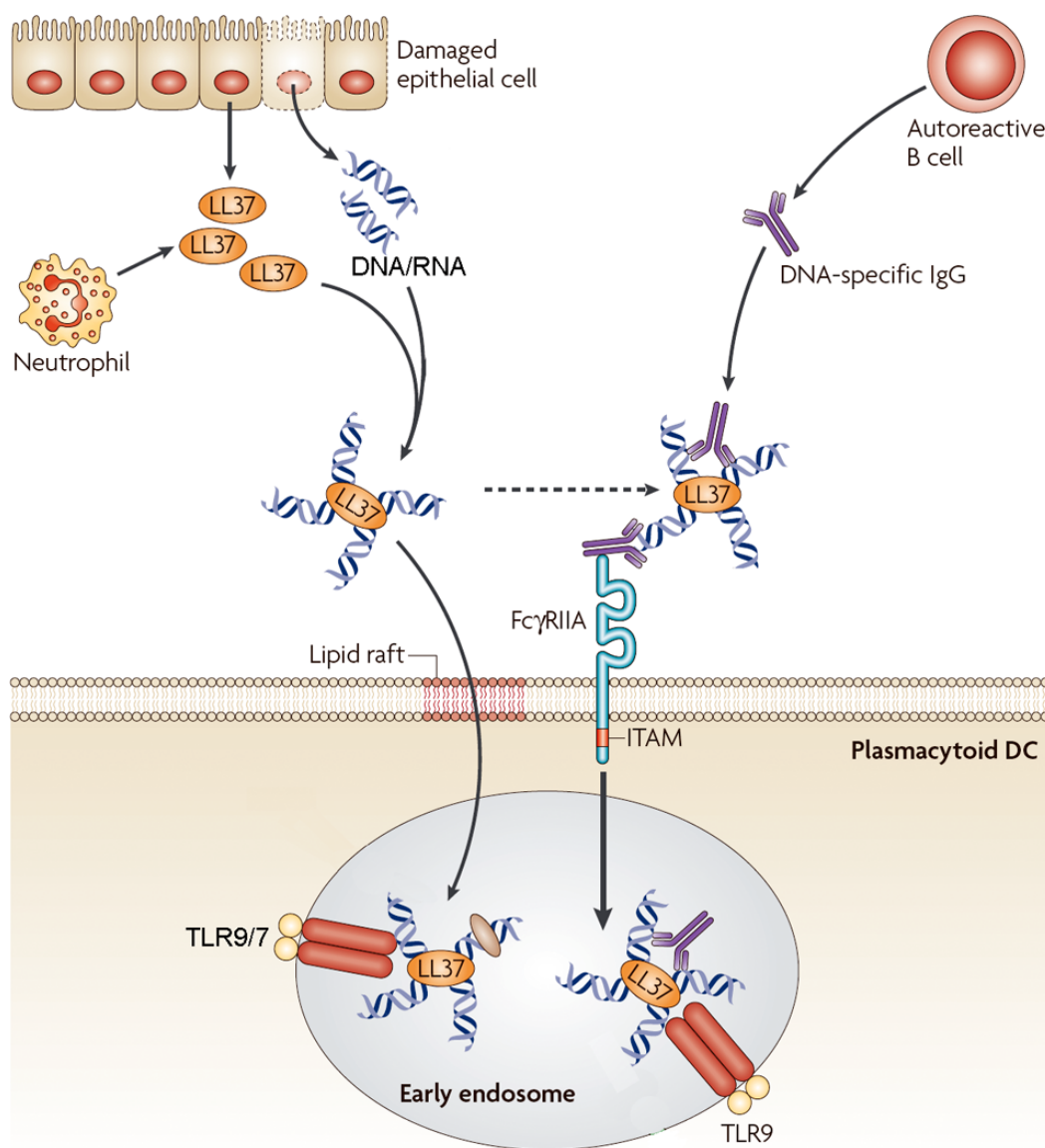
2.3.B; Lande R et al, 2010), but the question remained about the trigger of this process in NETosis. While hyper-activation of neutrophils in response to nonspecific stimuli like phorbol esters or microbial infection have been shown to induce the NETosis process in the original studies, a very recent study showed autoantibodies to neutrophil granule-derived enzymes like myeloperoxidase and elastase can also induce neutrophil death associated with release of NETs (Kessenbrock K et al, 2009). Interestingly other studies done in our lab revealed the presence of autoantibodies to the neutrophil-derived AMPs in the sera from SLE patients (Lande R et al, 2010). So we investigated whether these autoantibodies can also induce NETosis in neutrophils and found that the autoantibodies to the neutrophil-derived AMPs could indeed induce apoptosis in neutrophils and subsequent release of NETs (described in Section 2.3.B; Lande R et al, 2010). Thus taking all these findings together we could gather strong support in favor of our hypothesis that the neutrophil-NET-derived nucleic acid-AMP complexes are present in the lupus immune complexes and the presence of AMPs in these complexes determines the structural integrity and immunogenicity of these complexes.

**T**he antimicrobial peptide LL37 is typically expressed in the epithelial cells in different parts of the body or released by neutrophils and the expression occurs in the context of infection or other types of tissue injury. Although the exact mechanisms of LL37 expression are not yet fully understood, recent studies report the involvement of the vitamin D3 metabolic pathway and the hypoxia response pathways, in addition to role of microbial infection (Liu PT et al, 2006;

Schauber J et al, 2007; Peyssonnaux C et al, 2008). In the psoriatic lesions LL37 shows a sustained overexpression by the keratinocytes, which then can lead to continuous formation of the nucleic acid complexes that we described in our studies leading to innate immune activation through the toll-like receptors. The resulting uncontrolled and continuous activation of both pDCs and mDCs then may lead to the induction of high-levels of autoreactive T cells and development of the inflammatory process toward an overt autoimmune clinical condition. On the contrary, induction of LL37 expression in skin injuries is transient and may drive low levels of innate immune activation and inflammatory response helping in wound healing. However it is also possible that this transient LL37 expression and innate immune activation in skin injuries may induce low levels of autoreactive T cells, although the extent is insufficient to drive an overt autoimmune process. Recent studies report activation of T cells on injury, which then participate in wound healing (Toulon A et al, 2009). It may also be that the transient innate immune activation in tissue injury can only allow activation of T cells specific for high-avidity antigens derived from invading pathogens thus precluding the activation of autoreactive T cells.

**T**he notion derived from the studies we did is that any clinical condition, with availability of high levels of the antimicrobial peptide LL37 along with extracellular release of self nucleic acid, should be susceptible to this innate immune activation resulting from immune recognition of the self nucleic acid molecules. We found evidences that support this notion in the cutaneous inflammatory disease Psoriasis and in the systemic autoimmune disease SLE. Keratinocyte-

Figure 3.1



**Figure 3.1. Self nucleic acid sensing leading to pDC activation in tissue injury and autoimmunity.** The antimicrobial peptide LL37, secreted by epithelial cells in response to injury or tissue-infiltrating activated neutrophils, binds self-nucleic acid molecules released by dying cells, forming condensed complexes that are protected from extracellular nuclease degradation and delivered endosomal compartments of plasmacytoid dendritic cells (pDCs). A similar event is proposed to occur in the cutaneous autoimmune disease psoriasis, leading to the pathogenetic inflammatory process. In the systemic autoimmune disease SLE, anti-DNA autoantibodies produced by autoreactive B cells can bind to these self-DNA–LL37 complexes, thereby potentially enhancing their translocation into the pDC endosomes through Fc receptors. (Modified from Gilliet M et al, 2008, with permission from Nature Reviews Immunology)

derived LL37 in Psoriasis and the neutrophil-derived nucleic acid-peptide complexes in SLE seem to play a major role in the innate immune activation in these clinical conditions. Other chronic inflammatory diseases like rosacea (Yamasaki K et al, 2007), rheumatoid arthritis (Paulsen F et al, 2002), colitis ulcerosa (Schauber J et al, 2003), chronic nasal inflammatory disease (Kim ST et al, 2003), sarcoidosis (Agerberth B et al, 1999) and cystic fibrosis (Bucki R et al, 2007) have also been reported to be associated with persistent high levels of LL37. Thus one may speculate that in these clinical conditions also the self nucleic acid-LL37 complexes may form and play a role to sustain the inflammatory responses through TLR-mediated activation of dendritic cells (**Figure 3.1**). Thus one may envisage therapeutic strategies to inhibit the expression of LL37 or the formation of LL37-nucleic acids complexes that can be explored in a lot of these clinical conditions.

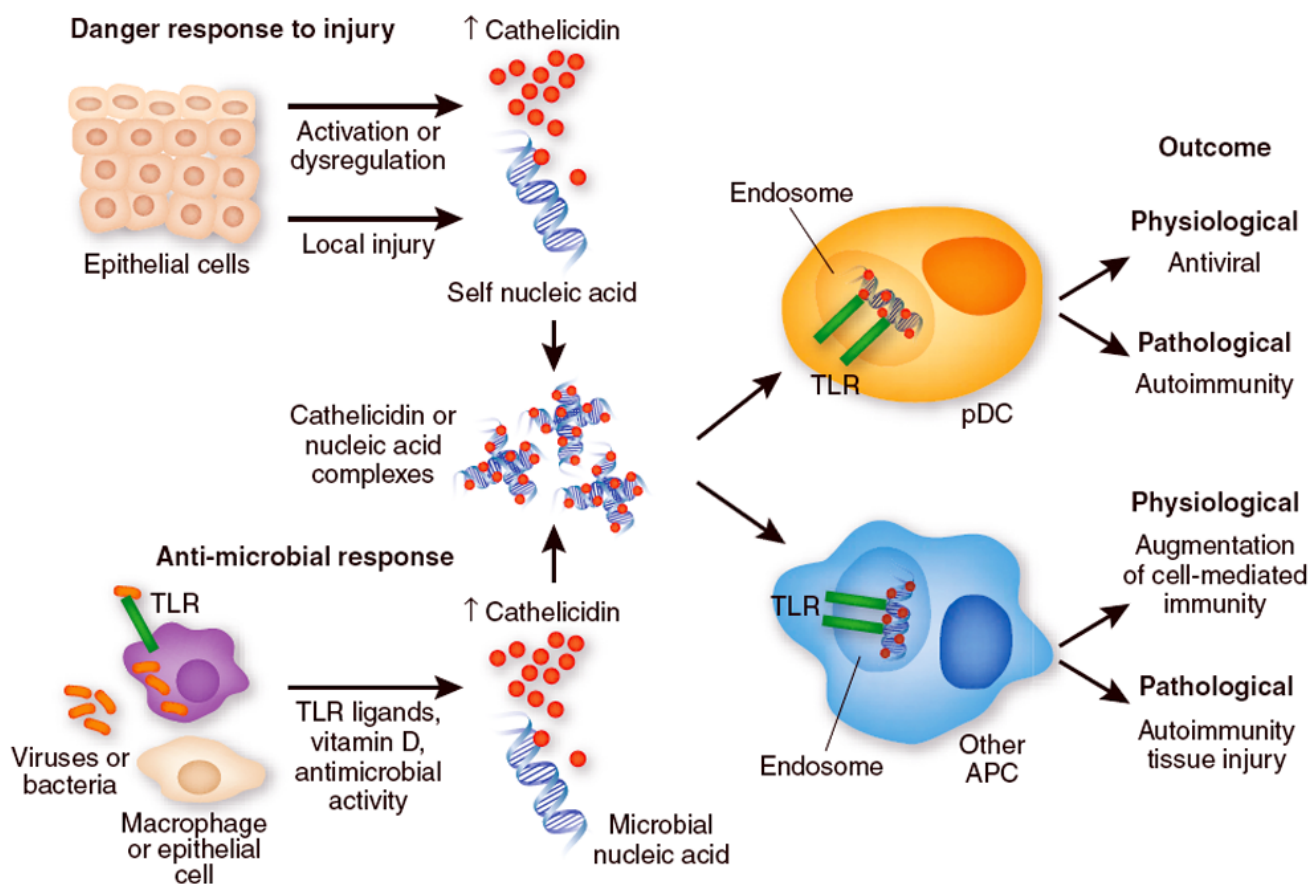
Other questions that remain to be addressed are involvement of other endogenous cationic peptides in the body in similar pathway of innate immune activation. Some of the other antimicrobial peptides like beta defensins also have cationic amphipathic properties, thus making them strong candidates for this pathway of self nucleic acid recognition. Defensins also have been found to be overexpressed in Psoriatic epidermis both in studies done by us (data not shown in this dissertation) and by others (Peric M et al, 2009). So it is important to look for similar interaction with nucleic acids using these peptides and to investigate whether they also participate in this break of innate tolerance to self nucleic acids thus contributing in autoimmune clinical contexts. Ongoing studies in our lab are

exploring these possibilities. Some of the RNA-complexes that we found *in situ* in Psoriatic lesional skin were found to be negative for LL37, creating the possibility of other peptides taking part in this complex formation.

**C**linical trials of therapeutic agents that target the inflammatory molecules in the body have confirmed major involvement of TNF- $\alpha$  in the pathogenetic process in Psoriasis, as inhibition of this cytokine action showed clinical improvements in patients (Mease PJ et al, 2000; Chaudhari U et al, 2001). But this sort of interventions target downstream genes in the pathway. A proximal target like the cationic peptide itself or measures that may affect clearance of extracellular nucleic acids should also be explored in psoriasis and other related clinical conditions. Although type I IFNs have been shown to be instrumental for the initiation of the disease process in Psoriasis (Nestle FO et al, 2005), a recent study found no clinical effect of an anti-IFN- $\alpha$  antibody (Bissonnette R et al, 2010). But this trial was conducted with patients with Chronic Plaque Psoriasis and the antibody used failed to show any inhibition of type I IFN gene signature. Although pDC-derived type I IFNs have been shown to play a defining role in the initiation of the disease process, at chronic phase of the disease recruitment and activation of the myeloid DCs seem to be the predominant feature. Thus one may speculate that self-RNA-LL37 induced induction of proinflammatory cytokines seem to gain more importance in the chronic phase of the disease and type I IFNs at this phase work mainly toward enhancement of the mDC maturation and the general inflammatory process for which we have support from our *in vitro* experiments.

**T**umor microenvironment is predominated by immunosuppressive cytokines and cellular effectors that prevent any efficient anti-tumor immunity to be operative. Activated effector cells like tumor antigen-primed dendritic cells or antigen-specific T cells have been in use for cancer immunotherapy trials (Finn OJ, 2008). But an intratumoral immunosuppressive milieu driven by the tumor cells and also by the immune cells that infiltrate the tumor comes in the way. Immuno-adjuvant therapies to potentiate directed immunotherapy have also been explored in association with specific immuno-therapies (Finn OJ, 2008). One of the strategies for such adjuvant therapies is using the TLR9 agonist CpG oligonucleotides to induce dendritic cell activation. In mouse models of tumors these TLR9 agonists have been shown to be active alone or in combination with cancer vaccines, tumor antigen specific antibodies, adoptive cellular therapies, radiotherapy and chemotherapies. Phase I and II clinical trials done with these molecules showed promise as single agents or as therapeutic vaccine adjuvants (Vollmer J et al, 2009). Keeping these studies in mind one might speculate about the potentials of an antitumor therapeutic strategy mediated through the pathway described here for immune recognition of self nucleic acids. Tumor cores are seat of continual cell death due to hypoxia and high cell turn over and thus rich in nucleic acids that are released extracellularly. Delivery of the cationic peptide molecules inside the tumor core, with the intention of triggering immunogenic complex formation with self nucleic acids released in the tumor stroma, and thus driving innate immune activation through toll-like receptors in the tumor infiltrating

Figure 3.2



**Figure 3.2. Endogenous cathelicidin mediated innate recognition of nucleic acids can play roles in both sterile and non-sterile inflammation.** The endogenous cathelicidin antimicrobial peptide LL37 can bind self nucleic acid molecules extracellularly released by dying host cells in case of sterile or non-sterile injury, leading to TLR-mediated activation of dendritic cells and immune response propagation. In case of infection the microbial nucleic acids can also potentially be bound by LL37 leading to efficient intra-endosomal transport and dendritic cell activation. The tissue injury processes themselves lead to over-expression of the cathelicidin peptide in the vicinity thus driving the inflammatory process. (Modified from Lee DJ et al, 2008, with permission from Nature Medicine)

antigen presenting cells, may be envisaged as a viable therapeutic strategy which remains to be explored.

On a broader perspective our studies describe a novel cell death sensing mechanism that can lead to a potent innate immune response by activating different subsets of dendritic cells through immune recognition of self nucleic acid molecules by toll-like receptors. Involvement of both DNA (Lande R et al, 2007; Ganguly D et al, 2009; Lande R et al, 2010) and RNA (Ganguly D et al, 2009) molecules in this pathway widens the downstream cascade. Protection of extracellular nucleic acids in presence of the cationic peptide leads to particulate complexes to form in the body thus constituting a potent danger signal for tissue injury and host cell death (**Figure 3.2**). In addition to innate immune activation in response to nucleic acid molecules of self-origin, whether this pathway may also operate in other clinical settings associated with microbial infection, taking part in the sensing of nucleic acids of microbial origin and resulting inflammatory processes, remains to be seen.

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## **Vita**

Dipyaman Ganguly was born in Berhampur, India on October 25, 1977, to Dilip Kumar and Sanghamitra Ganguly and lived most of his life in Calcutta (Kolkata), India. After graduating from Rahara Ramakrishna Mission Boys' Home High School in 1996, he entered the Calcutta Medical College & Hospitals of the University of Calcutta, India to study Medicine and received the MBBS degree in the year 2002. After completing medical education and clinical internship, Dipyaman worked as a Clinical Associate in the Molecular Immunogenetics Laboratory at the Institute of Genomics and Integrative Biology, Delhi, India. In 2003 Dipyaman joined the graduate program at the Indian Institute of Chemical Biology, Calcutta, India and received the PhD degree in Biotechnology from the West Bengal University of Technology, working with Dr. Santu Bandyopadhyay. In 2006 he enrolled at the University of Texas Houston Health Science Center, Graduate School of Biomedical Science to do a second PhD in Immunology and worked under the supervision and guidance of Dr. Michel Gilliet. Meanwhile in 2009 he married Amrita Goswami, an Electrical Engineer turned Information Technologist, who is also from India. Beyond Medicine and Biology Dipyaman also takes interest in skywatching, fine art photography and New-Wave films.

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