Old Herborn University Seminar Monograph

28. THE EPIDERMIS OF MAN: CO-EXISTING WITH COMMENSALS

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EPIDERMIS AS A DYNAMIC INTERFACE

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INTRODUCTION

Too long viewed as a mere battle-ground for the immune system, the epidermis is asserting its rightful place at the centre of cutaneous biology and pathophysiology. While immunologists seek ever finer distinctions between T cell subsets in inflammatory lesions, it is now increasingly clear that the protective requirements of the skin dictate virtually every metabolic process (including adaptive immune responses) in its underlying layers. True, there are 'outside-to-inside-back-to-outside' vicious cycles, whereby immune responses further compromise epidermal function, and there are also examples of primary immune disorders, such as autoimmune and bullous diseases, HIV infections, and superantigen-initiated flares of erythrodermic psoriasis, where a primary inflammatory infiltrate can produce downstream abnormalities in epidermal function (e.g., for HIV, see *Gunathilake*, 2010). But as the example of filaggrin-deficient atopic dermatitis eloquently demonstrates, most cutaneous immune phenomena occur downstream of primary epidermal insults, whether inherited or acquired, and these responses are recruited only when epidermal homeostatic responses fail to promptly re-establish normal cutaneous function. In this brief review, we will consider:

i) a new 'holistic' view of epidermal defence;
ii) a concise review of the structural basis for the barrier with an update on tight junctions and the corneocyte lipid envelope;
iii) intra-epidermal metabolic processes that are regulated by barrier requirements; and
iv) certain homeostatic signalling mechanisms that regulate these metabolic responses.

BRIEF REVIEW OF BARRIER STRUCTURE AND FUNCTION

The two-compartment model

The protective functions of the skin, including the permeability barrier, largely localize to the outer epidermis and stratum corneum (SC) (Table 1) (Figure 1). The SC is an anucleate structure, arranged in a 'brick and mortar' mosaic of flattened corneocytes ('bricks'), embedded in lipid-enriched extracellular matrix ('mortar') that is organized into parallel stacks of lamellar bilayers, enriched in ceramides, cholesterol, and free fatty acids (FFA) (*Elias* and *Menon*, 1991). These water repellent lipids restrict the outward flow of water, while also impeding the inward absorption of toxins, allergens, and microbial pathogens (*Prausnitz* et al., 2012). It is the secretion of the contents of multiple, small ovoid lamellar

Table 1: Defensive gradients in the outer epidermis

Functions:	Outer surface (sebaceous glands)	Stratum corneum	Stratum granulosum
Antimicrobial:	AMP, FFA (↓pH)	FFA (↓pH), AMP, SPI	AMP, TLR
Permeability barrier:	—	Cholesterol, Cer, FFA in lamellar bilayers	Tight junction (larger xenobiotes)
Antioxidant:	Vit. E	Vit. E, Sprr2d, Sprr2h, Slpi	SOD, CoQ, catalase, GluTR
UV-B:	—	t-UCA (melanin)	Melanin
Mechanical:	—	Cornified envelopes	—
Cohesion:	—	Lipids, Corneodesmosomes	Desmosomes, Adherens junctions
Cytokine activation:	—	IL-1α/β release	TNFα, IL-1α/β, GMCSF, IL-6, NGF, AR, VEGF
Neurosensory:	—	—	TRPVs, TRPM8
Hydration:	Glycerol	FLG → NMF; glycerol, urea	AQP channels, Urea transporters

Abbreviations: AMP, antimicrobial peptide; Cer, ceramide; CoQ, co-enzyme Q; FFA, free fatty acid; GluTR, glutamyl tRNA reductase; GMCSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; NGF, nerve growth factor; NMF, natural moisturizing factor; Slpi, serine leukocyte protease inhibitor; SOD, superoxide dismutase; SPI, serine protease inhibitor; TLR, toll-like receptor; TNF, tumor necrosis factor; TRPM8, transient receptor potential melastatin-8; TRPV, transient receptor potential vanilloid; t-UCA, trans-urocanic acid; VEGF, vascular endothelial growth factor.

bodies (LB) (*Elias* and *Menon*, 1991) that delivers both lipid precursors and hydrolytic 'processing' enzymes that generate the hydrophobic species, ceramides (Cer), free fatty acids (FFA), and cholesterol, that mediate the permeability barrier (Figure 2). These three lipids, along with as-yet unidentified amphiphilic molecules, are required for the organization of the secreted lipids into mature lamellar bilayers (*Elias* and *Menon*, 1991).

The corneocyte-bound lipid envelope (CLE)

The external surface of the cornified envelope (CE) is coated with a monolayer of ω-hydroxyceramides (ω-OH-Cer) that is covalently bound to peptides (1° involucrin) within the CE (*Zheng* et al., 2011; *Breiden* and *Sandhoff*, 2014; *Rabionet* et al., 2014) (Figure 3). Both the origin and the function of this structure are still uncertain. While most workers believe that it is formed from a pool of secreted acylCer, the CLE also could derive from the insertion of a myriad of lamellar body limiting membranes during the exocytosis of these organelles (*Elias* et al., 2014). We noted that the CLE fails to form in several inherited and acquired disorders that compromise steps that either generate acylCer, or oxidize the ω-OH-linoleate moiety of acylCer (Figure 2). Since all of these disorders are characterized by a faulty permeability barrier, poor SC hydration, and impaired desquamation, it is tempting (but still not certain) that the CLE is linked to one or more of these functions (*Elias* et al., 2014).

The tight junction (TJ) controversy

How should we interpret an ever-expanding literature that proclaims a

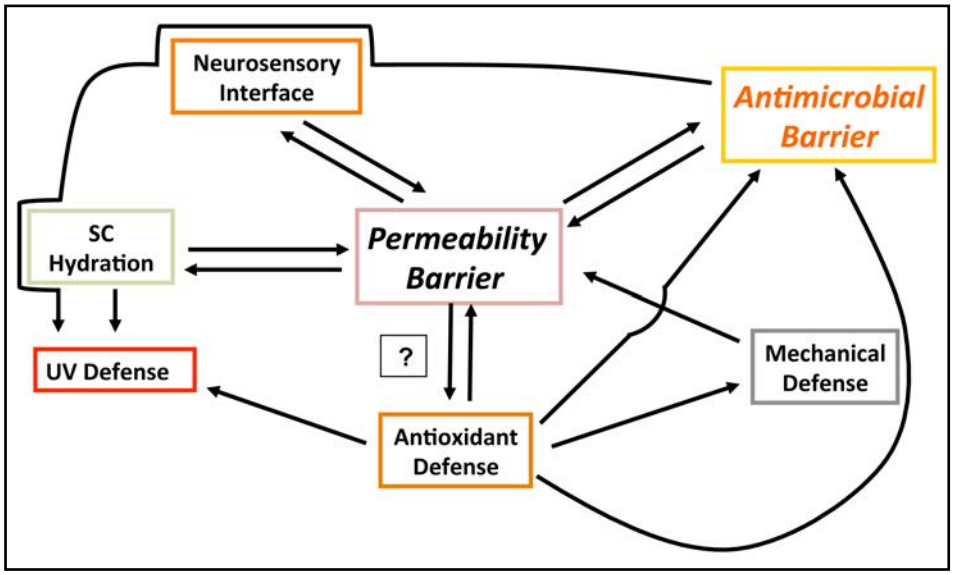

Figure 1: Protective (defensive) functions are related, co-regulated and interdependent.

potential role for TJ in normal permeability barrier function [e.g., (*Brandner* et al., 2002; *Kubo* et al., 2012)], as well as a potential role for abnormal TJ function in AD (De *Benedetto* et al., 2011)? We will attempt to navigate this heavily-invested subject as follows: First, complex TJ structures, such as those found in the kidney and gastrointestinal (GI) tract, do not occur in adult keratinizing epithelia (*Elias* et al., 1977). Second, with the exception of highly complex TJ in renal collecting tubules, where they comprise multitiered, overlapping sites of membrane fusion ('zonulae occludentes'), in other tubular epithelia, such as the trachea and GI tract, these junctions provide a relatively poor barrier against paracellular water movement (*Marchiando* et al., 2010; *Suzuki*, 2013). Much of the confusion in the skin-related literature has occurred because 'TJ *proteins*' are widely equated with 'TJ' (*Brandner* et al., 2002; *Furuse* et al., 2002; *Kubo* et al., 2012). Certainly, multiple TJ proteins heavily decorate the apical-lateral plasma membranes of cells in the outer stratum granulosum of normal adult epidermis, forming 'kissing points'. However, these focal attachments; i.e., 'maculae occludentes' (*Elias* et al., 1977), do not comprise true zonulae occludentes (=TJ), as occur in tubular epithelia. The most compelling evidence that these putative TJ play no direct role in the paracellular water barrier comes from solvent extraction studies, where removal of SC lipids by repeated, gentle, lipid solvent swabbing *completely abrogates* the permeability barrier (*Grubauer* et al., 1989). It should be noted that this observation also excludes a possible 'back-up' role for TJ-like structures in the *water* barrier, although it remains possible that true TJ eventually could begin to form in response to such repeated solvent wipes. Moreover, these incomplete structures could suffice to interdict the paracellular passage of larger xenobiotes, particularly when the overlying lipid-based barrier becomes defective, as occurs in atopic dermatosis (*De Benedetto* et al., 2011).

Yet, these structures, though insufficient to contribute directly to the nor-

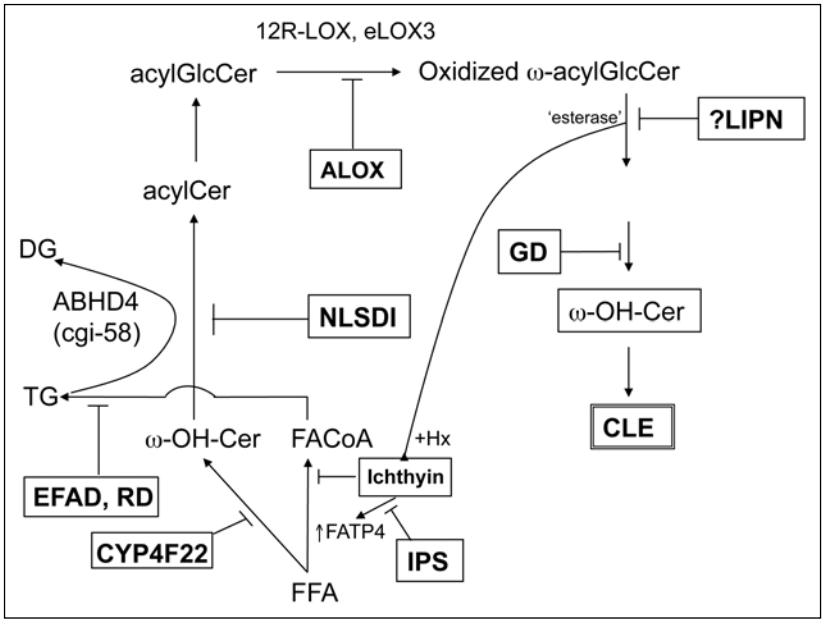

Figure 2: Pathways leading to CLE formation: insights from inherited and acquired lipid metabolic disorders. (Modified from: Elias et al., 2014).

mal water barrier, are nonetheless critical for the development of permeability barrier competence. Transgenic knockout of the key TJ protein, claudin 1, results in a fatal, post-natal permeability barrier abnormality (*Furuse* et al., 2002). Indeed, our recent studies show that replete TJ are present early in epidermal development, but they become functionally incompetent later in foetal life in parallel with establishment of the lipid-based barrier (*Celli* et al., 2012). Although an acquired reduction in the expression of the TJ protein, claudin 1, has been reported in atopic dermatitis (*De Benedetto* et al., 2011), treatment of cultured human keratinocytes with the Th2 cytokine, IL-4, instead *upregulates* claudin 1 expression, while simultaneously *downregulating* another TJ protein, occludin (Y. Hatano, personal communication). Moreover, occludin (but not claudin) protein levels decline in filaggrin-deficient human epidermis (*Gruber* et al., 2011). Hence, it is likely that abnormalities in TJ proteins in atopic dermatitis, should they occur, likely result from the Th2-dominant milieu, which is known to downregulate many other epidermal differentiation-linked proteins [e.g., (*Howell* et al., 2008)].

Since adult epidermis does not generate the types of complex zonulae occludentes necessary to impede paracellular water movement, attention should be focused instead on the possible functions of these incomplete junctions (maculae occludentes) in normal epidermis; and how acquired defects in such focal connections could contribute to disease pathogenesis. We believe that these structures perform important 'fence functions' in adult epidermis, including polarizing the direction of lamellar body secretion towards the apex of the outermost granular layer (*Elias* et al., 1998), while also restricting selected membrane transporters, such as the sodium-hydrogen antiporter 1 (NHE1), to the apical plasma membrane of these cells.

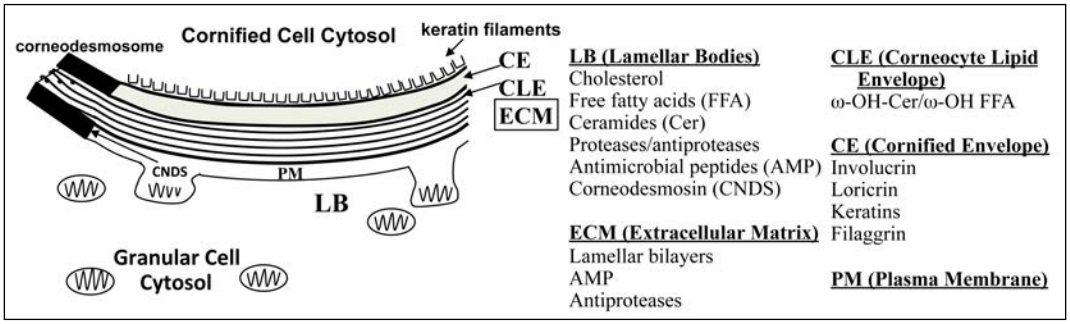

Figure 3: Diagram of stratum corneum membrane domains. (Modified from: Schmuth et al., 2008).

INTERDEPENDENCE OF, AND INTERRELATIONSHIP BETWEEN EPIDERMAL DEFENSIVE FUNCTIONS

While it is common practice to list the various defensive functions of the skin as discrete processes (Table 1), in most cases, these functions are interrelated, co-regulated, and interdependent. As is evident from Figure 1, more and more connections are emerging between these defensive functions, of which we will highlight only a few for consideration here. Best appreciated are the connections between the permeability barrier and antimicrobial defence. Shared structural and biochemical processes (*Elias*, 2007), as well as common metabolic processes, unite these two functions (Table 2).

Moreover, epidermal lamellar bodies provide a common delivery mechanism for components with overlapping functions, such as free fatty acids and antimicrobial peptides (AMP) (Table 2), of which at least one, the cathelicidin carboxyterminal peptide, LL-37, is required not only to restrict pathogen invasion, but also as an apparent structural component of lamellar bilayers (*Aberg* et al., 2008). In multiple clinical situations; in experimental perturbations; and after applications of therapeutic ingredients that either compromise or improve permeability barrier homeostasis, corresponding alterations occur in LL-37, and to a lesser extent, in hBD2 expression (*Aberg* et al., 2008; *Rodriguez-Martin* et al., 2011) (Figure 4).

Table 2: How permeability and antimicrobial barriers are linked

1. Co-localization of both functions to extracellular ('mortar') domains
2. Pathogens attempt to invade through SC extracellular domains
3. Some permeability barrier lipids (e.g., free fatty acids and sphingosine) exhibit potent antimicrobial activity
4. Certain antimicrobial peptides (AMP) localize to lamellar bodies (along with lipids), and are co-delivered to SC extracellular domains
5. Both AMP expression and secretion accelerate after permeability barrier disruption, paralleling up-regulation of lipid synthesis
6. At least one AMP (LL-37) is required for permeability barrier homeostasis
7. Certain serine proteases (e.g., secretory leukocyte protease inhibitor, SLPI) that regulate SC cohesion also exhibit potent antimicrobial activity.

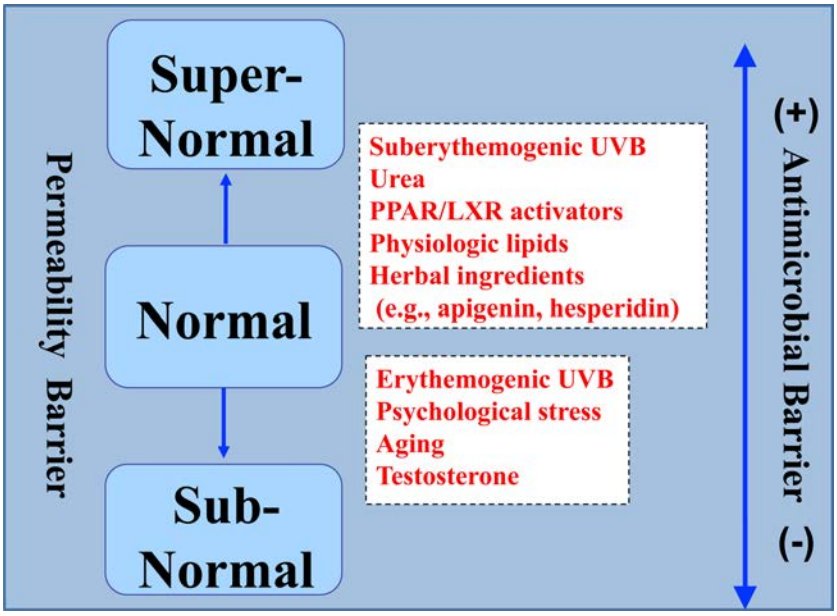

Figure 4: Parallel changes in barrier function and cathelicidin expression. (Modified from: Rodriguez-Martin et al., 2011).

Lamellar bodies also deliver proteases and anti-proteases that initially regulate SC cohesion, and then orchestrate the digestion of corneodesmosomes (*Caubet* et al., 2004; *Brattsand* et al., 2005) (Figure 3). But corneodesmosome degradation is only the first in a series of subsequent cellular events that leads to the eventual shedding of corneocytes from the skin surface (*Lin* et al., 2012) (Figure 5). Finally, as noted above, lamellar bodies also secrete at least two antimicrobial peptides, human beta-defensin2 (hBD2) and LL-37, into the SC extracellular domains (*Oren* et al., 2003; *Braff* et al., 2005; *Aberg* et al., 2007). Because they appear to be so intertwined, it becomes a matter of semantics as to whether not only these two, but also whether several other functions should be considered as discrete or interrelated processes (Figure 1).

The multiple functions that are impacted by the epidermal structural protein, filaggrin, serve as another illustrative example of the link between multiple defence functions. First, the full-length protein becomes a component of the corneocyte envelope (CE) (*Eckert* et al., 2004; *Presland*, 2009), contributing to epidermal mechanical defence (*Gruber* et al., 2011). We have shown that an intact CE is required for the supramolecular organization of secreted lipids into lamellar bilayers, as eloquently demonstrated in two disorders of cornification, transglutaminase 1-deficient lamellar ichthyosis (*Elias* et al., 2002), and loricrin keratoderma (*Schmuth* et al., 2004). But it is the subsequent, humidity-dependent proteolysis of FLG above the mid-SC (*Scott* and *Harding*, 1986), that impacts an even broader suite of functions (Figure 6). Following FLG hydrolysis, its constituent amino acids are further deiminated, both enzymatically and non-enzymatically, into a suite of polycarboxylic acids (= 'natural moisturizing factor') that not only account for much of SC hydration, but also contribute to defence against UV-B and to the acidification of the SC

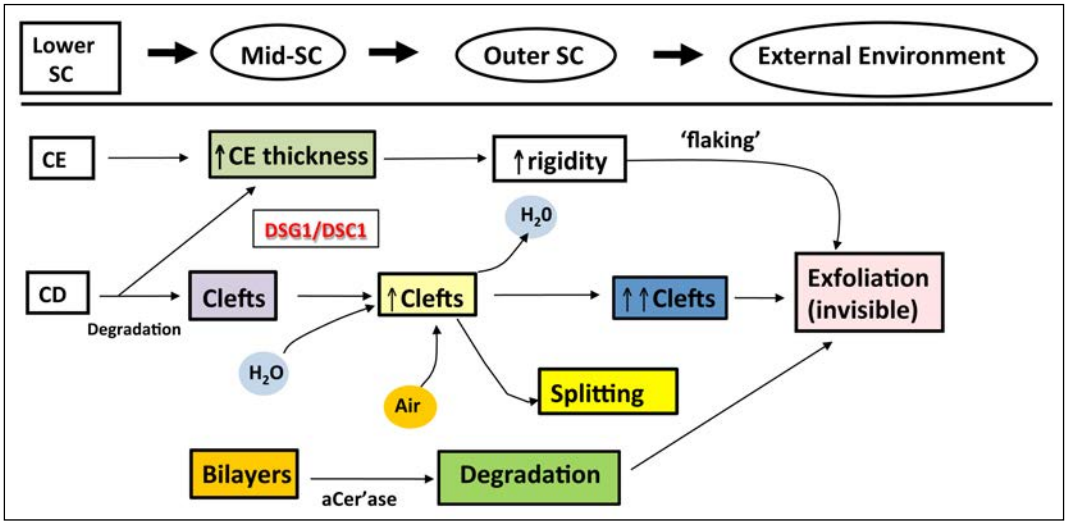

Figure 5: Basis for normal exfoliation: summary of observations. (Modified from Lin et al., 2012). Abbreviations: aCer'ase, acidic ceramidase; CD, corneodesmosome; CE, cornified envelope; DSC1, desmocollin 1; DSG1, desmoglein 1; SC, stratum corneum.

(Figure 6). The reduced pH of the SC in turn is critical for multiple functions, including not only antimicrobial defence, but also permeability barrier homeostasis (*Mauro* et al., 1998), SC cohesion, and pro-inflammatory cytokine activation.

We next highlight another recent example of linked functions that recently emerged from the laboratory of Sabine Werner (Inst. of Cell Biology, Zurich), who showed that a key transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2), regulates the expression of two cornified envelope precursors, small proline-rich proteins (Sprr2b and Sprr2h). This transcription factor also regulates expression of a potent antimicrobial protein, secretory leukocyte protease inhibitor (Slpi), which is also an inhibitor of serine proteases (kallikreins) that regulate SC cohesion (Figure 7). The cohesiveness of the SC in turn is critical for both permeability barrier function and antimicrobial defence. Together, these examples of functional links illuminate how discrete epidermal protective functions should instead be considered as components of a broader, protective 'superfunction' of the skin.

METABOLIC MECHANISMS THAT MAINTAIN EPIDERMAL HOMEOSTASIS

Life in a terrestrial environment requires constant vigilance, accompanied by responses, either draconian or subtle, to external perturbations that potentially threaten the organism with desiccation, microbial invasion, oxidant damage, UV-B-induced apoptosis, and/or impaired mechanical defence. Consider the most dramatic example, i.e., an external thermal burn, with its potentially devastating consequences. The foremost threat to such patients, of

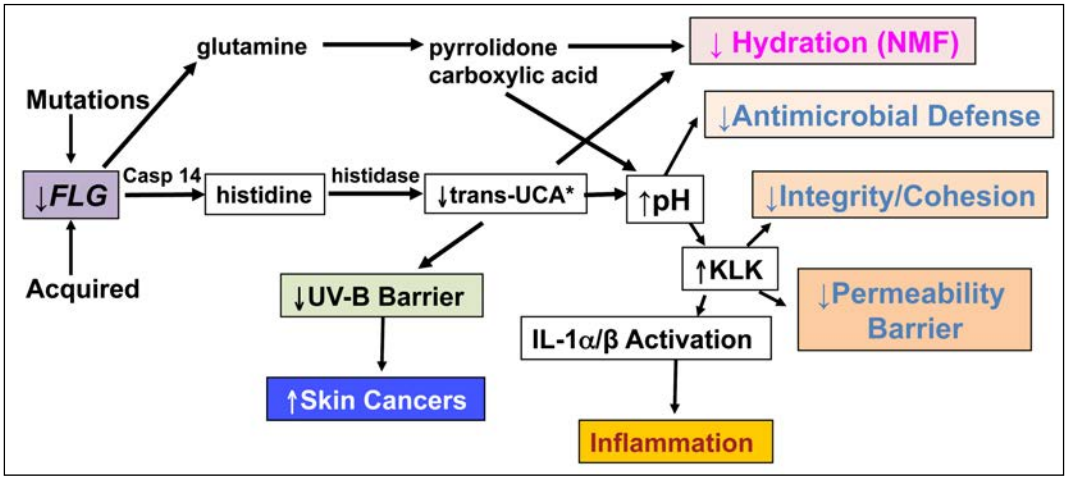

Figure 6: Multiple downstream consequences of filaggrin deficiency in atopic dermatitis: *Trans-urocanic acid (t-UCA) is the most potent endogenous UV-B filter in lightly-pigmented skin. Loss of t-UCA could account for the higher incidence of non-melanoma skin cancers in AD. (Modified from: Thyssen et al., 2013).
Abbreviations: Casp 14, Caspase 14; KLK, kallikrien, NMF, natural moisturizing factor

course, is rapid desiccation due to an unrestricted loss of internal fluids and electrolytes, as well as an increased susceptibility to pathogen invasion. Yet, even following such potentially catastrophic injuries, the skin attempts to repair itself. What is the driving force behind the repair of such wounds? Entire generations of surgeons and skin biologists have focused once again upon 'inside-to-outside' phenomena, related either to the initial inflammatory responses, platelet-derived growth factors, granulation tissue, collagen remodelling, and/or wound contracture as key 'drivers' of wound healing. Re-epithelialization often is noted only in passing as the inevitable downstream consequence of these earlier events. Few, if any of these investigators have considered the possibility that it could be the *imperative to re-establish permeability barrier homeostasis* that likely 'drives' much

Table 3: Chronology of Metabolic Response to Acute Barrier Disruption

Chronology:	0→20 min	20 min→2 hrs	30 min→6 hrs	6 hrs→12 hrs	16+ hrs
Event:	Secretion of preformed pool of lamellar bodies	Terminal differentiation (physiologic apoptosis)	↑Lipid synthesis + secretion	↑Lipid processing	↑DNA synthesis
Known signals:	$\downarrow Ca^{2+}$	KLK → PAR2	SREBPs; ↑IL-1α; $\downarrow Ca^{2+}$	?	AR, NGF, IL-1α
Effects of occlusion:	Blocks	Blocks	Blocks both lipid synthesis and transport	Blocks	Blocks DNA synthesis, AR, NGF and VEGF (but not cytokine) production

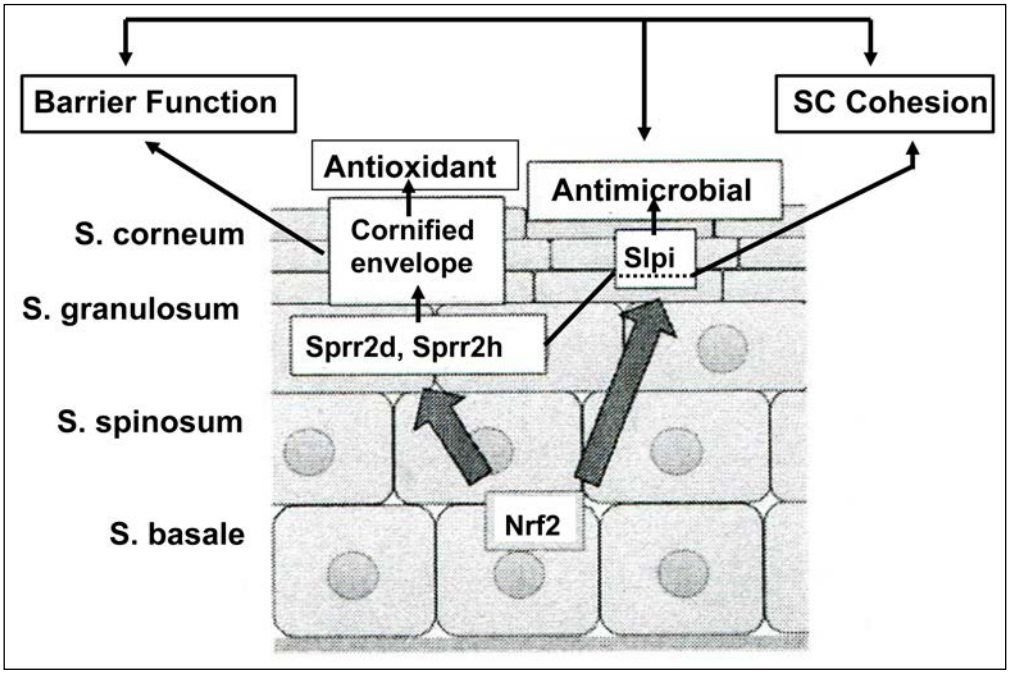

Figure 7: Nrf2 regulates not only antioxidant defense, but also barrier function and antimicrobial defense through increased cornified envelopes and secretory leukocyte protease inhibitor [Slpi] expression. (Modified from: Schäfer et al., 2012).
Abbreviations: Nrf2, nuclear factor erythroid 2-related factor 2; Slpi, secretory leukocyte protease inhibitor; Sprr, small proline-rich protein.

of the wound healing sequence, which includes re-epithelialization followed by stratification of epidermis into a functional stratum corneum. They need only observe that occlusion with vapour-permeable wraps delays wound healing, while applications of vapour-permeable wraps stimulate all of the processes described above, including re-epithelialization.

We view one of our standard laboratory models; i.e., sequential tape stripping, as a type of superficial wound. Tape stripping (no different than either detergent or solvent wipes) produces a defect in the permeability barrier, and all three of these unrelated, acute perturbations stimulate an identical series of metabolic responses in the underlying epidermis that rapidly re-establishes permeability barrier homeostasis in a predictable sequence, and with characteristic kinetics (Table 3). This approach (which we term the cutaneous stress test or 'treadmill of the skin') can be deployed to identify specific metabolic responses that bring about re-establishment of barrier homeostasis. The earliest response to acute barrier perturbations is the immediate secretion (within 15-20 minutes) of much of the pre-formed pool of lamellar bodies from cells of the outer stratum granulosum (SG) (*Elias* et al., 1998). After exteriorizing their cargo of lamellar body contents, these outermost SG cornify; i.e., they undergo physiologic apoptosis (*Demerjian* et al., 2008), followed immediately by the apical migration of subjacent SG cells (*Elias* et al., 1998) (Table 3).

Yet, barrier perturbations also stimulate injury responses that may be unrelated to the restoration of barrier

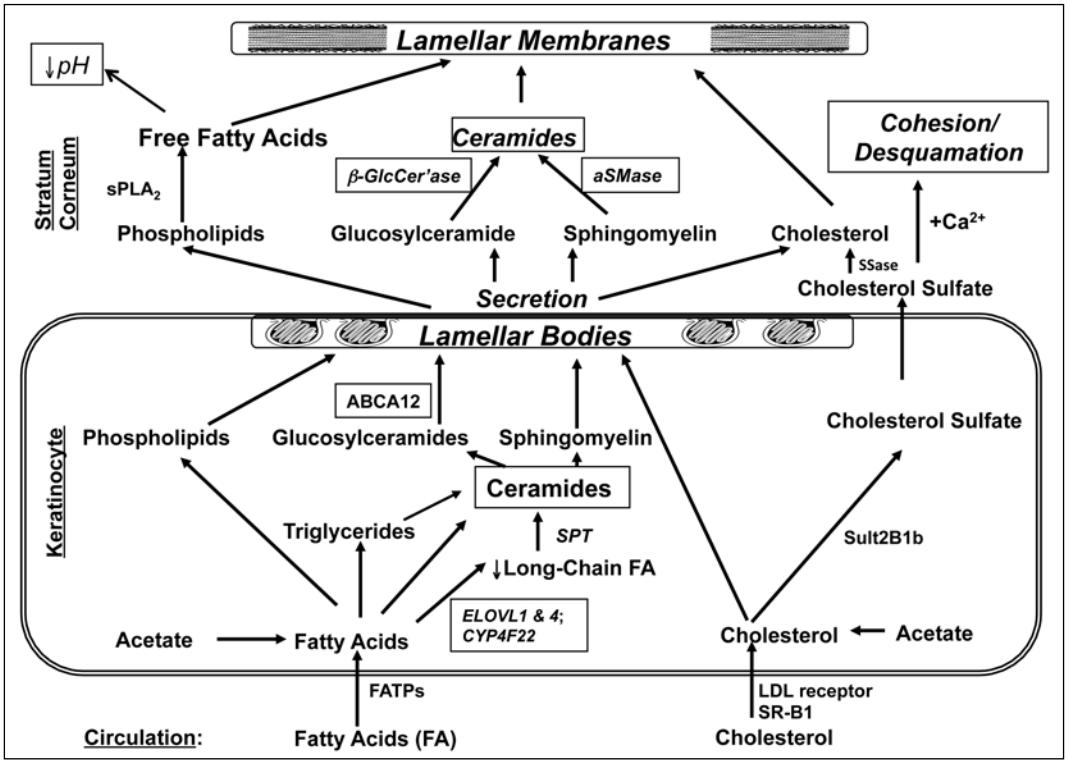

Figure 8: Lipid metabolic events leading to normal barrier formation. (Modified from: Feingold and Elias, 2014).
Abbreviations: β-GlcCer'ase, β-glucocerebrosidase; aSMase, acidic sphingomyelinase; FATPs, fatty acid transport proteins; sPLA2, secretory phospholipase A2; SSase, steriod sulfatase.

function. To distinguish between these two events, one can artificially restore barrier function with a vapour-impermeable wrap, such as a Latex® glove or a sheet of Saran® wrap. By sending a 'message' that the barrier function is now normal, these forms of occlusion shut down metabolic events that are solely directed at restoring barrier function, including virtually all of the changes shown in Figure 8 and Table 3 (*Feingold*, 2009). Yet, some responses, such as increased cytokine production (see below), are not blocked by occlusion. These could be dual-purpose; i.e., signals of both barrier homeostasis and an injury response. Finally, it should be noted that the same 'stress test' approach has allowed us to identify abnormalities in barrier function in:

i) developmental (neonatal and aged skin) settings (*Ghadially* et al., 1995; *Choi* et al., 2007);
ii) human populations, subjected to psychological stress (*Garg* et al., 2001) or endowed with different pigment types (Reed et al., 1995; Gunathilake et al., 2009); and
iii) disease settings (*Schmuth* et al., 2007; *Elias* et al., 2008).

Finally, the stress test led to the development of new generations of 'barrier repair' therapeutics (*Man* et al., 1996), as well as novel metabolically-based, drug delivery technologies (*Menon* and *Elias*, 2000).

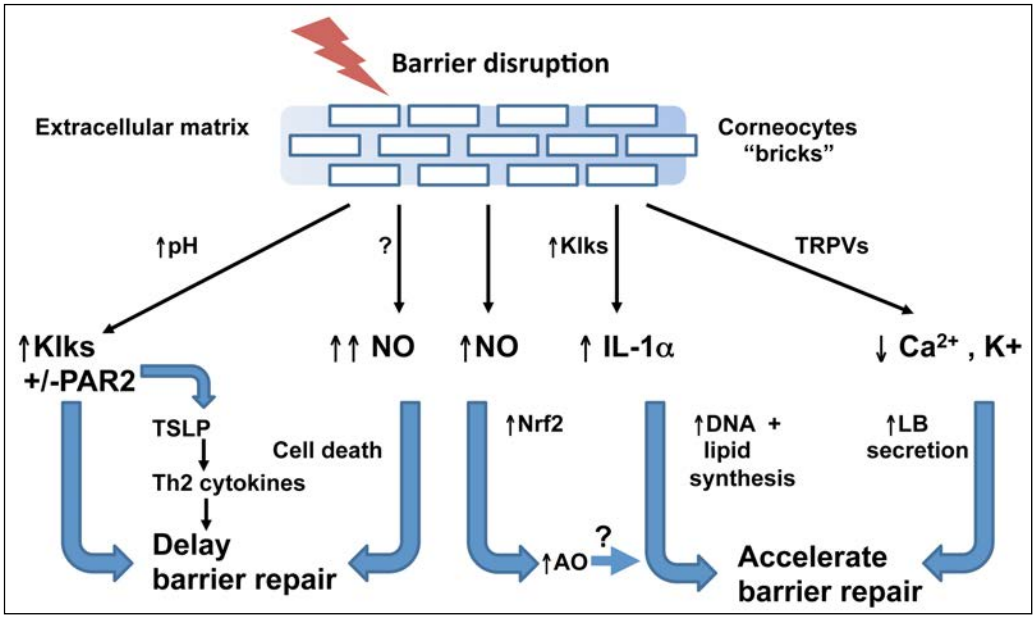

Figure 9: Regulation of permeability barrier repair. Examples of how disruption of the permeability barrier results in signals that can either accelerate or delay barrier repair. (Modified from: Feingold et al., 2007).
Abbreviations: AO, antioxidants; Ca, calcium;l IL-1α, interleukin-1alpha; K, potassium; Klks, kallikreins; LB, lamellar bodies; NO, nitric oxide; Nrf2; nuclear factor erythroid 2-related factor 2; PAR2, proteinase-activated receptor; TSLP, thymic stromal lymphopoietin.

SIGNALS OF BARRIER HOMEOSTASIS

It still is only partially understood how perturbations of the outer skin surface signal the underlying nucleated layers to initiate the metabolic responses that restore permeability barrier homeostasis. To date, several extracellular signalling mechanisms have been identified that are known to stimulate a broad array of metabolic responses in the underlying epidermis (Figure 9, Table 4). But it also should be noted that external perturbations 'turn on' intracellular signalling mechanisms (second messengers) that also regulate these metabolic responses (Table 5).

These include the 'liposensor' subclass of nuclear hormone receptors, PPARα, PPARβ/δ, PPARγ and LXR, which regulate the transcription of several genes that are critical for epidermal differentiation and lipid production (*Schmuth* et al., 2008) (Figure 10). Also carefully studied are sterol regulatory element binding proteins (SREBPs) that modulate epidermal sterol and triacylglyceride synthesis (*Harris* et al., 1998). Then, barrier disruption stimulates hyaluronic acid production which, depending upon fragment size, regulates epidermal proliferation, differentiation and cholesterol synthesis (*Bourguignon* et al., 2006), nitric oxide (NO) production, and endoplasmic reticulum (ER) stress responses. It should be noted, however, that egregious external insults result in cell death or apoptosis (Figure 9), by one or more of these mechanisms (*Park*et al., 2011). In addition, the sulphated sterol, cholesterol sulphate,

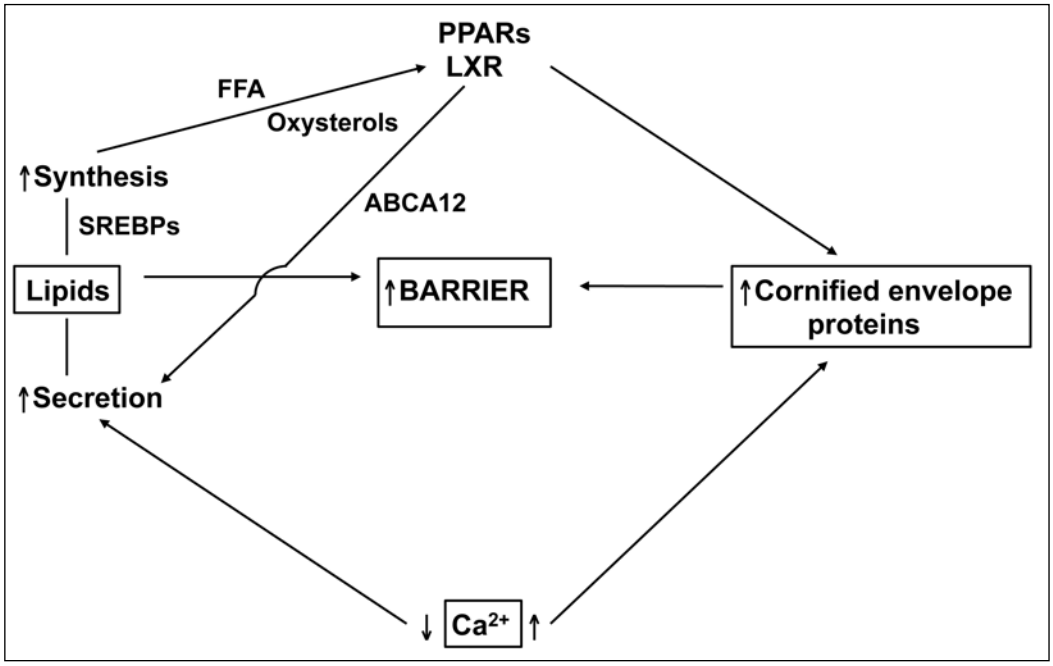

Figure 10: Speculative diagram that illustrates coordinate regulation of epidermal barrier homeostasis by changes in calcium, and activation of the liposensor sub-class of class-II nuclear hormone receptors (modified from: Elias and Feingold, 2001)
Abbreviations: ABCA12, ATP-binding cassette transporter A12; FFA, free fatty acid; LXR, liver-x receptor; PPAR, peroxisome proliferator-activated receptor; SREBP, sterol response element binding transcription factor.

which is generated late in epidermal differentiation, is a potent transcriptional regulator of epidermal differentiation (*Hanley* et al., 2001). Yet, new signalling networks, both extra and intracellular, that link external perturbations to metabolic response in the underlying epidermis continue to be discovered (Tables 4 and 5). While several of these signals broadly regulate epidermal differentiation and/or lipid production, perhaps in a redundant or overlapping fashion, others instead modulate more discrete metabolic pathways within the epidermis.

It is important to distinguish whether these signalling mechanisms represent purely homeostatic, or in part, injury-mediated responses. The 'gold standard' applies again - do these signals deploy following acute external perturbations, even when the barrier is immediately restored by occlusion (see also above)? Several cytokines, but not the growth factors NGF, AR and VEGF, continue to up-regulate, even in the face of barrier restoration by occlusion, indicating that they could represent, at least in part responses to injury, rather than purely homeostatic mechanisms alone (Tables 4 and 5).

Table 4: Signals that regulate permeability barrier homeostasis

Extracellular modulations:	Sensor	Signal	Homeostatic responses	Potential pathogenic signal*
External humidity:	TRPV4, TRPM8	Ca^{2+}	↑Lamellar body secretion; ↓-↑Epidermal differentiation	No
Osmolar stress (cell volume):	AQPs, UTs, TauT, TonEBP, Na-dependent myo-inosital transporter	Ca^{2+} BGT1 PSLC5A3	↑Epidermal differentiation; ↑Lipid synthesis; ↑AMP production, anti-apoptotic (↑HSPs)	No
Acidity:	TRPV1	Ca^{2+}	↑NHE1 + ? others	Yes (via SP→PAR2)
Barrier disruption:	ΔpH	Cytokines; Klk→PAR2	↑Epidermal proliferation; ↑lipid synthesis/secretion (IL-1α); terminal differentiation	Yes (inflammation, pruritis)
Heat:	TRPV3, Ca^{2+}	?Ca^{2+}	?	No
Injury (wounding):	TLR3	ncRNA	↑Lipid synthesis + secretion; ↑innate immunity	Yes (inflammation)

*Fail to downregulate with artificial barrier restoration following acute perturbation.
Abbreviations: AQP, aquaporin; BGT1, betaine/gamma-amino-n-butyric acid transporter 1; Chol, cholesterol; HSP, heat shock protein; Klk, kallikrein; ncRNA, non-coding RNA; NHE1, sodium-hydrogen antiporter 1; PAR2, protease-activator receptor 2; TauT, taurine transporter; TLR3, toll-like receptor 3; TonEBP, tonicity enhancer binding protein; TRPM8, transient receptor potential melastatin-8 ; TRPV, transient receptor potential vanilloid.

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Table 5: Second messengers of permeability barrier homeostasis

External perturbations	Signal	Homeostatic response	Potential pathologic signal
Barrier disruption, UV-B, oxidative stress	ER stress→↑Cer→↑S1P	↑epidermal CAMP (LL-37) production	Cell death, apoptosis, if excessive
—	↑Cholesterol sulfate→ PKGη, AP1 elements	↑epidermal differentiation	Abnormal SC cohesion & barrier function
Barrier disruption	SREBPs	↑sterol, triacylglycerol synthesis	No
Barrier disruption	PPARs, LXR	↑epidermal differentiation, lipid synthesis	No
Barrier disruption	HA→CD44 receptor	↑epidermal proliferation, differentiation, sterol synthesis	No
Barrier disruption→ oxidative stress	nitric oxide→↑cGMP, ↑Ca^{2+}	↓Barrier repair	Inflammation; Apoptosis

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MAST CELLS: LINKING ALLERGY AND MICROBIOME

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SUMMARY

It is now widely accepted that there is a relationship between the microbiota and development and severity of allergic disease. In investigating the mechanisms underlying microbiota modulation of allergic disease the focus has been on the induction phase of the disease; alterations in the phenotype and function of antigen presenting cells, induction of regulatory T cells and shifts in Th1/Th2 balance. However there is evidence that microbes can influence the effector phase of allergic disease. The position of mast cells on the frontline of defence against pathogens also suggests they may play an important role in fostering the host-microbiome relationship and maintaining the dynamic ecosystem of the super-organism. There is emerging evidence that the mast cell plays an important role in microbiota host communication and in particular, that certain non-pathogenic microbes and components of the microbiota can influence the development and severity of allergic disease by modulating mast cell function. Furthermore, it appears that different non-pathogenic bacteria can utilize distinct mechanisms to stabilize mast cells, acting locally though direct interaction with the mast cell at mucosal sites or attenuating systemic mast cell dependent responses, likely thorough indirect signalling mechanisms. Further investigation of mast cell regulation by non-pathogenic or commensal bacteria will likely lead to a greater understanding of host microbiota interaction and the role of the microbiome in development of allergy and other diseases.

INTRODUCTION

The Mast cell, long regarded as a key effector of human allergic disease and immune responses to helminthic parasites is now considered a primary inducer and amplifier of both innate and adaptive immune responses (*Vliagoftis* and *Befus*, 2005).

The presence of mast cells in particularly high numbers in the skin, airway and gut mucosa is indicative of a role as important gatekeepers/sentinels for fighting infectious organisms in these portals of entry. MC express a complement of Toll-like and other microbe associated pattern recognition receptors and activation by bacteria leads to signals that modulate both innate and adaptive immune responses that protect against infection. Mast cells participate in direct killing of organisms by phagocytosis and reactive oxygen species production and can produce anti-microbial peptides such as cathlicidins (*St John* and *Abraham*, 2013). Furthermore, mast cells have been found to produce extracellular traps that encompass and kill organisms in a similar manner to neutrophils (*von Kockritz-*

Blickwede et al., 2008). Mast cells also protect against infection indirectly processing and presenting bacterial antigens to T cells and recruit other inflammatory cells through the release of pro-inflammatory mediators (*Moon* et al., 2010).

Increased study and awareness of the important physiological role of the microbiota is leading to a shift in our perceptions of immunology. A realization that the relationship between the immune system and the external environment is not purely adversarial and that the immune system has evolved not only to protect against pathogens but, just as importantly, to promote a healthy microbiota. Thus the position of mast cells on the frontline of defence against pathogens also suggests they may play an important role in fostering the host-microbiome relationship and maintaining the dynamic ecosystem of the super-organism. There is emerging evidence that the mast cell plays an important role in microbiome host communication and in particular, that certain non-pathogenic microbes and components of the microbiota can influence the development and severity of allergic disease by modulating mast cell function.

MICROBIOTA AND ALLERGY: A ROLE FOR MAST CELLS?

The 'microflora hypothesis' proposes that perturbations in microbiota, because of dietary changes and increased antibiotic use in 'industrialized' countries, leads to disruption of the normal microbiota-mediated mechanisms of immunological tolerance in the mucosa. Consequently this dysregulation leads to an increased susceptibility to immunological disorders including allergy (*Noverr* and *Huffnagle*, 2005; *Shreiner* et al., 2008). Recent studies utilizing gnotobiotic mice have identified that key species within the microbiota, such as clostridia (*Atarashi* et al., 2011), single bacterial strains, or isolated components of these strains, can strongly influence the development of T cell phenotypes in the intestine (*Round* and *Mazmanian*, 2010). Overall evidence suggests that the microbiota of various tissue sites play a central role in governing susceptibility to the Th2 skewed inflammatory response associated with allergy. Certain components of microbiota are immunomodulatory, and colonization of specific subsets of microbes can induce immune signals that are either pro-inflammatory or tolerogenic in nature. Studies in animal models also lend support to the microflora hypothesis. In particular, a recent study by *Noval Rivas* et al. (2013) identified that mice genetically predisposed to food allergy (Il4raF709) exhibit a specific gut microbiota signature distinct from sensitization-resistant wild-type mice. Strikingly, adoptive transfer of microbiota from Il4raF709 but not wild type mice into naïve recipients lead to up-regulation of OVA-specific TH_2 and IgE responses and promoted anaphylaxis. This strongly suggests that dysbiosis provides one mechanism contributing to the pathogenesis of allergy.

Strong evidence for a causal relationship between specific microbiota changes and allergy comes from the study of the skin and allergic dermatitis. Overall, the skin of atopic dermatitis patients has been demonstrated to have greatly reduced microbiota diversity relative to the same skin area of healthy controls. *Staphylococcus aureus*, rarely present on healthy skin is found on the skin in more than 90% of atopic dermatitis patients and some

treatments to reduce *S. aureus* colonization decrease disease severity (*Leung* et al., 2008). Furthermore a recent analysis of the microbiota composition of lesional skin of moderate-to-severe paediatric atopic dermatitis patients revealed temporal shifts associated with disease flares and treatment (*Kong* et al., 2012). Specifically, the relative abundance of *S. aureus* and the skin commensal *Staphylococcus epidermidis* was increased with disease severity, whilst *Streptococcus, Propionibacterium*, and *Corynebacterium* genera were increased following therapy (*Kong* et al., 2012).

Significantly, another recent study by *Nakamura* et al. (2013) identifies mast cells as a mechanistic link between *S. aureus* and atopic dermatitis. These investigators identified that δ-toxin, produced by *S. aureus,* was a mast cell degranulating factor and that *S.aureus* isolates recovered from patients with atopic dermatitis produced large amounts of δ-toxin. Furthermore, immunoglobulin-E enhanced δ-toxin-induced mast cell degranulation in the absence of antigen. Skin colonization with *S. aureus,* but not a mutant deficient in δ-toxin, increased IgE and interleukin-4 production and promoted inflammatory skin disease. Crucially, the enhancement of IgE production and dermatitis by δ-toxin was abrogated in KitW-sh/W-sh mast-cell-deficient mice and restored by mast cell reconstitution (*Nakamura* et al., 2013). Thus it appears that *S. aureus* infection drives a Th2 type immune response with associated IgE and inflammatory cytokine production through direct action on mast cells.

COMMENSAL BACTERIA REGULATE MAST CELL FUNCTION

It has emerged in recent years that microbal exposure either naturally in the environment or through dietary supplementation in the form of probiotics, can protect against allergic disease. For example, there is evidence that the protective effect of the farming environment on development of atopic sensitization, hay fever, and asthma is related to the wider range of microbial exposures in children living on farms compared to urban dwelling peers (*Riedler* et al., 200). This is supported by animal studies demonstrating that specific bacteria that are abundant in cowsheds, *Acinetobacter lwoffii* F78 and *Lactococcus lactis* G121, can attenuate allergic responses in mice (*Debarry* et al., 2007).

Clinical trials indicate that feeding mothers with *Lactobacillus rhamnosus* GG in the pre and early post-natal period may be effective in the treatment and prevention of early atopic disease in children (*Kalliomaki* et al., 2001, 2003). In a similar vein, *Lactobacillus fermentum* was shown to be beneficial in improving the extent and severity of atopic dermatitis in young children (*Weston* et al., 2005). It should be noted that there have also been a number of clinical trials showing no effect of the same probiotic strains on the incidence or severity of allergic disease (*Vliagoftis* et al., 2008).

Animal models have provided strong evidence indicating that oral administration of certain microbes can have systemic effects on immune responses, it has been shown that perinatal treatment with *L. rhamnosus GG* suppresses the development of experimental allergic asthma in adult mice (*Blumer* et al., 2007; *Feleszko* et al., 2007). *L. rhamnousus JB-1*, could attenuate allergen induced allergic air-

way response in adult mice (*Forsythe* et al., 2007; *Karimi* et al., 2009) and others have since confirmed these anti-allergic effects in the airway using a variety of bacteria (*Lim* et al., 2009; *Adam* et al., 2010; *Li* et al., 2010). In addition, several strains of lactobacillus have been shown to attenuate allergic dermatitis when administered orally to mice (*Inoue* et al., 2007; *Hacini-Rachinel* et al., 2009; *Won* et al., 2011). A number of mechanisms have been identified that may contribute to the ability of these bacteria to attenuate allergic inflammation including altered antigen presentation by dendritic cells (*Hart* et al., 2004; *Stagg* et al., 2004), Th1 polarization (*Adel-Patient* et al., 2005; *Hisbergues* et al., 2007) or the induction of regulatory T cells (*Karimi* et al., 2009, 2012). More recently there has been evidence that certain Lactobacilli may influence the effector phase of adaptive inflammation (*Schiffer* et al., 2011). Specifically it is emerging that inhibition of mast cell responses is a component of the immunomodulatory effects of certain bacteria and may be a contributing factor to the ability of candidate probiotic organisms to attenuate allergic inflammation (*Kim* et al., 2008; *Magerl* et al., 2008; *Oksaharju* et al., 2011; *Forsythe* et al., 2012a).

The earliest evidence that commensal bacteria may communicate with mast cells came from the observations that non-pathogenic *E. coli* strains could inhibit mast cell activation following direct co-culture *in vitro* and *ex vivo* following injection of the bacteria into the peritoneal cavity of mice (*Magerl* ete al., 2008). *Wesolowski* and *Paumet* (2014) recently provided some insight into the mechanism underlying the inhibitory effect of *E. coli* on mast cell degranulation. They determined that co-culture with *E. coli* disrupts the sequence of events leading to secretory granule fusion with the cell membrane. Specifically the interaction between IKKβ and SNAP23 is inhibited, resulting in the hypophosphorylation of SNAP23. Subsequent formation of the ternary SNARE complex between SNAP23, Syntaxin4 and VAMP8 is strongly reduced leading to impaired VAMP8-dependent granules release (*Wesolowski* and *Paumet*, 2014).

Lactobacilli have also been demonstrated to directly inhibit mast cell activation *in vitro*, however this ability appears to be highly strain specific (*Kawahara*, 2010; *Oksaharju* et al., 2011; *Schiffer* et al., 2011; *Forsythe* et al., 2012a). Overnight incubation with a strain of *L. casei* inhibits IgE-dependent mouse mast cell and human basophil activation (*Schiffer* et al., 2011). Inhibition could be induced by both viable and irradiated *L. casei*, suggesting the no involvement of secreted metabolites. A role for a structural component of the bacteria, rather than a secreted metabolite, is further supported by the observation that inhibition was also prevented when mouse mast cells were separated from bacteria by a semi-permeable membrane, indicating a requirement for *L. casei*/mast cell contact (*Schiffer* et al., 2011). However, the mechanism underlying the ability of lactobacilli to inhibit mast cell function is unknown and it may be that different strains employ distinct approaches to suppress cell activity. Indeed, *L. casei* induced inhibition of mast cells was not mediated by TLR or NOD1/2 receptors, while another study demonstrated that inhibition of IgE mediated mast cell activation by a strain of *L. reuteri* was at least partially mediated by TLR2 (*Schiffer* et al., 2011).

It is important to note that while evidence suggests that direct inhibition of mast cells by non-pathogenic bacteria *in vitro* requires cell contact with the microbe, many of these bacteria when delivered orally, or into the peritoneal

cavity, can inhibit systemic mast cell dependent responses. For example the requirement for direct bacteria mast cell interaction cannot readily explain the ability of *L. casei* to inhibit IgE mediated passive systemic anaphylaxis when delivered i.p. (*Schiffer* et al., 2011). Indeed evidence suggests systemic mast cell suppression may be mediated by a mechanism distinct for the effect of direct mast cell bacteria interaction.

The *Lactobacillus rhamnosus* strain JB-1 does not inhibit mast cell degranulation following direct *in vitro* co-culture (*Forsythe* et al., 2012a). However feeding JB-1 to rats inhibited peritoneal mast cell response to stimulation *ex vivo* and attenuates the passive cutaneous anaphylaxis response. These observations strongly suggest an indirect mechanism of action involving additional cell types (*Forsythe* et al., 2012a).

The exact nature of this mechanism and the potential intermediary cells are currently unknown. It has been reported that direct exposure of human peripheral blood mast cells to a *Lactobacillus rhamnosus* strain lead to a down-regulation of FcεR1 expression on the cell surface (*Oksaharju* et al., 2011). However, as treatment with JB-1 also inhibits degranulation in response to non-IgE mediated activation it is unlikely that changes in expression of this receptor account for the observed inhibition of degranulation. It was demonstrated that feeding *Lactobacillus rhamonosus* was also associated with inhibition of IK_{Ca} current in peritoneal mast cells (*Forsythe* et al., 2012a). KCa3.1 channel current is critical to the function of many immune cells (*Beeton* et al., 2001; *Wulff* et al., 2003, 2004; *Chandy* et al., 2004) KCa3.1 opening is not required for, but potentiates, mast cell secretion (*Duffy* et al., 2004; *Shumilina* et al., 2008). The IK_{Ca} opener 1-EBIO enhances IgE-dependent $Ca2^{+}$ influx and degranulation in response to a submaximal stimulus (*Duffy* et al., 2004), while mice from KCa3.1 deficient (KCa3.1-/) demonstrate attenuated degranulation in response to FcεR1 mediated activation (*Shumilina* et al., 2008). Indeed the degree of attenuation in response to IgE mediated activation of mast cells following *L. rhamnosus* feeding was similar to that observed in KCa3.1 deficient mice (*Shumilina* et al., 2008). The activation of a range of G_s-coupled receptors including β_2-adrenoceptors, A_{2A} adenosine receptors and EP2 prostaglandin receptors can lead to inhibition of the IK_{Ca} current (*Duffy* et al., 2005,2007, 2008). It is possible that other G_s-coupled receptors may also inhibit KCa3.1 opening and a variety of immune or neuronal derived mediators could be responsible for *L. rhamnosus* effects on the ion channel.

POTENTIAL MECHANISMS OF SYSTEMIC MAST CELL STABILIZATION BY BACTERIA

Galectins

The work of *de Kivit* et al. (2012) suggests galectins may play a role in the mechanism thorough which modulation of gut bacteria results in a systemic alteration in mast cell function. Galectins are secreted by keratinocytes, intestinal epithelial cells (and various immune cells, including dendritic cells, macrophages and mast cells) (*Hirashima* et al., 2004; *Rabinovich* et al., 2009; *Larsen* et al., 2011; *Smetana* et al., 2013).

Galectins have been described to be involved in many physiological processes, including cell signalling, cell

adhesion, chemotaxis and cell apoptosis. In particular, Galectin-9 strongly and specifically binds IgE, a heavily glycosylated immunoglobulin, and that this interaction blocks IgE-antigen complex formation thus preventing degranulation. Interestingly Galectin-9 can be expressed by mast cells and immunological stimulation has been demonstrated to augment Galectin-9 secretion from the cells indicating that Galectin-9 is an autocrine regulator of mast cell function (*Niki* et al., 2009).

A diet containing prebiotic galacto- and fructo-oligosaccharides and a strain of *Bifidobacterium breve* protected against acute allergic symptoms and suppressed mast cell degranulation in whey-sensitized mice. The anti-allergic effects of the synbiotic treatment were correlated with increased galectin-9 expression by intestinal epithelial cells and increased levels of galectin-9 in serum (*de Kivit* et al., 2012). Galectin-9 is soluble-type lectin that recognizes β-galactoside containing glycans. Crucially, serum derived from whey-sensitized synbiotic-treated mice was able to suppress IgE-mediated mast cell degranulation and the extent of this suppression was correlated with serum galectin-9 (*de Kivit* et al., 2012). *In vitro* studies of mast cell degranulation involving RBL-2H3 cells demonstrated that Galectin-9 suppressed IgE mediated degranulation of the cells stimulated. This inhibitory effect was completely abrogated in the presence of lactose, indicating lectin activity of Galectin-9 is critical. Whether the increase in galectin-9 production applies universally to probiotic and prebiotic treatments that stabilize mast cells remains to be determined.

Quorum sensing molecules

In addition to inducing the release of signalling molecules in host cells bacteria themselves can produce an array of molecules that can influence immune cell activity without the need for direct contact. Many species of bacteria communicate with each other through a cell density dependent signalling system, termed quorum-sensing (*Walters* and *Sperandio*, 2007; *Kendall* and *Sperandio*, 2007). These signals are mediated by small hormone-like molecules and allow a population of bacteria to coordinate their gene expression and activities. It is becoming evident that certain components of the quorum sensing system can also influence the behaviour of eukaryotic cells (*Rumbaugh*, 2007; *Lowery* et al., 2008). Many Gram-positive bacteria use small peptides to coordinate their activities (*Kong* et al., 2012). Gram-negative bacteria appear to use several types of small molecules. Of these the best-described signalling system is the N-acyl-homoserine lactone (AHSL) system. AHSLs are fatty-acid-based signalling molecules and mediate bacterial processes by interacting with inducible transcriptional regulators (*Walters* and *Sperandio*, 2006; *Subramoni* and *Venturi*, 2009). These molecules differ significantly only in the constituents of their fatty acid derived acyl chains. Homologs of the AHSL synthase (LuxI-type synthase) and AHSL receptor (LuxR-type receptor) regulate responses to the structurally distinct AHSL.

It has been demonstrated that AHSL, and in particular N-(3-oxododecanoyl)-L-homoserine lactone (3OC12-HSL) which functions as a transcriptional regulator in *Pseudomonas aeruginosa*, can also alter mammalian cell responses. 3OC12-HSL can promote the up-regulation of pro-inflammatory cytokines and chemokines in lung epithelial cells induce apoptosis of neutrophils and macrophages and inhibit lymphocyte proliferation and TNF production (*Ritchie* et al., 2003,

2007; *Vikström* et al., 2006; *Zimmermann* et al., 2006). There have been somewhat contradictory reports regarding the effect of AHSL on mast cell function suggesting that 3OC12-HSL may attenuate mast cell responses by inducing apoptosis or enhance mast cell degranulation (*Wu* et al., 2001; *Li* et al., 2009). These differential effects may depend on the phenotype of the mast cell and the concentration of AHSL. Nevertheless, inter-kingdom communication, with bacteria derived signalling molecules influencing mast cell activity, is a potential means through which specific microbes may influence allergy development or disease severity and deserves further investigation.

Immunoregulation of Mast cells

There good evidence linking the immunomodulatory function of certain commensal bacteria, and components thereof, to induction of Treg and their associated cytokines (*Feleszko* et al., 2007; *Karimi* et al., 2009; *Round* and *Mazmanian*, 2010). *Mazmanian* et al. (2008) demonstrated that oral ingestion of polysaccharide A (PSA) derived from *Bacteroides fragilis* protects animals from experimental colitis through induction of IL-10-producing CD4+ T cells. *Bifidobacterium infantis* induces expression of Foxp3+ T cells that protect mice against *Salmonella typhimurium* infection (*O'Mahony* et al., 2008), while early life treatment of mice with *L. Rhamnosus GG* leads to an attenuated allergic airway response in adult animals that is also associated with an increase in $Foxp3^+$ T cells (*Feleszko* et al., 2007). *L. rhamnosus JB-1* significantly increases the proportion of $CD4^+CD25^+Foxp3^+$ Treg cells in the mesenteric lymph nodes and spleen of non-sensitized adult mice (*Karimi* et al., 2009). In OVA-sensitized mice challenged with inhaled antigen this increase in Foxp3 was also observed in the mediastinal lymph nodes indicating that *L. rhamnosus* induced Treg can migrate to the airways in response to inflammation. Although the suppressive activity of Treg cells requires prior activation through their T-cell receptor, once activated, Treg cells can suppress in an antigen-non-specific way called ‘‘bystander suppression’’ (*Tang* et al., 2008). Recently, it has been shown that constitutive $Foxp3^+$ Treg can control mast cell activation and IgE-dependent anaphylaxis in mice (*Kanjarawi* et al., 2013). Inhibition of mast cell degranulation by Tregs appears to require OX40/OX40 ligand interactions. It is known from *in vivo* transfer studies that a population of Treg cells can create a regulatory milieu that promotes the outgrowth of new populations of Treg cells with antigen specificities distinct from those of the original Treg population (*Tang* et al., 2008). It now appears that the stabilization of mast cells and reduction of mast cell dependent systemic responses following exposure to specific bacteria or shifts in microbiota composition may be a consequence of the T cell driven regulatory milieu**.**

MICROBIOTA-MAST CELL COMMUNICATION BEYOND ALLERGY

In addition to being at the frontline of host defence mast cells can translate signals between the nervous immune and endocrine systems (*Theoharides*, 1996; *Forsythe* et al., 2012b).

Considering effects beyond immunity, it should be noted that much of our earliest understanding of the relationship between the nervous and immune systems came from the study of mast

cell-nerve interactions. Indeed, mast cells have been described as neuro-immuno-endocrine master-players (*Theoharides*, 1996). Mast cells can be activated by a range of neurotransmitters and hormones while, in turn, a variety of molecules, including histamine and serotonin, synthesized and released by mast cells can influence neuronal activity and endocrine function (*Frieling* et al., 1991, 1993). Similarly, mast cell derived cytokines including TNF and growth factors, such as NGF, modulate the threshold for activation of local neurons and promote nerve fibre growth (*Leon* et al., 1994; *van Houwelingen* et al., 2002; *Arnett* et al., 2003; *Kakurai* et al., 2006).

Mast cells have been demonstrated to influence behaviour of mice (with mast cell deficiency resulting in a more anxious phenotype) (*Nautiyal* et al., 2008) and to participate in regulation of the HPA axis (*Matsumoto* et al., 2001). There is also strong evidence for mast cells as important participants in visceral hypersensitivity and pain perception, particularly in irritable bowel syndrome (*Barbara* et al., 2007; *Wood*, 2007).

There are marked parallels between the neuroendocrine role of mast cells and the physiological effects described following exposure to non-pathogenic commensals/probiotic, (decreased intestinal permeability, myenteric nerve activation, anti-nociception, HPA regulation, and changes in anxiety-like behaviour) (*Kamiya* et al., 2006; *Kunze* et al., 2006; *Wang* et al., 2010; *Bravo* et al., 2011) and future studies designed to test potential causal relationships between neuroendocrine regulation and the ability of certain gut bacteria to modulate mast cell function will be of great interest.

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OUR MICROBIAL SELF: ESSENTIAL FUNCTIONS FOR COMMENSAL BACTERIA ON THE SKIN

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INTRODUCTION

It is increasingly well accepted that the community of microbes that normally inhabit our body influences human immune behaviour, but it remains incompletely understood how this occurs or how this information can be used therapeutically. We have been particularly interested in the analysis of potentially mutually beneficial relationships between the most commonly isolated skin bacterium, *Staphylococcus epidermidis*, and the skin. Interactions between *S. epidermidis* and our immune system enhances skin protection by suppressing uncontrolled inflammatory reactions and stimulates host antimicrobial peptide (AMP) production (*Lai* et al., 2009, 2010). These specific beneficial effects on immunity are now just two of several actions that have been associated with the presence of a normal microbiome (*Wanke*, 2011; *Naik* et al., 2012). However, it was unclear how cutaneous microbes can exert such effects while residing on top of the epidermis, not in contact with living cells. To address this important question, we have recently shown that low numbers of bacteria penetrate the intact epidermal barrier in mice and in humans (*Nakatsuji* et al., 2013). This means that the skin acts as a filter (rather than a barrier), and controls the balance of the dermal microbial communities. In this paper we suggest that the presence of microbes below the surface of the skin can provide a rational explanation for how microbes influence normal skin homeostatic functions. In particular, we propose that the pathophysiology of several skin diseases can be explained by disruption of balance between the barrier and the microbiome.

COMMENSAL MICROBES MAINTAIN SKIN HOMEOSTASIS

Several recent studies have shown that the skin commensal bacterial community modulates the host immune system. In a 2009 publication in *Nature Medicine*, our group first demonstrated a beneficial interaction between *S. epidermidis*, a predominant commensal species on normal human skin, and skin inflammatory responses (*Lai* et al., 2009). We showed that a unique lipoteichoic acid (LTA) produced by *S. epidermidis* was recognized through a MyD88-dependent process to activate TNF-a receptor–associated factor-1 (TRAF1). This event inhibited excess skin inflammation during skin injury. In separate but related work, we also demonstrated that a small molecule of <10 kDa secreted from *S. epidermidis* increased expression of human β-defensins (hBDs) in murine skin or human keratinocytes (*Lai* et al., 2010).

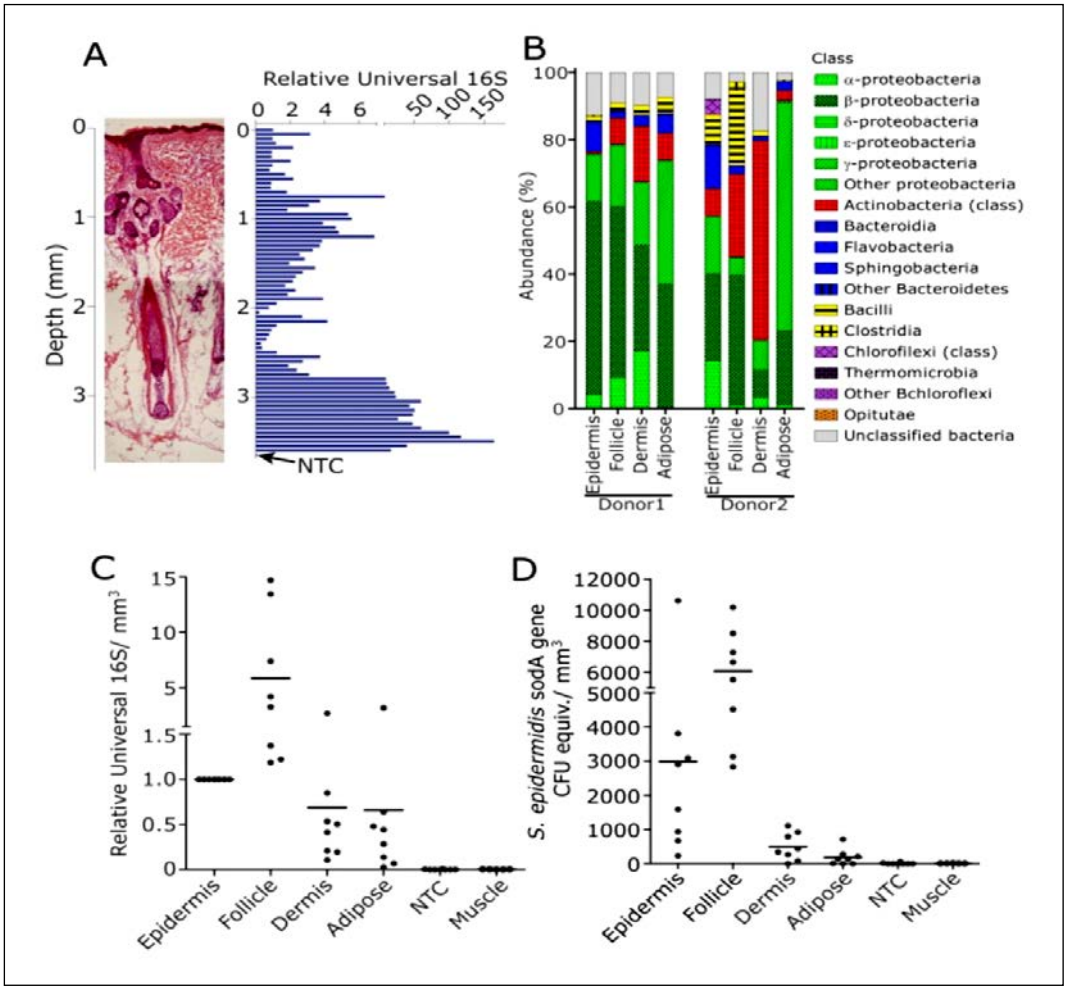

Figure 1: Bacterial DNA is present in normal human dermis. (Taken from: Nakatsuji et al., 2014). **(A**) 16S rRNA quantified with real-time qPCR from total DNA extracted from sequential 50 µm horizontal sections of human facial skin, H&E staining of the sectioned biopsy is shown in the same scale as the Y-axis of the graph. **(B)** Pyrosequencing for diversity analysis in Laser Capture Microdissection (LCM) sections of isolated epidermis, follicle, dermis and adipose tissue. Bacterial class is identified by colour. 2 representative patients are shown. **(C)** qPCR for total bacteria by universal 16S rRNA gene from LCM samples as prepared in (B) (n=8). **(D)** qPCR for *S. epidermidis* from LCM of same as (C). NTC=non-tissue control from same slide of LCM. Muscle is additional normal human tissue control that shows no bacterial 16S signal is detected in this tissue processed along slide of skin biopsy.

Following our discoveries, other groups also have shown that a product secreted from *S. epidermidis* strongly sensitizes the host innate immune response to pathogens (*Wanke* et al., 2011). In cultured human keratinocytes, activation of TLR2 by peptidoglycan, the major constituent of Gram-positive cell wall, strengthens the cellular tight junctions (*Yuki* et al., 2011). More recently, it was demonstrated that skin commensal bacteria enhanced cutaneous T-cell functions via activation of IL-1 signalling (*Naik* et al., 2012). Indeed, germ-free mice are more susceptible to infections in the

skin than conventionally housed mice (*Naik* et al., 2012). Furthermore, additional work has suggested that a shift in the microbiome will be detrimental not only because of the loss of beneficial species, but also because of the addition of pathogenic species. Colonization of mice with *S. aureus* producing delta-toxin led to exacerbation of Th2 response and elevated IgE resembling AD (*Nakamura* et al., 2013). This response was dependent on mast cells residing in the dermis. A more detailed review of this topic can be found in a recent review (*Sanford* and *Gallo*, 2013).

THE EPIDERMIS IS A MICROBIAL FILTER, NOT A BARRIER

Inherent to the observations described above is the conclusion that bacteria must somehow interact with the cells of the immune system to subsequently influence their action. However, this rational conclusion contradicted the prevailing belief that the structure of the skin's stratum corneum, and tight junctions between epithelial cells, prevent entry of bacteria under normal conditions. In fact, topical drug delivery is severely limited by the size of molecules, with a typical upper limit of entry to be only about 500 Daltons. The question remained then: how does the microbiome exert control on cells located below the epidermis?

The obvious answer to this question was that the epidermis was not absolute barrier to bacteria or products of the microbiome that can diffuse to deeper levels. To determine if bacteria enter the dermis we first made sequential horizontal sections of normal human facial and palm skin (bearing no hair follicles) and determined abundance of microbial DNA at different depths with qPCR using universal 16S rRNA primers (*Horz* et al., 2005) (Figure 1A). The existence of bacterial 16S rRNA genes was seen deep in subcutaneous tissues. In contrast, the qPCR signal was negative in simultaneously-prepared control samples from OCT tissue embedding compound surrounding the tissue, and downstream of cutting by the cryostat blade, [non-tissue control (NTC)]. These indicated that the detection of the bacterial 16S rRNA gene was not due to contamination from reagents, sample processing and sample crosstalk. Notably, however, 16S rRNA genes were detected in skin samples beneath the maximal depth of follicles in facial skin and below the eccrine glands in palm skin. Appendageal structures were expected to contain a microbial community, but our first experiments suggested that bacterial DNA may exist outside of the follicle or sweat glands. Subsequent studies using a laser capture microdissection (LCM) technique to isolate dermis away from appendages confirmed the existence of microbes in dermis and adipose. We also used the combination of LCM and pyrosequencing and real-time qPCR with 16S rRNA primers or genus- and species- specific primer/probes to quantify and identify the bacterial DNAs existing in each skin compartment. Universal 16S rRNA and *S. epidermidis*-specific genes were consistently detected by real-time qPCR in LCM-isolated sections from dermal and adipose tissue, (Figure. 1B,C, D). To rule out that the qPCR signals from these regions were due to contamination of these samples, NTC samples were prepared by LCM in the same manner as the pyrosequencing analysis. In addition, LCM section of human skeletal muscle biopsies served as another control. We also used multiple

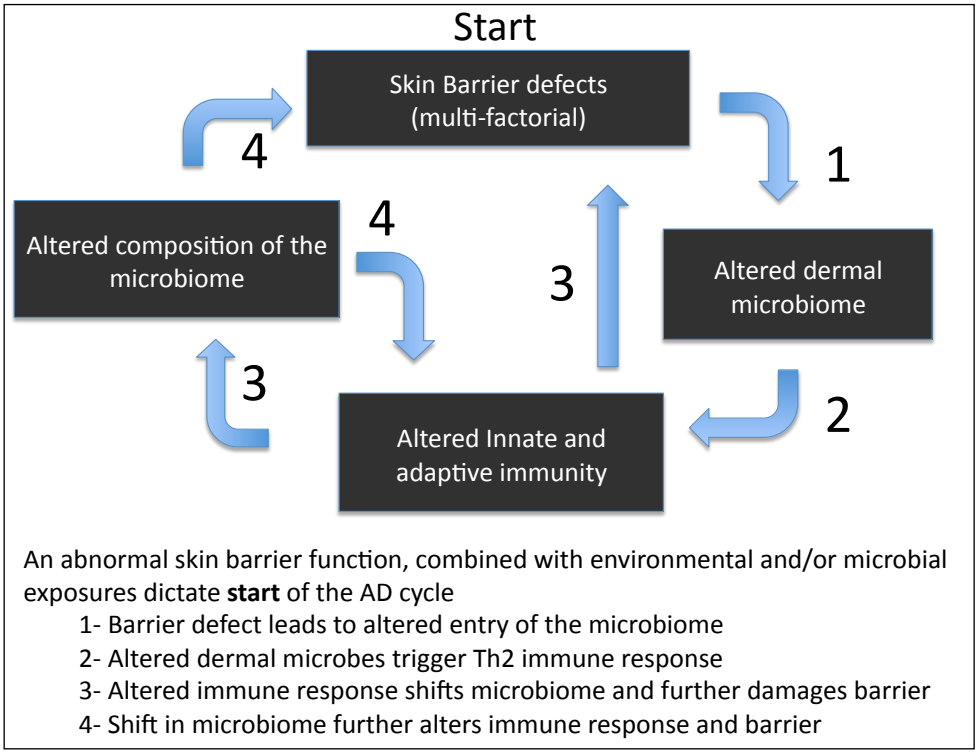

Figure 2: A model to connect the function of the skin barrier in Atopic Dermatitis (AD) with the function of the microbiome

other techniques to further confirm the results. Due to space limitations we will not show the data here. To summarize: bacteria were also detectable by Gram-staining inboth reticular dermis and dermal adipose, by immunostaining for *S. epidermidis*, *Pseudomonas* spp. and anti-lipopolysaccharide (LPS). Also, co-immunostaining with anti-CD11c failed to detect these bacterial products within these cells by confocal imaging, indicating that the presence of bacteria in the dermis was not associated with classical phagocytic immune cells that could have engulfed bacteria in transit from the surface into dermis. Bacterial 16S rRNA was also detectable by *in situ* hybridization with an oligonucleotide probe EUB338 (*Amann* et al., 1990) in dermal adipose tissue. In summary, we have optimized several independent techniques (qPCR for horizontal sections, Gram stain, several immunostains, *in situ* hybridization, LCM and pyrosequencing, and LCM and qPCR) to detect bacterial RNA, DNA and antigens in the dermis.

A MODEL FOR THE LINK BETWEEN HOST BARRIER DEFECTS AND THE MICROBIOME

Patients with atopic dermatitis (AD) have known defects in both physical and antimicrobial barriers. Therefore, if these barrier defects influence microbe entry into the skin, and the presence of microbes in the dermis alters skin im-

mune responses (as our data suggest), our observations provide an explanation for how barrier defects may result in abnormal inflammation. This is not infection, but rather a change in an important environmental signal. Such an altered signal could perpetuate a chronic cycle: an inherent barrier defect can lead to an altered microbial community causing altered immunity, altered immunity may then alter the surface microbiome and barrier, and the dysbiosis of the dermal microbial community then may perpetuate the disease by further harming the barrier and triggering inflammation. An illustration of our hypothesis for the interaction between these variables is seen in Figure 2. The significance of this discovery for human disease is that our model explains how several different genetic or environmental differences in barrier function may result in dysbiosis and skin inflammation.

A fundamental assumption of this model is that the presence of bacteria, or bacterial products, in the dermis is a critical factor in both the acute response (steps 1-3) and the chronic responses (steps 3-4). Although there is good evidence now that the microbiome influences immunity there has not been adequate work to show how this links to the known physical barrier defects in AD. Furthermore, there is increasing evidence that specific members of the microbiome (in particular *S. aureus*) may exacerbate the allergic response (*Nakamura* et al., 2013). Therefore, it is important to understand mechanisms that regulate the entry of microbes into the skin and explore how this influences the immune response. Furthermore, showing which bacteria can best minimize *S. aureus* colonization and suppress allergic disease may be of great clinical importance.

The concept of a dermal microbiome, and the proposed link between barrier and the immune response characteristic of AD, suggests that interactions take place between microbes and live cells below the epidermal barrier (Figure 2). Our current hypothesis extends this and suggests that the dysbiosis of the skin surface microbiome alters the immunological phenotype by penetrating into the dermis. This hypothesis does not necessarily disprove the notion that the dermis is sterile. The dermal microbiome may or may not be alive. The critical prior finding is that the products of microbes (bacterial 16S rRNA, multiple species-specific genomic DNA and antigens) are located in contact with live cells below the stratum corneum. Notably, the microbial products observed deep in the skin are generally not present within classical $CD11c^{+}$ phagocytic immune cells, suggesting the microbes enter across the epidermal barrier rather than carried in by phagocytosis. The capacity of animal models to experimentally manipulate dermal microbe entry permits us to test how this affects skin immunity. Such observations can provide an important step towards explaining the association between skin barrier defects and inflammation in AD.

A CHANGE IN THE BARRIER RESULTS IN A CHANGE IN DERMAL MICROBES

Loss-of-function mutations found within the filaggrin gene (*FLG*) represent a significant genetic factor predisposing to AD (*Palmer* et al., 2006; *Smith* et al., 2006; *Sandilands* et al., 2007; *Bisgaard* et al., 2008). It was

thought that *FLG* mutations facilitate penetration of environmental pollutants, irritants, and allergens passing the epidermal barrier, resulting in development of uncontrolled allergic reactions (*Bisgaard* et al., 2008; *Fallon* et al., 2009).

In preliminary studies we used *FLG* null mutants in a Balb/c background ($FLG^{ft/ft}$ Balb/c), which have been bred to remove the matted mutation (*ma*) that also was present in the originally described flaky-tail mice. This mouse is a specific *FLG* mutant, and has no spontaneous phenotype. Prior work has shown that the $FLG^{ft/ft}$ mouse will develop an AD-like phenotype if manipulated by external injury from tape-stripping, and an immunological stimulus from repeated cutaneous ovalbumin (OVA) stimulation (*Fallon* et al., 2009). Therefore, we performed a preliminary experiment to study microbe entry under each condition. We found that the $FLG^{ft/ft}$ Balb/c mouse has enhanced barrier defect as classically measured by transepidermal water loss (TEWL). This correlated with increased penetration of *Staphylococcus aureus* into the dermis measured by a newly developed technique of tracking entry of a candidate microbe applied to the surface. *S. aureus* was only slightly increased on the surface in $FLG^{ft/ft}$ Balb/c or in comparison to PBS-sensitized group, (all groups were tape-stripped). Notably, *S. aureus* was greatly increased in sub-epidermal dermal and adipose tissue of OVA-sensitized $FLG^{ft/ft}$ skin compared to identically treated WT. These data support our working hypothesis.

ANTIMICROBIAL PEPTIDES ARE AN IMPORTANT COMPONENT OF THE EPIDERMAL BARRIER

Our labs work over the past 20 years has shown how AMPs are an integral part of the host innate immune system. The secretion or release of these peptides provides direct disinfecting action against various infectious pathogens. Critical roles of AMPs on the skin innate immune defence have been shown by our group using cathelicidin knockout mice ($Camp^{-/-}$). We discovered that these mice were susceptible to pathogen infections, thus showing how important AMPs are in mammalian immunity (*Nizet* et al., 2001). Subsequently, we and others found that the induction of some AMPs such as cathelicidin and hBD-2 and hBD-3 is low in AD (*Ong* et al., 2002; *Nomura* et al., 2003; *Lande* et al., 2007). This diminished induction of AMPs linked to a higher propensity to *S. aureus* colonization and may explain why infections occur more frequently in AD. Similar to findings in the *Flg-/-* mouse we saw a significant difference in the *S aureus* in dermal and adipose compartments between $Camp^{-/-}$ and WT. This suggests *Camp* participates as part of the filter regulating dermal microbial abundance.

CLINICAL EVIDENCE FOR AN ABNORMAL MICROBIAL BARRIER IN ATOPIC DERMATITIS

In addition to our new data in mouse models, we have also obtained preliminary clinical data from patients that support our hypothesis. A pilot study using lesional and non-lesional forearm skin from patients with AD demonstrated important differences in the dermal microbial community of AD. In

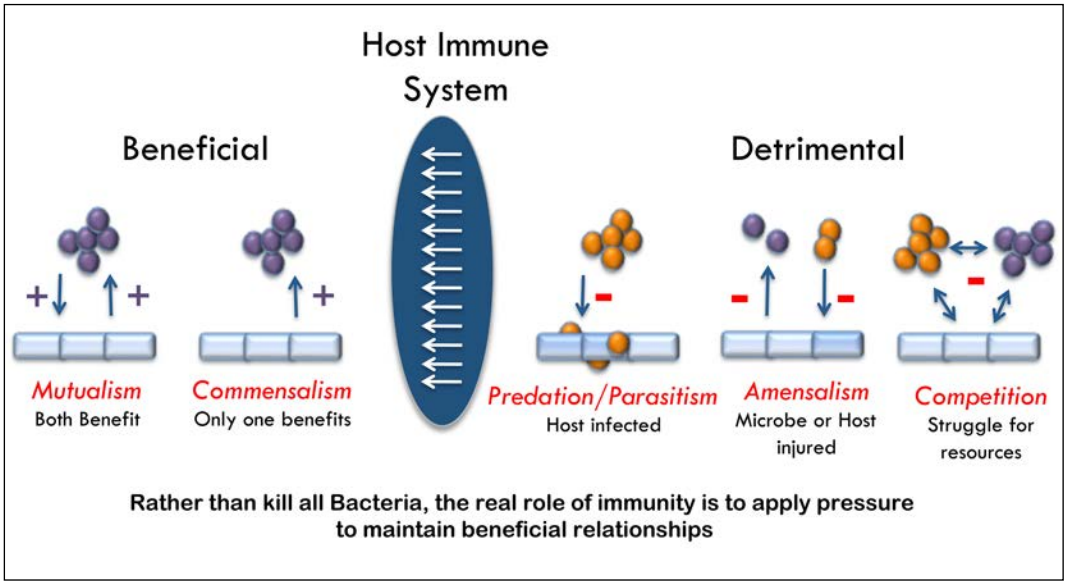

Figure 3: Potential relationships between microbes and us: A new way to think about the immune system. (Adapted from: Schommer and Gallo, 2013).

non-lesional skin of AD, the microflora in the epidermis and dermis primarily consisted of the phyla of *Proteobacteria* and/or *Actinobacteria*. Importantly, the diversity in the dermis of non-lesional AD skin was different that seen in any prior analysis from normal patient skin. Also, we measured a higher relative abundance of bacteria in AD lesional compared to normal skin or non-lesional skin. In lesional skin, proportions of *Firmicutes* were higher in the epidermis of lesional sites than non-lesional sites, a finding consistent with previous observations of the superficial community of AD skin and the increased abundance of *S. aureus* (*Kong* et al., 2012). Furthermore, we found a similar trend in the bacterial communities in the dermis in AD as in the epidermis. Real-time qPCR analysis indicated that *S. aureus* (a prominent member of the *Bacilli* class) was detected both in epidermis and dermis of lesional skin, but not in non-lesional skin**.** In addition, total bacterial abundance in the dermis of lesional skin was much higher than that of non-lesional skin. We also detected increased level of *Propionibacterium acnes* (an *Actinobacteria*) in lesional sites, and the level was higher in dermis than epidermis. (Of note: the skin surface is cleaned for standard surgical prep prior to biopsy and was not clinically infected).

CONCLUSIONS

In summary, rapidly advancing knowledge about the composition and function of the microbiome is redefining conceptions about our immune system. Rather than a permanent hostile relationship, we probably co-exist in a mutualistic relationship. Under such a relationship, both parties benefit. Of course, this is not the only possible interaction between microbes and the

host, and all of the commensal bacteria that have been described to benefit us can also harm us. Figure 3 presents a summary of the types of relationships that can exist between host and microbe. This figure also illustrates our new concept for the function of the immune system. Given our current knowledge regarding the functions of the microbiome, it is more reasonable to consider the host immune system as a mechanism to apply pressure to maintain beneficial relationships between bacteria and the host. In fact, given the abundance and distribution of bacteria both at the surface and within us, it is most logical now to consider the human body as a heterogeneous collection of organisms hoping to work towards a common good. Thus, the concept of **the microbial self** emerges from the paradigm-shifting observations that bacteria enter deep into the dermis. We hypothesize that this event is central to how the microbiome exerts influence on the immune system, and that the entry of some bacteria will exacerbate disease while others may rescue it. The near future will bring new insight into how the combination of epithelial function and the skin microbiome influences skin immunity. The implications of this are great, and hopefully will provide new therapeutic approaches to human disease.

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CRUCIAL ROLE OF MPRF-MEDIATED DEFENSIN RESISTANCE IN *STAPHYLOCOCCUS AUREUS* NASAL COLONIZATION

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SUMMARY

Major bacterial pathogens such as *Staphylococcus aureus* are facultative pathogens and are members of the microbiotas of human body surfaces. *S. aureus* uses the MprF protein to achieve resistance to host-derived cationic antimicrobial peptides (CAMPs) such as defensins and cathelicidins. While the importance of MprF for *S. aureus* invasive infections is well documented it remains unclear if CAMP resistance is also a prerequisite for the capacity of *S. aureus* to colonize its preferred niche in healthy humans, the anterior nares. The cotton rat model of nasal colonization was used to investigate the potential role of MprF as a colonization factor. Of note, a *S. aureus mprF* mutant turned out to have a reduced capacity to colonize cotton rat noses compared to the parental strain. This finding underscores the role of CAMPs in shaping the composition of mirobiotas at mammalian body surfaces and they demonstrate that CAMP resistance mechanisms are promising targets structures for antimicrobials that would not only interfere with infection but also with colonization by bacterial pathogens.

INTRODUCTION

Bacterial pathogens with resistance to the most effective available antibiotics are increasingly spreading around the world causing severe morbidity and a vast number of deaths. Methicillin-resistant *Staphylococcus aureus* (MRSA) remains the most frequent cause of antibiotic-resistant infections on intensive care units followed by vancomycin-resistant enterococci and enterobacteria producing extended-spectrum b-lactamases (ESBL) or carbapenemases, which have emerged in recent years and are on the rise (*Boucher* et al., 2009). Of note, most of these pathogens have their reservoirs in the microbiotas of human body surfaces such as the intestine (enterobacteria) or nose (*S. aureus*). Only certain individuals are colonized by such antibiotic-resistant pathogens and colonized patients at risk e.g. on intensive-care units or under immunosuppression often suffer from severe infections caused by an endogenous strain (*Weidenmaier* et al., 2012; *Biehl* et al., 2014). Why only certain persons are colonized and how antibiotic-resistant bacteria can prevail in human microbiotas has remained unclear.

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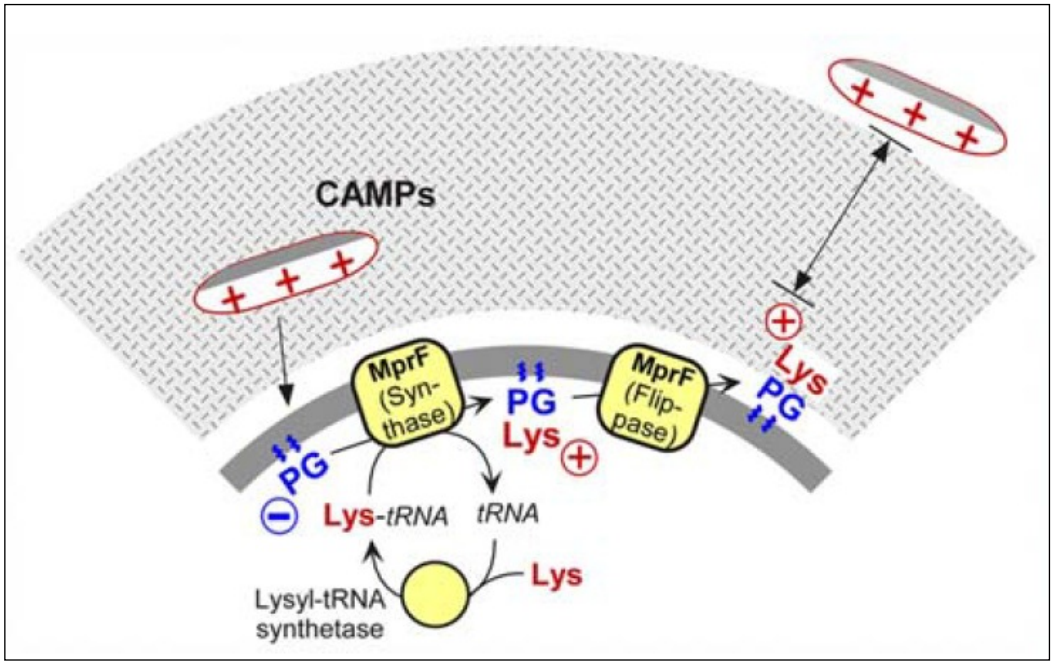

Figure 1: CAMP resistance mediated by MprF. (From: Ernst and Peschel A, 2011).

The capacity of a given bacterial strain to persist in a microbial community is governed by its competitive fitness in the interaction with other microbes and with the host. The underlying mechanisms are complex and have been hardly explored. They may include capabilities to (i) bind to a limited number of host attachment sites, (ii) take up and metabolise scant nutrients, or (iii) produce antibacterial substances referred to as bacteriocins that inhibit competitors. In addition, (iv) intrinsic mechanisms conferring resistance to host antimicrobial peptides such as defensins may contribute to bacterial fitness in the human nose.

Defensin-like 'endogenous antibiotics' include defensins, cathelicidins, RNase 7, or dermcidin (*Hancock* and *Sahl*, 2006). They play key roles in human antibacterial host defence and have also been found to shape the microbiota in the human gut (*Salzman* et al., 2010). Such peptides are released by immigrating neutrophils and secreted e.g. by epithelial cells. Most human antimicrobial peptides have a cationic net charge, which increases their affinity for the generally anionic bacterial cell surface molecules (*Peschel* and *Sahl*, 2006). Such cationic antimicrobial peptides (CAMPs) kill bacteria by damaging essential membrane-associated machineries (*Wenzel* et al., 2014). Peptides with related physicochemical properties and modes of action are also produced by bacteria of human microbiotas (referred to as bacteriocins; e.g. epidermin) (*Arnison* et al., 2013) or are used as therapeutic antibiotics (e.g. daptomycin) (*Bayer* et al., 2013). Antimicrobial peptides produced by human or bacterial cells have been described also in the fluids found in the human nose (*Cole* et al., 1999) but it remains unclear how important they may be for the ecology of the nasal microbial habitat.

Pathogens often have mechanisms to resist antimicrobial peptides and such mechanisms are crucial for resist-

ing bacterial capacities to withstand host defence (*Peschel* and *Sahl*, 2006). *S. aureus* uses several intrinsic mechanisms for resisting CAMPs. They are based on modification of polyanionic cell envelope components such as teichoic acids, membrane lipids, or peptidoglycan to reduce the affinity for host antimicrobials by altering the surface net charge (*Peschel* et al., 1999, 2001; *Munch* et al., 2012). The underlying mechanisms have been found to be crucial for the capacity of *S. aureus* and many other pathogens to colonize or cause infections. The multiple peptide resistance factor MprF is a bifunctional membrane protein that (i) modifies anionic phosphatidylglycerol (PG) with lysine to produce lysyl-PG and (ii) translocates Lys-PG from the inner to the outer leaflet of the cytoplasmic membrane (Figure 1) (*Ernst* et al., 2009). MprF is found in many Gram-positive (staphylococci, listeria, group-B streptococci) and Gram-negative pathogens (pseudomonads, burkholderiae, many plant pathogens such as *Agrobacterium*) and is thought to be an attractive target for future anti-virulence therapeutics (*Ernst* and *Peschel*, 2011). MprF mutants of *S. aureus* and other pathogens are profoundly virulence-attenuated but a potential role of this protein in colonization of human body sites has remained unknown.

MATERIALS AND METHODS

The cotton rat model was used as described earlier (*Baur* et al, 2014; *Krismer* et al., 2014). Briefly, cotton rats (*Sigmodon hispidus*) were anesthetized and instilled intranasally with 10 µl of 1×10^8 colony-forming units (CFU) of *S. aureus*. Six days after bacterial instillation the animals were euthanized and noses were removed surgically. The noses were vortexed in 1 ml of PBS containing 0.5% tween for 30 s. Samples were plated on appropriate agar plates [B-medium, sheep blood containing 250 µg/ml streptomycin and HiCrome Aureus Agar (Fluka)] and the bacterial CFU was determined. All animals received drinking water with 2.5 mg/ml streptomycin continuously, starting three days prior to the experiment to reduce the natural nasal flora. All guidelines of the US Department of Agriculture and the Biosynexus Inc. Institutional Animal Care and Use Committee were followed.

RESULTS AND DISCUSSION

When inoculated with *S. aureus* the noses of mice quickly loose the bacteria and stable colonization can hardly be established in these animals. In contrast, cotton rats are susceptible to many human respiratory pathogens and the histology of the cotton rat nares is similar to that in humans, as they both have squamous and pseudostratified epithelial areas (*Burian* et al., 2010). Several *S. aureus* factors have been described as determinants for nasal colonization using this model: wall teichoic acid (WTA), a surface-exposed structure of Gram-positive bacteria (*Weidenmaier* et al., 2004), the iron-regulated surface determinant A (encoded by isdA), catalase, alkyl hydroperoxide reductase, the autolysin SceD (encoded by sceD) (*Clarke* et al., 2006; *Cosgrove* et al., 2007), and the methionine-biosynthetic enzyme MetI (*Krismer* et al.,

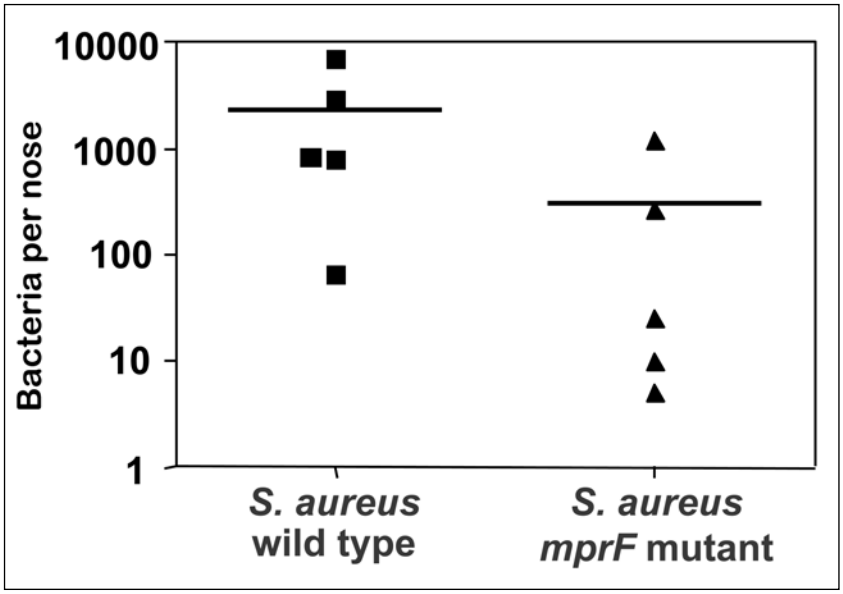

Figure 2: Impact of MprF on *S. aureus* nasal colonization in cotton rats.

2014). Several key virulence and colonization factors were found to be expressed at similar levels in human and cotton rat noses (*Burian* et al., 2010; *Krismer* et al., 2014) and antibodies directed against the epithelial cell receptor SREC I block *S. aureus* binding to human and cotton rat nasal epithelial cells (*Baur* et al., 2014). All these evidences indicate that the cotton rat represents a reliable and representative model for experimental nasal colonization studies.

In order to study if MprF may play a role in *S. aureus* nasal colonization the recently described *S. aureus* SA113 *mprF* deletion mutant (*Peschel* et al., 2001) and its parental wild-type strain were instilled into the noses of cotton rats and the numbers of *S. aureus* bacteria per nose were determined seven days later (Figure 2). While the wild type colonized the animals at numbers of several thousand bacteria per nose the *mprF* mutant was recovered at ca. ten times lower numbers. Thus, the MprF-mediated resistance mechanism enables *S. aureus* to persist in the nose while its absence leads to a strongly abrogated colonization capacity.

Defensins and other CAMPs are found in nasal fluids of *S. aureus*-colonized humans but it has remained unknown if they contribute to limiting the growth of nasal bacteria. Our results indicate that MprF is not only avirulence but also an important colonization factor for *S. aureus*. The occurrence of MprF in many other human commensals, opportunists, and aggressive pathogens suggests that it may be a general bacterial mechanism allowing growth in host environments with high CAMP concentrations. It remains unclear if only host-derived CAMPs limit bacterial growth in the nose or if bacteriocins produced by other bacteria of the nasal microbiota may also be a reason for *S. aureus* to require CAMP resistance mechanism during its commensal life style. A second CAMP resistance mechanism, the teichoic acid-modifying DltABCD machinery, has also been found to contribute to the nasal colonization capacity of *S. aureus* (*Weidenmaier* et al., 2004).

However, since teichoic acids also play a critical role in the attachment of *S. aureus* to nasal epithelia cells the potential importance of DltABCD-dependent CAMP resistance for *S. aureus* nasal colonization has remained unknown.

S. aureus carriage in ca. 30% of the human population represents a major risk factor for severe invasive infections (*von Eiff* et al., 2001). Therefore, patients are decolonized with the antibiotic mupirocin before they are admitted to elective surgery. With increasing resistance to mupirocin among clinical *S. aureus* alternative antibiotics are urgently needed (*Arnison* et al., 2013; *Hetem* and Bonten, 2013). Our discovery of a crucial role of MprF in nasal colonization puts MprF on the list of potential targets for new antimicrobials that would not kill *S. aureus* directly but render the bacteria susceptible to endogenous human CAMPs, bacteriocins, and antibiotics such as daptomycin.

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VITAMIN D, SUNLIGHT AND OUR SKIN

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SUMMARY

This paper reviews historical, clinical, animal and molecular-based studies linking sunlight, vitamin D and the immune system to maintaining the barrier defence and health of the skin. It focuses on the discovery that vitamin D regulates the expression of antimicrobial peptides important in skin immunity with an emphasis on the cathelicidin antimicrobial peptide. The dysregulation of antimicrobial peptide expression, altered composition of microbiota in skin and use of UVB heliotherapy and vitamin D are discussed. Findings from recent and past studies indicate that vitamin D mediated regulation of the cathelicidin gene may provide approaches for therapy of skin infections and disorders. Also, there is emerging evidence that cutaneous synthesis of vitamin D and induction of the cathelicidin antimicrobial peptide gene may provide a mechanism by which sunlight and vitamin D may modulate the skin microbiota.

INTRODUCTION

Historically the ancient Egyptians, Babylonians, Assyrians, Greeks and Romans recognized the health benefits of the sun. Sunbathing to restore health or heliotherapy was promoted by Hippocrates and extoled by Herodotus. Pliny the Elder remarked "Sol est remediorum maximum" or the "sun is the best remedy" (*Levine*, 1971). Sunlight effectively treated rickets and in the 19th and 20th centuries and heliotherapy at sanatoriums was standard treatment for tuberculosis (*Howson*, 1928; *Koch*, 1901; *Rajakumar,* 2003). Niels Ryberg Finsen won the 1903 Nobel Prize in Medicine and Physiology for his discovery that heliotherapy with artificial sun light effectively treated lupus vulgaris a cutaneous infection with *Mycobacterium tuberculosis* (*Mtb*) (*Grzybowski* and *Pietrzak*, 2012). It wasn't until the 1920s that vitamin D, a major curative component created by the sun, was identified through a series of elegant studies by Adolf Windaus, Harry Goldblatt, Harry Steenbock, Alfred Hess and Mildred Weinstock (*Norman*, 2012). Hess and Weinstock verified light produced vitamin D by demonstrating that ultraviolet light irradiation of small portions of skin from rachitic rats cured other groups of rachitic rats fed the skin. This was not observed with non-irradiated skin (*Norman*, 2012). These discoveries led to the fortification of foods with vitamin D and the eradication of rickets in the United States and the development of a high-dose oral therapy with vitamin D for lupus vulgaris in the 1940s (*Dowling*, 1946; *Gaumond*, 1948). Furthermore, these discoveries identified vitamin D as a likely explanation for the healing properties of the sun.

THE VITAMIN D PATHWAY

A vitamin is required for normal physiological processes, is not synthesized by the body and must be acquired regularly from the diet. By this definition vitamin D is a misnomer as the diet is a poor source and the most effective way to acquire vitamin D is through synthesis in the skin or consumption of a purified supplement. Briefly, natural sunlight or artificial ultraviolet B rays cleave the B-ring of 7-dehydrocholesterol in the skin to produce cholecalciferol or vitamin D_3. Vitamin D_3 is absorbed into the blood and circulates to the liver where it is hydroxylated by the cytochrome p450 enzyme CYP27A1 to calcidiol or 25-hydroxyvitamin D_3 [$25(OH)D_3$]. This form circulates in the blood and is measured in the serum as an indicator of vitamin D status. Calcidiol is converted to its bioactive form, calcitriol or 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], by the mitochondrial 1α-hydroxylase enzyme CYP27B1 in the kidney. $1,25(OH)_2D_3$ binds to a transcription factor called the vitamin D receptor (VDR). This steroid-hormone nuclear receptor binds to specific sites in the genome and interacts with cofactors to activate and/or repress the expression of target genes (*Mangelsdorf* et al., 1995; *Christakos* et al., 1996).

The renal synthesis of calcitriol is essential for efficient uptake of dietary calcium for bone health. A drop in circulating Ca^{2+} levels stimulates the production of parathyroid hormone (PTH) that, in turn, induces CYP27B1 expression by primary renal tubules. This increases $1,25(OH)_2D_3$ production, which activates Ca^{2+} transporter expression through the VDR in the small intestine. The increase in circulating Ca^{2+}, suppresses PTH production. In a negative feedback loop, activated VDR binds to the *CYP27B1* promoter and represses its expression. Also, VDR induces fibroblast growth factor-23 in osteocytes which inhibits secretion of PTH and inhibits CYP27B1 and stimulates CYP24A1 a mitochondrial enzyme that catabolizes both $1,25(OH)_2D_3$ and $25(OH)\ D_3$ to limit $1,25(OH)_2D_3$ levels and prevent hypercalcaemia (*Zierold* et al., 1995; *Saito* et al., 2003; *Paz* et al., 2007).

An abundance of epidemiological, clinical and basic research has highlighted the potential roles of vitamin D in preventing cancer, autoimmune disorders, cardiovascular disease and infections (*Grober* et al., 2013). The synthesis of calcitriol in non-renal tissues and cells is likely important for mediating these additional health benefits (*Hewison* et al., 2004). The extra-renal synthesis of $1,25(OH)_2D_3$ occurs in lung, colon, parathyroid glands, bone, skin and macrophages. In immune cells it is considered important for optimal immune response at sites of infection (*Hewison* et al., 2004).

VITAMIN D AND IMMUNITY

The historical connection between sources of vitamin D and successful treatment of tuberculosis highlighted an important early link with immune function. The connection of vitamin D to immunity was strengthened by the discovery that the VDR is expressed in T and B cells, monocytes, macrophages, dendritic cells (DCs) and neutrophils (*Bhalla* et al., 1983; *Provvedini* et al., 1983; *Mangelsdorf* et al., 1984; *Brennan* et al., 1987; *Kreutz* et al.,

1993; *Deluca* and *Cantorna,* 2001; *Takahashi* et al., 2002; *Adorini* et al., 2004;). $1,25(OH)_2D_3$ inhibits Th17 development, increases the *frequency* of Th2 and regulatory T-cells, decreases Th1 development, and modulates T-cell proliferation and cytokine expression (*Lemire* et al., 1995; *Penna* and *Adorini,* 2000; *Boonstra* et al., 2001; *Daniel* et al., 2008). $1,25(OH)_2D_3$ also promotes tolerance in dendritic cells and T-cells and inhibits B-cell differentiation into plasma cells (*Mathieu and Adorini,* 2002; *Adorini* et al., 2004; *Chen* et al., 2007). Overall vitamin D appears to mediate an anti-inflammatory response and promote tolerance in the adaptive response.

In addition to responding to circulating $1,25(OH)_2D_3$, dendritic cells, macrophages and T-cells actively metabolize $1,25(OH)_2D_3$ (*Hewison,* 2012). Extra-renal production of $1,25(OH)_2D_3$ by macrophages from some granulomatous disease patients was reported (*Barbour* et al., 1981; *Adams* et al., 1983). *In vitro* studies with non-disease macrophages suggested that CYP27B1 activity was induced as part of the normal immune response (*Koeffler* et al., 1985; *Reichel; Koeffler* and *Norman,* 1986). DCs confer specific homing properties upon T cells during the adaptive immune response. Sigmundsdottir and colleagues demonstrated the importance of local production of active vitamin D on T-cell homing to the skin. DCs derived from the skin were able to synthesize $1,25(OH)_2D_3$ from vitamin D_3. This induced expression of CC chemokine receptor 10 in T cells and suppressed expression of gut-homing receptors, which enabled T cells to migrate toward the chemokine CCL27 that is secreted by keratinocytes in the epidermis. Their findings support a model that DCs utilize locally produced vitamin D to program T-cell epidermal tropism (*Sigmundsdottir* et al., 2007).

The production of potentially high local levels of $1,25(OH)_2D_3$ could be important for intracrine and paracrine pathways that may influence the interactions between vitamin D, the immune system and pathogens (*Hewison,* 2012). During the mid-1980s, a link between vitamin D-deficiency and impaired immune defence to *Mtb* was proposed and it was demonstrated that both $25(OH)D_3$ and $1,25(OH)_2D_3$ increased the ability of human monocytes to control *Mtb* proliferation (*Davies,* 1985; *Rook* et al., 1986). Nearly 20 years later the mechanism for this increased killing by human monocytes/macrophages was elucidated. Several groups discovered that vitamin D increased expression of the cathelicidin antimicrobial peptide (*CAMP*) gene (*Wang* et al., 2004; *Gombart; Borregaard and Koeffler,* 2005; *Weber* et al., 2005). This vitamin D-mediated induction was subsequently shown to occur through activation of Toll-like receptor 2/1-signalling using the synthetic 19-KD *Mtb*-derived lipopeptide (*Liu* et al., 2006). Activation of macrophages induced CYP27B1 and VDR expression and increased conversion of $25(OH)D_3$ to $1,25(OH)_2D_3$. The increase in ligand activated VDR, in turn, induced CAMP. Liu and colleagues further showed in an *in vitro* assay that insufficient levels of serum $25(OH)D_3$ resulted in a lack of CAMP induction upon activation of macrophages, consistent with the observation that vitamin D-deficiency correlates with increased susceptibility to *Mtb* infection (*Liu* et al., 2006).

The human ß-defensin 2 or *DEFB4* gene was also identified as a vitamin D inducible antimicrobial peptide gene, but its induction by vitamin D or TLR2/1 activation was not as dramatic or robust as that of CAMP (*Wang* et al., 2004; *Liu* et al., 2006). Subsequent

work showed that co-treatment of monocytes with IL-1 and $1,25(OH)_2D_3$ induced *DEFB4*. This induction was concomitant with binding of both NF-κB and VDR to the *DEFB4* promoter (*Liu* et al., 2009). In the absence of $1,25(OH)_2D_3$, the intracellular pattern recognition receptor nucleotide-binding oligomerization domain protein 2 (NOD2) by muramyl dipeptide (MDP) activates NF-κB and there is a modest induction of the *DEFB4* gene (*Voss* et al., 2006; *Wang* et al., 2010); however, pre-treatment with $1,25(OH)_2D_3$ followed by MDP leads to a robust, synergistic induction of the *DEFB4* gene (Fig. 1B) (*Wang* et al., 2010). The MDP signal was amplified because $1,25(OH)_2D_3$ strongly induced expression of NOD2 in primary human monocytic and epithelial cells (*Wang* et al., 2010). Taken together, activation of the vitamin D pathway alone is not sufficient to induce robust expression of *DEFB4* and additional signalling pathways are required (*Liu* et al., 2009; *Wang* et al., 2010). It was further demonstrated that knockdown of either DEFB4 or CAMP expression decreased killing of *Mtb* by macrophages indicating their importance to fighting the infection (*Liu* et al., 2009). An important mechanism for killing *Mtb* appears to be the ability of vitamin D to induce autophagy and the requirement of CAMP induction to promote this process (*Hoyer-Hansen* et al., 2005; *Wang* et al., 2008; *Yuk* et al., 2009). Additional findings support a paracrine macrophage-lung epithelial cell signalling pathway that is driven by IL-1β and $1,25(OH)_2D_3$ (*Verway* et al., 2013). In this model, $1,25(OH)_2D_3$ increased IL-1β secretion in *Mtb*-infected macrophages. The secreted IL-1β induced *DEFB4* expression from airway epithelial cells, which enhanced control of *Mtb* growth in co-cultured macrophages *in vitro* (*Verway* et al., 2013). Taken together these studies support an important role for vitamin D in modulating the immune response to infection.

SKIN, VITAMIN D, ANTIMICROBIAL PEPTIDES AND BARRIER DEFENCE

The epidermis is comprised of four layers of keratinocytes at different stages of differentiation: the stratum basale (basal layer), stratum spinosum (spinous layer), stratum ganulosum (granular layer) and the stratum corneum (cornified layer). The basal layer contains stem cells that proliferate and differentiate into the cells of the upper layers. As the cells leave the basal layer they express keratins, involucrin and transglutaminase K in the spinous layer. Above this, the granular layer contains keratohyalin granules that contain profilaggrin, loricrin and lamellar bodies which dump their contents composed of lipids and antimicrobial peptides into the extracellular space between the granular and cornified layers to generate the permeability barrier of the skin (*Muehleisen* et al., 2012). The cornified layer is comprised of cells that form an impermeable outer layer of dead cells that creates a barrier to invasion. Disruption of the barrier triggers induction of antimicrobial peptides and CAMP is critical for protecting the skin against infection as demonstrated by enhanced susceptibility of the *Camp* knockout mouse to Group A *Streptococcus* (*Nizet* et al., 2001). The activation of the vitamin D pathway through TLR-signalling has been described in epithelial keratinocytes. Fol-

lowing wounding of the skin, the expression of TGF-β induces CYP27B1 to produce $1,25(OH)_2D_3$ and activate intracrine expression of CAMP and TLR2 to combat infections that could occur with epidermal injury (*Schauber* et al., 2007). TGF-β and $1,25(OH)_2D_3$ also induce expression of 5-lipo-oxygenase which catalyses the synthesis of leukotrienes in monocytes (*Harle* et al., 1998). Leukotrienes are involved in leukocyte chemo-attraction at sites of infection and phagocytosis of bacteria and trigger processing of hCAP18 to LL-37 by neutrophils (*Peters-Golden* et al., 2005; *Wan* et al., 2007). In addition, to directly killing pathogens, LL-37 recruits immune cells and contributes to neo-angiogenesis and wound healing (*Koczulla* et al., 2003; *Heilborn* et al., 2003).

The peptide LL-37 is the predominant form found in neutrophils, but it is a minor constituent of the forms found in and on the human skin and surface (*Yamasaki* et al., 2006). Processing of hCAP18 in the granular layer by kallikreins, both of which are stored in lamellar granules, allows release of the peptides into the cornified layer (*Yamasaki* et al., 2006). Another source of cathelicidin on the skin's surface is secretion of LL-37 from the eccrine gland in sweat and sebocytes (*Murakami* et al., 2002; *Lee* et al., 2008). The composition of the peptides may impact the antimicrobial versus host cell signalling properties of LL-37. The shorter peptides have more potent antimicrobial properties whereas the longer LL-37 form has more potent inflammatory properties through stimulating host immune cell receptors and inducing chemokine release and immune chemo-attraction (*Braff* et al., 2005). These "alarming" properties provide an additional mechanism by which vitamin D could modulate immune response (*Yang* and *Oppenheim,* 2004). Dysregulation of cathelicidin expression and processing has been implicated in atopic dermatitis, psoriasis and rosacea (*Lande* et al., 2007; *Yamasaki* et al., 2011; *Kopfnagel* et al., 2013). In psoriasis, the overexpression of LL-37 increases TLR9 activation in plasmacytoid DCs (pDCs) contributing to the development of disease. Paradoxically, an effective treatment is topical administration of vitamin D analogues which can induce *CAMP* gene expression in healthy skin (*Weber* et al., 2005). It has not been determined if topical vitamin D affects LL37 levels in psoriasis patients, but vitamin D does interfere with the capacity of pDCs to induce T cell proliferation and secretion of INF-γ, thus limiting inflammation (*Karthaus* et al., 2014).

The epidermis is the key source of vitamin D for the body and keratinocytes possess all of the machinery (enzymes CYP27A1 and CYP27B1) to metabolize vitamin D to its bioactive form, $1,25(OH)_2D_3$ (*Muehleisen* et al., 2012). Keratinocytes express the VDR and $1,25(OH)_2D_3$ regulates the proliferation and sequential differentiation of keratinocytes into the cells that form the upper epidermal layers (*Muehleisen* et al., 2012). Loss of CYP27B1 or VDR leads to hyper-proliferation of the basal layer and defects in differentiation of the upper layers, which ultimately abrogates permeability barrier formation and the immune response (*Panda* et al., 2001; *Dardenne* et al., 2001; *Xie* et al., 2002). The skin is capable of epidermal synthesis of $1,25(OH)_2D_3$ upon exposure to UVB rays (*Lehmann* et al., 2003). This could be biologically important for regulation of cell proliferation, differentiation and immunity in the skin. Indeed, Mallbris and colleagues demonstrated that exposure to a single dose of UVB, but not UVA significantly upregulated CAMP expression in the skin of eight healthy

fair-skinned volunteers (*Mallbris* et al., 2005). The induction of vitamin D target genes in the skin by UVB exposure could affect the immune status and barrier function of the skin. It also raises the question, what is the impact on the composition of the microbiota?

SUNLIGHT, VITAMIN D, CATHELICIDIN AND MICROBIOTA

Microbiota studies are most advanced with respect to the gut. The gut is home to roughly 10^{14} bacteria comprised of 500-1,000 distinct species (*Gill* et al., 2006). This complex community is critical for host metabolism, barrier function and crosstalk with the immune system (*Hooper* et al., 2002; *Turnbaugh* et al., 2006; *Hooper and Macpherson,* 2010). Intestinal homeostasis is essential for health of the host as disruption of this balance, or dysbiosis, is associated with chronic intestinal inflammation, disorders of the gut, obesity, metabolic syndrome (MetS) and impaired barrier defence and immunity (*Sartor,* 2008; *DuPont* and *DuPont,* 2011; *Tilg* and *Kaser,* 2011; *Tehrani* et al., 2012). Maintenance of gut homeostasis depends on adequate epithelial barrier defence that is affected by interactions between the immune system, microbiota and diet. The host innate immune system is a key component in maintaining a "good fence" between the host and its microbiota and knocking-out specific genes in mice has highlighted the role of innate immunity in determining the composition of these intestinal "neighbours" (*Salzman* et al., 2010; *Vijay-Kumar* et al., 2010; *Caricilli* et al., 2011; *Kellermayer* et al., 2011). Activation of TLRs promotes epithelial cell proliferation and secretion of IgA and AMPs into the gut lumen (*Abreu,* 2010). Mice lacking TLR-5 or TLR-2 develop many features of metabolic syndrome and this correlates with altered composition of the gut microbiota and transplantation of these microbiota into wild-type germ-free mice confers many of the hallmarks of metabolic syndrome to the recipients (*Vijay-Kumar* et al., 2010; *Caricilli* et al., 2011; *Kellermayer* et al., 2011). Nucleotide-binding, oligomerization domain 2 (NOD2)-deficiency leads to altered composition of the host microbiota, both in mice and in humans, which may explain NOD2's role in the aetiology of Crohn's disease (*Rehman* et al., 2011). TLR and NOD2 deficiency impacts a multitude of signalling pathways in the cell and there is a large gap in our knowledge of the molecular mechanisms downstream of TLRs that regulate the composition of the microbiota; Because AMPs directly kill microorganisms, they are ideal mediators to consider. Salzman and colleagues demonstrated that deficiency of Paneth cell defensins or expression of a human-specific Paneth cell defensin (α-defensin 5, *DEFA5*) in mice resulted in significant defensin-dependent changes in the composition of the microbiota (*Salzman* et al., 2010). There were significant losses in segmented filamentous bacteria in DEFA5-expressing mice and a reduction in interleukin-17 (IL-17)-producing lamina propria T-cells (*Salzman* et al., 2010). The role of CAMP in modulating the gut microbiota is not known, but we have compared the metabolomic profile of faecal material from wild type and CAMP knockout mice and identified differences in metabolism in bile acids, phospholipids and amino acids that involve gut microflora. These preliminary data suggest that loss of CAMP may affect the com-

position of the microbiota and thus, gut metabolism (our unpublished observations).

Like the gut, the skin has a thriving microbial ecosystem of diverse ecological niches from moist to dry areas, sebaceous areas and areas with varied densities of hair, skin folds and thicknesses. This varied geography leads to differences in microbiota composition in the different body regions (*Hannigan* and *Grice,* 2013). Changes in microbiota composition are associated with these diseases and may contribute to the disease condition. *Staphylococcus aureus* colonization of the skin is associated with atopic dermatitis and treatment with antimicrobials decreases severity, but a microbial cause is not clear (*Huang* et al., 2009). It has been observed that a decreased bacterial diversity correlates with disease severity (*Kong* et al., 2012). In psoriasis, decreased representation of *Propionibacterium* and increased representation of Firmicutes in plaques is observed when compared to normal or uninvolved skin (*Fahlen* et al., 2012). The connection between aberrant AMP expression, shifts in the microbiota composition and disease pathology remains to be elucidated.

Altered host immune function is associated with changes in the skin microbiome. In patients with primary immunodeficiency diseases that share the hallmark of atopic dermatitis, the skin displayed increased permissiveness with altered microbial population structures, decreased site specificity and colonization with species not found on controls. Increased fungal diversity and the increased presence of *Candida* and *Aspergillus* were consistent with the increased susceptibility of these patients to fungal infections (*Oh* et al., 2013). In mice, the systemic inhibition of complement signalling led to significant changes in the skin microbiota (*Chehoud* et al., 2013). This was concomitant with a decrease in pattern recognition receptors, antimicrobial peptides, cytokines and chemokines. These findings highlight the importance of host-microbe interactions in skin homeostasis. They also demonstrate that changes in host immunity can lead to changes in the composition of the microbiota. Considering the effect of vitamin D on adaptive and innate immune responses, exposure of the skin to sunlight could lead to changes in host immunity and in turn impact the composition of the microbiota.

UVB exposure either through sunlight or artificial light causes immune suppression in the skin (*Field* et al., 2005). Phototherapy containing UVB rays is used to treat both inflammatory conditions of atopic dermatitis and psoriasis. In the case of atopic dermatitis this can lead to a reduction of infection. In both conditions, a reduction in inflammation is observed with an increase in regulatory T cells and improvement of the epidermal barrier and restoration of cutaneous homeostasis (*Tartar* et al., 2014). As dysregulation of CAMP has been implicated in these disorders, it would be of great interest to determine if regulation of CAMP by vitamin D synthesized in the skin contributes to the restoration of homeostasis. It has been demonstrated that oral vitamin D supplements increased CAMP expression in the skin of atopic patients; however, improvement in barrier defence or changes in microbiota composition were not determined (*Hata* et al., 2008). Narrow-band UVB treatment of children with atopic dermatitis and controls with vitiligo caused a decrease in cutaneous *Staphylococcal* populations and in atopic patients the number of *S. aureus* strains that produced toxin were reduced to the levels found on the controls. This study

shows that UVB can cause shifts in the microbial composition of the skin, but did not address mechanism (*Manco* et al., 2006). The immune suppression caused by UVB is counterintuitive to the reduced infections in patients. The fact that UVB also induces CAMP expression may decrease inflammation while boosting the innate immune system and protecting the skin from infection (*Mallbris* et al., 2005; *Zasloff,* 2005).

THE VITAMIN D-CATHELICIDIN PATHWAY IS HUMAN AND PRIMATE-SPECIFIC

As discussed previously, the use of transgenic and knockout mouse models for immune mediators has provided insight between the interactions of the host and its microbiota, both in terms of host genes that impact the composition of the microbiota and how the microbiota impacts the host immune system. The vitamin D-cathelicidin pathway is human and primate-specific and not conserved in mice and other mammals (*Gombart* et al., 2005, 2009). Therefore, it would be difficult to model in animals. We generated a transgenic mouse line that carries a genomic copy of the human gene (manuscript in preparation). This mouse was then crossed on to the CAMP knockout mouse background (*Nizet* et al., 2001) to create a mouse that carries only the human CAMP gene. The human CAMP gene is expressed in epithelial barrier tissues and responsive to $1,25(OH)_2D_3$ *in vitro* and *in vivo* (manuscript in preparation).

We and others have identified an important difference between humans and mice in regards to vitamin D metabolism. It was recently reported that mouse macrophages do not express CYP27B1 even with LPS stimulation as observed in humans (*Kapetanovic* et al., 2012; *Ooi* et al., 2014). In mice, the source of CYP27B1 in the immune system was reported only in CD8+ T cells (*Ooi* et al., 2014). We have observed this lack of CYP27B1 expression in our "humanized" mouse model (manuscript in publication). Also, we found that it is not possible to induce vitamin D target genes like human CAMP with TLR activation in mouse macrophages in the presence of $25(OH)D_3$, although this occurs in humans as described previously (*Liu* et al., 2006). These findings would argue that utilization of vitamin D by the mouse immune system is very different from humans and that the mouse is not an ideal model with respect to vitamin D and the immune system. Nevertheless, the *CYP27B1* gene been reported to be expressed in the human and mouse skin (*Zehnder* et al., 2001; *Flanagan* et al., 2001; *Bikle* et al., 2004) with one report of no expression (*Kutuzova* et al., 2006). If the vitamin D pathway is better conserved between mice and humans in the skin, then we believe that our model could be useful for understanding the role of vitamin D and CAMP in skin barrier function and interactions with the microbiota. The mouse *Camp* knockout and "humanized" *CAMP* mouse should allow investigators to determine if the *CAMP* gene is important in determining the composition of the skin microbiota and may shed light on the role of sunlight and vitamin D on the health the skin and its commensals.

CONCLUSION

The role of sunlight and vitamin D on the form and function of the skin involves numerous mechanisms. It is clearly important in the differentiation of keratinocytes into the different layers that form the epidermis and the epidermal permeability layer. The homing of immune cells, the degree of inflammation and the expression of AMPs are all controlled by the vitamin D pathway. Wounding activates the vitamin D pathway and the up-regulation of pathogen sensors and the cathelicidin antimicrobial pathway are keys in fighting infection and promoting healing. The capacity of the skin to synthesize bioactive $1,25(OH)_2D_3$ and increase expression of the cathelicidin suggests that sun exposure may play a role in determining the composition of the microbiota; however, this remains to be determined. Animal models are not adequate as the vitamin D-cathelicidin pathway is conserved only in humans and non-human primates; therefore, either "humanized" mouse models or studies in humans are needed. We have developed a "humanized" cathelicidin transgenic mouse, but important differences between humans and mice with respect to the use of vitamin D by the immune system could limit its utility. Elucidating the importance of sunlight, vitamin D and its impact on the skin microbiome will require careful studies and recognition of limitations in both model systems.

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STAPHYLOCOCCUS AUREUS AND THE SKIN MICROBIOME IN ATOPIC DERMATITIS

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SUMMARY

The knowledge accumulated in our understanding of atopic dermatitis has been acquired with progress in epidemiology, genomics and immunology. In this context, the reputed role of microbial organisms such as *Staphylococcus aureus* has always been regarded according to their pathogenic characteristics. More recently however, due to modern metagenomic analysis, it became evident that skin is colonized by a dense community of commensal microorganisms of which the diversity may be key for a normal skin. Thus, a new picture emerged, in which a balanced and diversified microbiome seems to engage in a dialogue with epithelial cells to control the growth of potential pathogens. In this context, the Toll-like receptor 2 (TLR2) seems to represent a pivotal structure for the recognition of microbial signals. TLR2 may also be critical for mounting an adequate Th17 driven antimicrobial response in the skin. Although we are just starting to explore und understand this complex interaction, it is speculated that a directed manipulation of the skin microbiome, aimed at restoring the default diversity could represent an interesting therapeutic approach

INTRODUCTION

Atopic dermatitis is a genetic complex disease, which display a highly complex phenotype (*Bieber*, 2008). In the recent years, progress in our understanding the pathophysiology of this disease has forwarded two main aspects, which seem to be mirrored by recent genetic inside. Indeed, on one hand this disease evolves on the background of an intrinsic epidermal barrier defect which is genetically determined (*Irvine* et al., 2011) and on the other hand is tightly related to a chronic inflammation (*Gittler* et al., 2013) emerging on the background of the so called atopic sensitization, i.e. IgE-mediated allergic reactions (*Wu* and *Zarrin*, 2014). From an immunological point of view, the initial Th1 versus Th2 dogma, which has dominated our understanding of the pathophysiology of atopic dermatitis, has more recently evolved in a more diverse landscape including the role of regulatory T-cells, Th22-cells and Th17-cells (*Eyerich* and *Novak*, 2013). The latter cells have been in the focus of our interest in the last years since they have been recognized as pivotal in immunity against *Staphylococcus aureus* (*Lee* et al., 2010; *Miller* and *Cho*, 2011). Indeed, this bacterium has been known since many years to be responsible for a substantial colonization of the involved as well as the uninvolved skin of atopic dermatitis (*Hauser* et al., 1985; *Boguniewicz*, 2012). Thus, although atopic skin is reproducibly heavily colonized by *S. aureus*, his real pathophysiological role remains elusive.

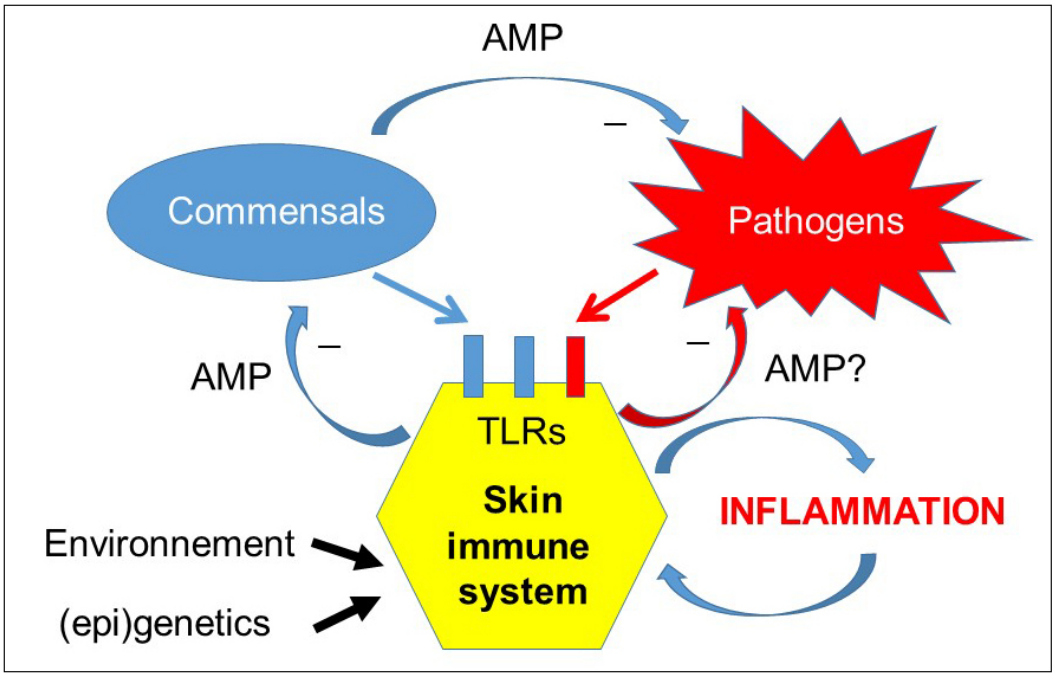

Figure 1: The cross-talk between the commensal, the putative pathogen bacteria and the skin immune system. The microbiomic balance on the skin: a good working "ménage à trois" (AMP = anti-microbial peptides; TLR = Toll-like receptors)

STAPHYLOCOCCUS AUREUS IN THE "PRE-MICROBIOMIC ERA"

So far, the observation of the colonization with *S. aureus* in atopic dermatitis has generated a number of hypotheses with regard to its potential pathophysiologic role in the initiation and maintenance of the chronic inflammation. Many in vitro and animal models have highlighted the numerous possible immunological impact points of *S. aureus* and their products on the skin immune system (*Higaki* et al., 1986; *Lever* et al., 1988; *Neuber* et al., 1991; *Hofer* et al., 1995; *Strange* et al., 1996; *Herz* et al., 1998; *Leung* et al., 1998; *Bunikowski* et al., 1999; *Morishita* et al., 1999; *Lin* et al., 2000; *Matsui* et al., 2000; *Zollner* et al., 2000; *Hikita* et al., 2002; *Matsui* and *Nishikawa*, 2002; *Wedi* et al., 2002; *Heaton* et al., 2003; *Lehmann* et al., 2004; *Breuer* et al., 2005; *Cardona* et al., 2006; *Langer* et al., 2007; *Machura* et al., 2008). Following mutually non-exclusive acting points have been identified so far: (i) polyclonal stimulation of T-cells (*Hemady* et al., 1983; *Skov* and *Baadsgaard*, 1995; *Strickland* et al., 1999), (ii) induction of *S. aureus*-specific IgE (*Walsh* et al., 1981; *Abramson* et al., 1982; *Henocq* et al., 1982; *Friedman* et al., 1985; *Nordvall* et al., 1992; *Bunikowski* et al., 1999; *Lin* et al., 2000; *Rossi* et al., 2004), (iii) induction of cytokines and chemokines from keratinocytes (*Vu* et al., 2010; *Takai* et al., 2014), (iv) induction of corticoid resistance by T-cells, (v) induction of homing receptor CLA on migrating T-cells in the skin (*Torres* et al., 1998).

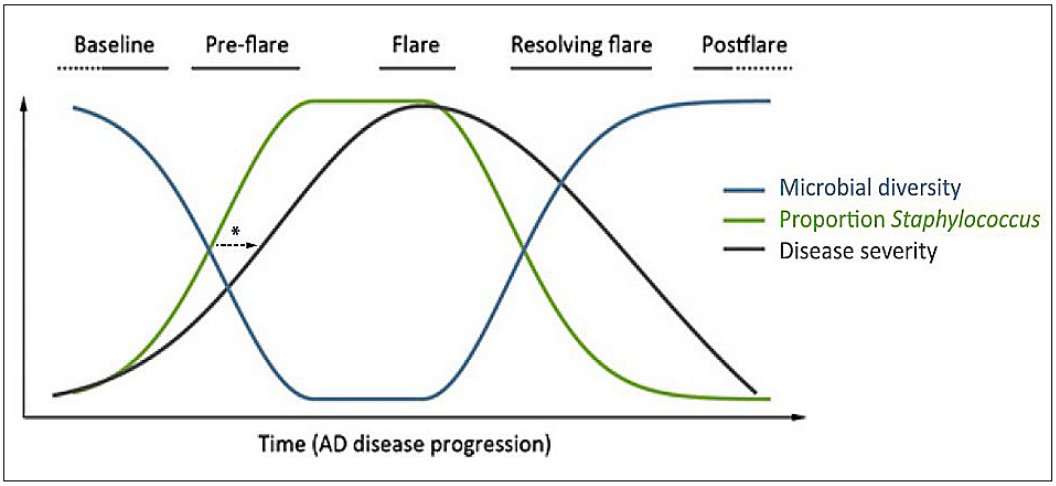

Figure 2: A putative temporal scenario of the microbiomic diversity and staphylococci growth with regard to the evolution of a flare in atopic dermatitis (from: Kong et al., 2012).

All these effects can act in concert to induce and possibly maintain a chronic inflammatory reaction in atopic skin. Moreover some *in vivo* experiments have shown that the application of *S. aureus* in patch test model on the skin of atopic dermatitis can induce an allergic reaction or inflammation similar to that observed in the disease itself (*Strange* et al., 1996; *Langer* et al., 2007). However, our view of the role of microbial agents on the skin was mainly dictated by the limited information provided through conventional technics in culturing skin-derived microbes. Indeed only one to 2% of the overall microbiomic colonization of the skin was evaluated by these conventional culture technologies.

STAPHYLOCOCCUS AUREUS IN THE "MICROBIOMIC ERA"

More recently, in the context of the human microbiome project (HMP), high throughput technologies based on 16 S RNA gene analysis have shown the tremendous diversity and a completely different picture of the microbiome resident in our body and on normal skin as well as the changes observed during different skin conditions (*Grice* et al., 2009; *Group* et al., 2009; *Costello* et al., 2009). Thus, a new picture emerges where the microbiome is now considered as an integral part of the epidermal barrier on the skin and the diversity of the microbiome seems to be key to understand the normal physiology and pathophysiology of this organ as well as for those of most other organs (*Kuo* et al., 2013a). To summarize this highly complex field of research, it can currently be postulated that normal skin is the product of a steady cross-talk between the skin immune system, the commensal microbes and the potentially pathogens on the skin, e.g. a kind of good working "ménage à trois" (*Zeeuwen* et al., 2012, 2013; *Nakatsuji* et al., 2013; *Grice*, 2014) (Figure 1). Thus, the high diversity of the skin microbiome seems to be key for a healthy skin. Most importantly, the so-called normal skin in

atopic dermatitis already harbours a reduced diversity of the microbiome, which is dominated by *S. aureus* as expected from the conventional culture technologies (*Kong* and *Segre*, 2012; *Kong* et al., 2012; *Oh* et al., 2012; *Chen* and *Tsao*, 2013). However, in the context of lesional skin, this altered diversity is even worsening and could precede the appearance of flares (*Kong* et al., 2012) (Figure 2). According to this hypothesis, the growth of *S. aureus* could indeed have a strong impact on the generation of flares and/or the perpetuation of chronic inflammation. However, we have completely underestimated the role of the other microbes present on the skin such as *Acinetobacter* or *Staphylococcus haemolyticus*. Indeed commensal microbes are in "steady dialog" with the potential pathogens and seem to control their growth by producing anti-microbial peptides. Among them, *Acinetobacter* also may display protecting effects against sensitization through the skin as well as allergic inflammation (*Fyhrquist* et al., 2014).

However, the reasons for this decreased diversity in the microbiome on normal looking and uninvolved atopic skin remains elusive. When considering the two main aspects in the pathophysiology of the disease, one may argue that the genetically driven intrinsic changes in the barrier function themselves could be pivotal for the overall changes in the microenvironment and favour the preferential growth of *S. aureus* under these conditions. This genetic control of the skin microbiome is most likely to that observed for the gut microbiome (*Goodrich* et al., 2014). On the other hand, it appears that in patients with atopic dermatitis, in contrast to other diseases such as psoriasis, an adequate Th17 response may be missing in the skin (*Toda* et al., 2003; *van Beelen* et al., 2007; *Eyerich* et al., 2009; *Auriemma* et al., 2013). Therefore, it can be speculated that the adaptive immune system in these individuals fails to shape and mount an efficient immune response against *S. aureus* (*Hayashida* et al., 2011). When considering the immune phenomenons underlying an appropriate Th17 response (*Miller* and *Cho*, 2011), recognition structures of the innate immune system such as Toll-like receptors (TLR) could play a key role in this context. Indeed, preliminary experiments in our group have shown that TLR2 is significantly downregulated on epidermal dendritic cells but not on keratinocytes in atopic individuals (*Iwamoto* et al., submitted). Moreover, these cells seem unresponsive to TLR2-ligands and are finally not able to induce a correct Th17 response. Whether this alteration in the dendritic cell biology is intrinsic to this lineage and/or the results of a peculiar microenvironment in the skin potentially induced by microbiomic signals is currently under investigation.

On the other hand, the possible role of TLR2 in atopic dermatitis has been highlighted many years ago in the context of single nuclear polymorphism of this structure reported in atopic individuals (*Prescott* et al., 2008; *Kormann* et al., 2009; *Oh* et al., 2009; *Liu* et al., 2011; *Potaczek* et al., 2011; *Fuertes* et al., 2013). Moreover, there are some conflicting results reported about the functionality of TLR2 expressed on keratinocytes or monocytes in patients suffering from atopic dermatitis (*Hasannejad* et al., 2007; *Sumegi* et al., 2007; *Niebuhr* et al., 2010; *Vu* et al., 2010; *Kuo* et al., 2013b; *Takai* et al., 2014). In any case, TLR2 has been recognized as a central recognition structure potentially involved in mechanisms leading to downregulation of atopic inflammation as shown in animal models.

CONCLUSION

Recent progress in our understanding of the microbiome on the skin in atopic dermatitis has substantially changed our view on the potential role of *S. aureus* in this condition. Moreover, the pivotal role of TLR2 on dendritic cells and their putative involvement in the induction of adequate Th17 response is now in the focus of our interest. Whether the microenvironment in atopic dermatitis provides a particular niche for the growth of *S. aureus* or inversely *S. aureus* induces a particular microenvironment in the skin of these patients remains unclear. Furthermore, the possibility that the microbiome on the skin has not only an impact on the immune system but also may display some impact on the epigenetic regulation in the skin has to be considered for our understanding on the natural history of this disease. Finally, there is growing evidence that the alteration of the diversity of the microbiome on the skin represents an interesting target for new approaches aimed at a manipulation and/or correction of the diversity, potentially associated with the use of microbiome-derived compounds able to prevent or to correct the sensitization and the chronic inflammation in these individuals.

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IMMUNOPATHOGENESIS OF PSORIASIS: ANTIMICROBIAL PEPTIDES TAKE CENTRE STAGE

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SUMMARY

Psoriasis is a chronic-relapsing inflammatory skin disease characterized by the aberrant activation of dendritic cells that stimulate pathogenic Th17 cells. Two main unsolved issues in the field are the nature of the initial trigger factor for the pathogenic Th17 cascade and the nature of the auto-antigen recognized by these Th17 cells. We found that plasmacytoid dendritic cells (PDC) and their activation to produce type I IFN are critical initial events that drive the development of Th17-mediated inflammation in psoriasis. Activation of PDC is driven by antimicrobial peptides such as LL37, hBD2, hBD3 and Lysozyme, which are overexpressed in psoriatic skin and which can trigger activation of TLR7 and TLR9 in PDC by forming complexes with self-nucleic acids released into the extracellular environment during cell turnover. In addition, we found that the anti-microbial peptide LL37 represents a direct auto-antigen recognized by pathogenic Th17 cells in psoriasis. Thus antimicrobial peptides are on one hand the innate trigger of dendritic cell activation, and, on the other hand, represent the antigenic stimulus and target for pathogenic Th17 cells in psoriasis.

PSORIASIS: A CHRONIC INFLAMMATORY DISEASE OF THE SKIN MEDIATED BY TH17 CELLS

Psoriasis is a common chronic inflammatory skin disease that affects 2 to 3% of the worldwide population (*Lowes* et al., 2007; *Griffiths* and *Barker*, 2007; *Nestle* et al., 2009). In its most prevalent form plaque psoriasis manifests as scaly erythematous plaques that may cover large body areas (Figure 1). Over the past years it has become clear that plaque psoriasis is mediated by T cells producing high levels of Th17 cytokines (*Zheng* et al., 2006; *Zaba* et al., 2007; *Lowes* et al., 2008). The pathogenic Th17 cells are stimulated in the dermis by aberrantly activated conventional dendritic cells producing TNF-α and IL-23 and subsequently migrate into the epidermis where they recognize a yet unknown auto-antigen. As a consequence, pathogenic Th17 cells produce IL-17 and IL-22, which are directly responsible for the keratinocyte hyperproliferation and the development of the psoriasis plaque. The pathogenic role of the Th17 cells in psoriasis is now validated by mouse model of psoriasis, the efficacy of targeting IL-23 or IL-17, and the discovery of genetic polymorphism in the IL-23A and IL-23R genes associated with the development of psoriasis (*Zheng* et al., 2006; *Zaba* et al., 2007; *Lowes* et al., 2008).

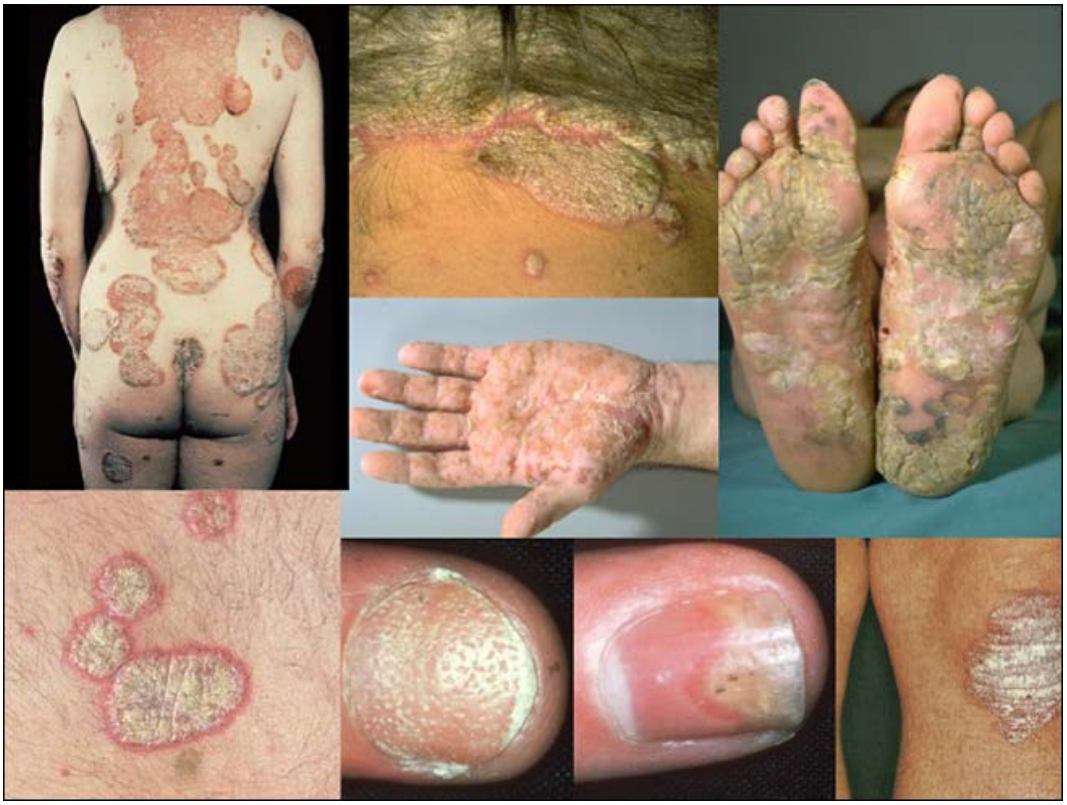

Figure 1: Skin manifestations of psoriasis.

ROLE OF PLASMACYTOID DENDRITIC CELLS AND TYPE 1 INTERFERONS IN THE INITIATION OF PSORIASIS

The role of innate events initiating the pathogenic Th17 cell cascade in the psoriatic skin has been poorly investigated. In 2003 we made an interesting clinical observation in a patient treated topically with the TLR7 agonist imiquimod (Aldara™) for what was thought to be a bowenoid keratosis. After 10 weeks of treatment, the patient showed an enlargement of the lesion along with surrounding satellite lesions consistent with the development of a psoriasis plaque (*Gilliet* et al., 2004). Toll-like receptor 7 (TLR7) is an endosomal receptor for viral single-stranded RNA that is specifically expressed by a subset of human dendritic cells called plasmacytoid dendritic cells (PDC) (*Gilliet* et al., 2008). PDCs are key effectors in antiviral immunity because they express TLR7 along with TLR9, a specific receptor for viral DNA. Upon viral infection, PDC expressing TLR7 and TLR9 sense viral RNA and DNA when brought into the endosomal compartment during the process of infection (*Gilliet* et al., 2008). In response to TLR7 and TLR9 activation, PDC's produce large amounts of type-I IFN (approximately 100 fold than any other cell type of the human body). Type-I IFN produced by PDC provide cell resistance to viral infection but also critically shape antiviral immune responses by maturing conventional dendritic cells expanding memory T cells and

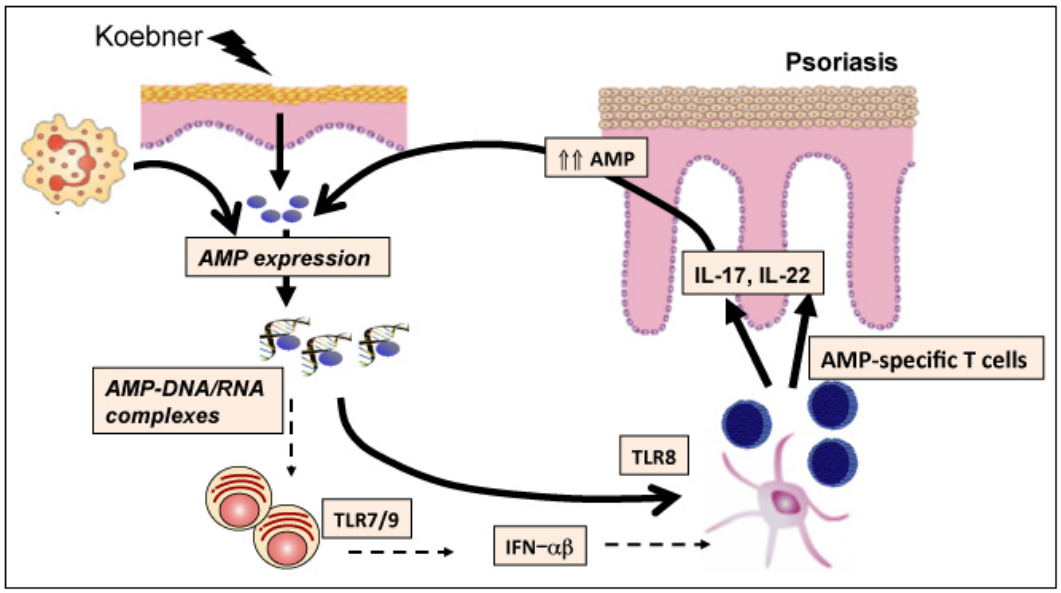

Figure 2: Immunopathogenesis of psoriasis.

activating cytotoxic NK cells (*Gilliet* et al., 2008). Whereas PDC are absent in healthy skin under homeostatic conditions, large numbers of PDC infiltrating the dermis were found in imiquimod treated skin (*Gilliet* et al., 2004) and in developing psoriatic skin lesions (*Nestle* et al., 2005). Not only were these PDC accumulating in developing psoriatic skin lesions, but they were also found to produce large amount of type-I IFN (*Nestle* et al., 2005). The role of PDC and type-I IFN's in the pathogenesis of psoriasis was assessed in a xenotransplant model of human psoriasis that is based on transplantation of un-involved skin of a psoriatic patient on an immunosuppressed (AGR 129) mouse. In this model, the engrafted human skin developes spontaneously into a fully-fledged psoriatic plaque within 35 days upon transplantation, a process mediated by resident Th17 cells. Injection of either neutralizing anti-IFNAR (type I IFN receptor) antibodies or an anti-BDCA2 antibody, which targets specifically human PDC's and blocks their ability to produce type-I IFN, completely inhibited the Th17 cell-dependent development of psoriasis (*Nestle* et al., 2005), indicating that PDC and their activation to produce type-I IFN is an upstream event in the immunopathogenesis of psoriasis (Figure 2).

ANTIMICROBIAL PEPTIDES DRIVE THE ACTIVATION OF PLASMACYTOID DENDRITIC CELLS TO PRODUCE TYPE-I INTERFERONS IN PSORIASIS

But how are PDC's activated to produced type-I IFN in psoriasis, a chronic inflammatory disease not linked to viral infection? To address this question we used fractions derived from HPLC of psoriatic scales to activate

PDC's isolated from peripheral blood. These experiments, followed by extensive biochemical characterization of the IFN inducing fractions using mass spectrometry and sequencing, allowed us to identify lL-37, human β-defensin 3, human β-defensin 2 and lysozyme as activators of PDC's (*Lande* et al., 2007, 2014a). These PDC activators are all cationic antimicrobial peptides, endogenous antibiotics that are normally not expressed in normal skin but produced by keratinocytes or released by infiltrating neutrophils in injured skin. A common feature of these antimicrobial peptides is their cationic and amphiphatic structure, which allows them to associate with bacterial membranes and to form pores in them, thus allowing killing of the microbe (*Zasloff*, 2002). In psoriasis LL-37, hBD2 and hBD3, as well as lysozyme are overexpressed throughout all epidermal layers but some staining can be found in the dermal compartment, where PDC's are located (*Lande* et al., 2007, 2014a), suggesting their role in the activation of PDC's and in the triggering of psoriasis. But how can antimicrobial peptides activate PDC's? PDC's are activated by viral RNA and DNA brought into TLR7 and TLR9 containing endosomal compartments during the infectious process. By contrast, self-RNA and self-DNA released in the context of cell turn-over are unable to activate PDC's because these nucleic acids are rapidly degrading in the extracellular environment and therefore fail to be internalized by PDC's. We found that, via their positive charges, antimicrobial peptides can form complexes with extracellular self-DNA and self-RNA fragments and protect them from extracellular degradation (*Lande* et al., 2007, 2014a; *Ganguly*, 2009). These complexes acquire net positive charges, which allows them to associate with anionic proteoglycans in the membrane of PDC's. As a consequence the nucleic acid complexes are endocytosed reaching intracellular TLR7 and TLR9 compartments, where they trigger the production of type I IFN's. Thus, cationic antimicrobial peptides IL-37, hBD2, hBD3, and lysozyme can break innate tolerance to self nucleic acids and lead to innate immune activation of PDC's via TLR7 and TLR9. Interestingly, we also found that RNA-AMP complexes can directly activate conventional DC's via TLR8 (*Ganguly*, 2009). Based on these findings, we propose the following model: antimicrobial peptides, induced during mechanical stress of the skin (Koebner phenomenon) form complexes with extracellular self-nucleic acids. These complexes trigger activation of TLR7 and TLR9 in plasmacytoid dendritic cells to induce type-I IFN and/or TLR8-expressing conventional dendritic cells that directly stimulate activation of pathogenic Th17 cells (Figure 2).

OVEREXPRESSION OF ANTIMICROBIAL PEPTIDES DRIVES SUSTAINED PLASMACYTOID DENDRITIC CELL ACTIVATION, LEADING TO PSORIASIS

In healthy individuals, skin injury is linked to a well-controlled and transient activation of PDC's by AMP-nucleic acid complexes, which promotes short-term inflammation and wound re-epithelialization (*Gregorio* et al., 2010). In psoriasis patients, skin injury leads to an exaggerated and persistent activation of PDC's due to an overexpression of AMP's. This leads to chronic inflammation, which ultimately drives epidermal hyperproliferation and develop-

ment of the psoriatic plaque (*Lande* et al., 2010). But what drives the constant overexpression of antimicrobial peptides in psoriasis? One important factor is the high levels of Th17 cytokines in the plaque, which contribute to the constant activation of keratinocytes to produce antimicrobial peptides. Indeed both IL-17 and IL-22 alone or in combination have been shown to trigger expression of antimicrobial peptides in keratinocytes (*Wolk* et al., 2004; *Liang* et al., 2006). Another interesting element is the identification of human β-defensin copy number polymorphism associated with the development of psoriasis, providing a genetic basis for the AMP overexpression in psoriasis (*Hollox* et al., 2008).

ANTIMICROBIAL PEPTIDES AS AUTO-ANTIGENS RECOGNIZED BY PSORIATIC T-CELLS

Because antimicrobial peptides are taken up by dendritic cell subsets we asked ourself whether these antimicrobial peptides could serve as auto-antigens and are presented to auto-immune T-cells in the psoriatic plaque. To address this question we used peripheral blood mononuclear cells from 52 psoriatic patients and stimulated them with either LL-37 or a scrambled form of the LL37 peptide. We found in approximately 40% of the patients that LL-37 induced a T-cell proliferation which was not present in control populations including healthy donors, scleroderma patients, erysipelas patients and atopic dermatitis patients (*Lande* et al., 2014b). LL-37 reactive T-cells did not only proliferate, but did also produce IFN-γ, and TH17 cytokines, IL-17 and IL-22 (*Lande* et al., 2014b). LL-37 reactive T-cells were both of CD4 and CD8 phenotype. Several CD4 and CD8 T-cell lines and clones were obtained and MHC restriction was demonstrated. Interesting, HLA-Cw6, that is found in 50% of psoriasis patients and is highly associated with the development of psoriatic disease, was found to be an excellent binder of LL-37. We also generated tetramers, which were able to detect LL-37 specific T-cells in the circulation of psoriasis patients (*Lande* et al., 2014b). A significant correlation between the presence of circulating IL-37 specific T-cells and the disease activity was observed. In addition, about 80% of patients with severe psoriasis (PASI >10) displayed circulating IL-37 specific T-cells. These findings alone with the fact that LL-37 specific T-cells produce pathogenic TH17 cytokines and are present in skin lesions suggests that LL-37 specific T-cells may be pathogenic T-cells in psoriasis. Accordingly, patients undergoing disease remission during anti-TNF treatment displayed decreased proliferative activity and tetramer staining of their LL37-specific T cells. Furthermore, LL37-specific T cells lost skin homing receptors CCR10, CLA, CCR6, and their ability to produce IL-17 and IL-22 (*Lande* et al., 2014b). More recent *in vivo* mouse studies, based on the repetitive injection of antimicrobial peptides into mouse skin, demonstrated a direct pathogenic role of AMP-specific T-cells. The injection of AMP induced the expansion of AMP-specific T cells in the skin and the development of a psoriatic phenotype, which was entirely T cell dependent.

CONCLUSION AND OPEN QUESTIONS

Cationic antimicrobial peptides are endogenous antibiotics that are expressed in injured skin. In addition to providing protection against microbial invasion we identified a unique pro-inflammatory function of these peptides through their ability to transport self-nucleic acids into intracellular compartment and promote activation of nucleic acid-recognizing Toll-like receptors. In psoriasis, cationic antimicrobial peptides LL37, hBD2, hBD3 and lysozyme are overexpressed by keratinoytes and drive a constant activation of dendritic cells including plasmacytoid dendritic cells producing type-I IFN and conventional dendritic cells producing IL-23 and TNF (Figure 2). On the other hand antimicrobial peptides may also represent auto-antigens presented by the activated dendritic cell subsets to autoimmune T cells, leading to their activation and expansion (Figure 2). Activated antimicrobial peptide-specific T cells may then migrate into the epidermis where they recognize the antimicrobial peptide expressed by keratinocytes and produce IL-17, IL-22 leading to the typical epidermal hyperproliferation that drives psoriasis (Figure 2).

A number of key questions remain to be answered. First, what is the structural requirement of antimicrobial peptide to be immunogenic and are all the antimicrobial peptides that drive activation of dendritic cells also T cell autoantigen? Second, do antimicrobial peptide-specific T cells escape central tolerance, or are these low-affinity T cells that are stimulated in the periphery in the context of a strong innate activation of dendritic cells? Third, what are the exact mechanisms underlying the concerted overexpression of several antimicrobial peptides in psoriasis? Is there a role for the microbiome? Finally, how can we target a broad range of antimicrobial peptides to block their immunogenicity for the treatment of psoriasis?

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THE NERVOUS SYSTEM AND IMMUNITY OF THE SKIN

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INTRODUCTION

One might imagine that the central nervous system (CNS) could play an important role in the defence of the skin. The skin and the underlying dermis are densely innervated by afferent and efferent neurons. The CNS can detect and map a site of injury on the skin surface through its afferent circuits, and deliver a neutrally mediated response to that site through its efferent arms. Furthermore, once an injury is perceived by the CNS anywhere on the skin surface, the nervous system could activate defensive signals throughout the body, including uninjured skin in anticipation of potential downstream consequences of microbial spread from the site of injury. Finally, the nervous system can act within seconds, faster than a microbe can propagate.

I believe the role played by the nervous system in the immunity of the skin can be appreciated through a careful analysis of two human diseases, infected ulcers on the feet of the diabetic, and erysipelas, superficial skin infection caused by Group A *Streptococcus.* In diabetes, the nervous system fails to protect the skin, while in erysipelas, the nervous system is being put on high alert. Here, I will tease out the role of the nervous system in the pathophysiology of each of these conditions, highlighting unappreciated immune functions of the nervous system, some of which are amenable to therapeutic intervention in these and other human diseases.

NEURAL DISCHARGE OF ANTIMICROBIAL PEPTIDES FROM GRANULAR GLANDS ON THE FROG'S SKIN

The skin of most species of frog contains granular glands, secretory structures that release a cocktail of defensive substances onto the surface of the animal's skin (*Zasloff*, 1987, 2002). The substances include antimicrobial peptides that constrain the skin microbiome, along with compounds that dissuade macroscopic predators. The glands are innervated by adrenergic nerves, which when stimulated by pain (or an electrical discharge) will cause the granular glands to empty their contents onto the skin surface. Indeed, in these animals the application of either adrenaline or nor-adrenaline directly onto the skin surface results in an almost immediate covering of the skin surface by a viscous hydrophobic secretion containing concentrations of antimicrobial peptides sufficiently high to kill almost any microorganism the frog would face at the site of an open skin wound. Thus, in the frog we see a straightforward example of how the nervous system can be engaged to provide immune defence of the skin.

THE INFECTED DIABETIC FOOT ULCERS

The diabetic is at risk of developing life threatening infected wounds on the skin of the lower extremities. The wounds appear initially as clinically unremarkable erythematous lesions, progress to superficial non-healing ulcers, and then can progress to deep, soft tissue wounds that penetrate the underlying bones. Gangrene and subsequent amputation of portions of the foot occur in about 50,000 diabetics annually, despite our awareness of the prevalence of the condition, and adequate management of the metabolic aspects of diabetes.

Why do wounds such as these occur in the diabetic foot? The "textbook" explanation places the proximate cause on the loss of sensory nerve functions that occurs as the diabetic ages. Normal levels of pain are not perceived in the lower extremities and, as a consequence, injury to the skin and soft tissues are not recognized and are untreated. In addition, the diabetic loses the usual moment-to-moment positional redistribution of weight bearing on the surfaces of the feet that occurs subconsciously in response to sensory input, leading to pressure-induced impaired vascular perfusion ("pressure sores") in certain areas of the foot that bear weight chronically. Accordingly, physicians advise individuals with diabetes to regularly examine their feet and to wear footwear that permits distribution of weight as evenly as possible across the surface of the foot.

What remains poorly understood is the underlying pathophysiology of the infections that occur in these wounds. The infections are poly-microbial with no specific pathogen identified as responsible for the progressive, invasive destructive process that descends from the initial superficial skin erosions through the dermis and muscle and then into bone (*Ge* et al., 2001). The wounds are often not particularly inflamed. Antibiotic treatment generally involves chronic systemic therapy and frequently fails to control the infection.

Recent discoveries on the mechanisms by which antimicrobial peptides are expressed and regulated on human skin suggest novel roles of the nervous system in skin immunity, and new insights in the pathophysiology of the diabetic foot ulcer that could have far reaching therapeutic consequences.

The initial neuropathy that develops in the diabetic is of a sensory type involving fine un-myelinated fibres ("C fibres") and can be demonstrated as a loss in pin prick sensation, and decrease in the sensation to heat and cold (*Ørstavik* et al., 2006). The cause of this neuropathy is unknown. Degeneration of sensory nerve fibres can be demonstrated within biopsies of the skin and soft tissues of the lower extremities (*Shun* et al., 2004). In addition, at about the same time as the onset of the neuropathy, a characteristic "diabetic dermopathy" is seen on the skin of many diabetic patients, characterized by melanotic papules (*Kiziltan* et al., 2006). The sensory innervation of the epidermis gradually becomes profoundly damaged as the course of diabetes continues.

The epidermis of man (and mouse) is designed both to restrict water loss and constrain or shape the microbiome that populates the skin. These functions are accomplished, in part, by the coordinated delivery of antimicrobial peptides and lipids that are packaged in structures called lamellar bodies (*Braff* et al., 2005). The lamellar bodies are produced by the keratinocytes as they begin to mature and move from their basal position in the epidermis. The

antimicrobial peptides and lipids penetrate the inter-keratinocyte spaces, as a "mortar", sealing the spaces between the flattening "brick-like" keratinocytes that comprise the superficial layers of the skin. What is remarkable, and somewhat counter-intuitive, is that upon injury to the skin, such as stripping the skin surface with tape, the recovery of the antimicrobial and permeability barrier requires the surface to dry. Within several hours lamellar bodies "ripen" and are delivered to the surface. Furthermore, signals are sent to the basal cells to initiate transcription and translation of antimicrobial peptides, insuring that adequate stores of these substances are available. However, if the surface is covered with a plastic film that maintains the moisture barrier, the barrier does not recover, and the induction of antimicrobial peptides is not observed (*Aberg* et al., 2008). Most likely, the osmotic pressure of the extracellular compartment in the injured skin is detected, and the receptors responsible for detection of the drift in osmolarity are one of the transient receptor potential vanilloid (TRPV) channels (*Liedtke*, 2006). These are also the receptors that sense temperature, and are expressed on the sensory nerve endings that lie at the junction between the epidermis and dermis. When activated these channels release calcium, and result in both transmission of an afferent stimulus to the brain as well as an efferent response from the nerve ending. The sensory nerve endings in human skin include peptidinergic nerves that release Substance P and Calcitonin Gene Related Peptide (CGRP) (*Schulze* et al., 1997). Each of these peptides has receptors on the keratinocyte. It is likely that activation of these receptors induces the antimicrobial/lipid barrier response and release of new, ripe lamellar bodies after surface injury. Furthermore, each of the two neuropeptides has physiological properties that are important in tissue injury defence and recovery/healing. Substance P, for example, has direct antimicrobial activity, induces capillary leakage and vasodilatation, is a chemo-attractant of neutrophils and macrophages, degranulates mast cells, and induces expression of pro-inflammatory adhesion proteins on endothelial cells, epithelial, and phagocytic cells (*Brogden* et al., 2005). In addition, Langerhans cells, which taste and present antigens within the epidermis after superficial injury, are activated by Substance P (*Staniek* et al., 1997). CGRP is a potent vasodilator of the arterioles of human skin (*Brain* et al., 1986). Both Substance P and CGRP stimulate the proliferation of fibroblasts and keratinocytes and promote healing in *ex vivo* models of human cutaneous wounds (*Cheret* et al., 2014).

The sensory nerves of the skin that express TRV1 can also communicate directly with dermal dendritic cells (*Riol-Blanco* et al., 2014). When the skin of a mouse is exposed repeatedly to imiquimod, TRV1+ sensory nerves in the skin stimulate dermal dendritic cells to secrete IL23, which in turn appears to induce the expression of IL22 and IL17 in nearby γδ intra-epithelial lymphocytes. These cytokines, in turn, should induce robust keratinocyte antimicrobial peptide expression, as well as keratinocyte proliferation. This recently reported discovery adds another dimension to the nervous system in the immune defence of the skin.

Sensory nerves within the epidermis are also capable of recognizing the presence of bacteria, by a direct sensing mechanism. A recent study in mice has demonstrated that *S. aureus* expressing lytic toxin reduces the threshold of heat and pain stimuli in afferents draining the sites in which microbes had been introduced (*Chiu* et al., 2013).

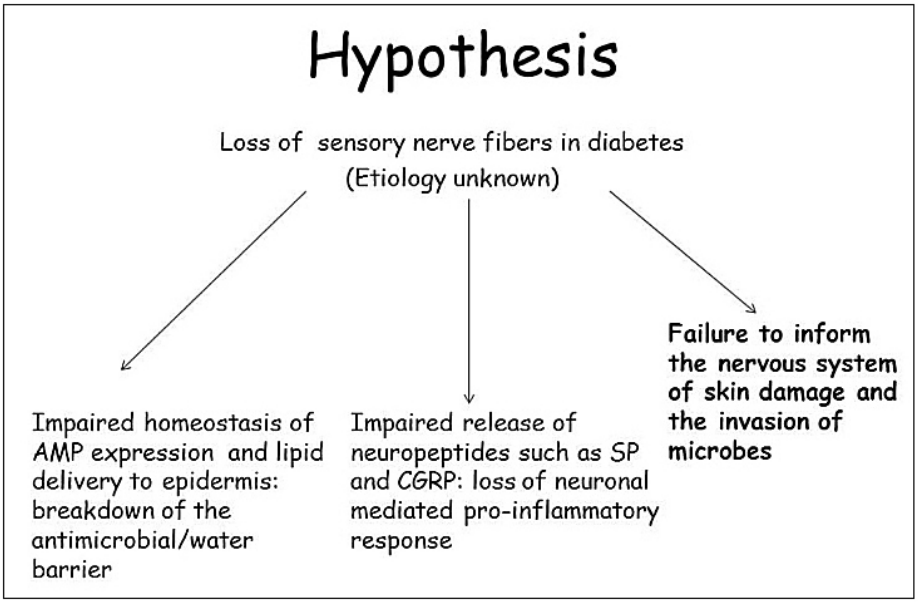

Figure 1: Hypothesis on the events leading to the non-healing skin ulceration and soft tissue destruction in the diabetic patiënt.

The receptors involved have not been identified, but they appear not be related to any of the known Toll-like receptors (TLRs). Furthermore, stimulation of the sensory nerves by this toxin leads to the release of CGRP, as measured in the dorsal root ganglion. This study demonstrates, however, that the nervous system can detect the presence of bacteria and therefore can "know" the anatomical location of an infection and could in principle utilize this information in ways that optimize the immune response.

In the diabetic who has developed a sensory neuropathy, these defensive and protective functions of the nervous system are lost. We hypothesize that the breakdown of the immune functions supported by the sensory nerves of the skin leads to the non-healing skin ulceration and soft tissue destruction in the diabetic (Figure 1).

ERYSIPELAS AND THE REGRESSION OF SARCOMA

Erysipelas is a superficial self-limiting infection of the epidermis and dermis by Group A *Streptococcus*. The infection is associated with a rapidly rising fever and a painful red indurated thickening of the skin. It begins as a small lesion, which grows in area over 4-6 days. The margins are raised and have a firm border. The fever and systemic symptoms of illness subside when the growth of the rash stops. Of particular interest is the curious finding that streptococcal organisms can rarely be identified in the bloodstream in this infection, unlike what is observed in a deeper streptococcal soft tissue infection (*Linder* et al., 2010). It is this observation associated with erysipelas that I believe suggests an underlying immune role being played by the nervous system in defence of the skin.

As I have noted above, the nervous

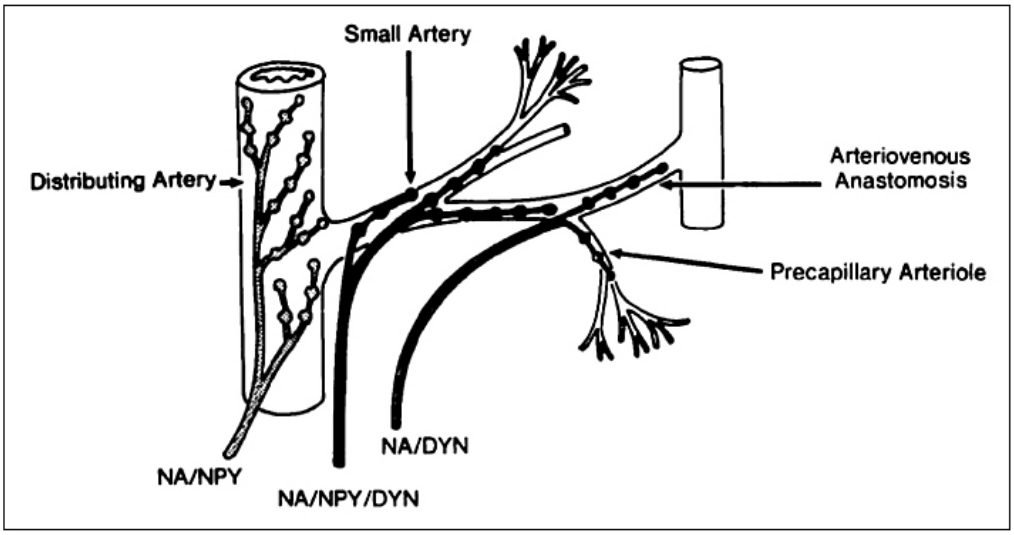

Figure 2: Diagram summarizing the microvasculature in the guinea pig ear (from: *Gibbins* and *Morris*, 1990).

system has the ability to detect the presence of microbes in the skin and underlying tissues through activation of sensory nerves. A question that we can ask is how this information is utilized by the central nervous system. In the case of erysipelas I would suggest that the response to the superficial streptococcal infection is a highly selective vasoconstriction in which the capillaries that perfuse the site of infection are made less accessible to the bacteria in their midst, in order to prevent their entry into the intra-vascular space. In addition, I suggest that the nervous system also responds by reducing the flood of blood through distant capillary beds to reduce the likelihood that a microbe that has entered the blood stream can escape and infect a distant tissue compartment. In other words, the nervous system shuts down access of streptococci both into and out from the vascular space. Local antimicrobial defences then deal with the primary skin infection. The spleen and other organs with phagocytic capacity eliminate bacteria that have gained intravascular access.

The nervous system is anatomically designed to regulate the flow of blood through capillary beds through control of pre-capillary sphincters. What is surprising is that the pre-capillary sphincter appears to be innervated by a specific nerve, different from that which controls blood flow through proximal arterioles (*Gibbins* and *Morris*, 1990) (Figure 2). As demonstrated in the vascular bed of the ear of the guinea pig the nerves that innervate the most distal portions of the arterial bed, the precapillary sphincter and the arteriovenous anastomosis, are distinctly different (noradrenaline/dynorphin containing) than those that innervate the more proximal arteriole (noradrenaline/Neuropeptide Y (NPY)/dynorphin) or the distributing artery (noradrenaline/NPY) (*Gibbins* and *Morris*, 1990). Thus, we can imagine that the central nervous system has the capacity to direct the vasoconstriction of specific pre-capillary beds by directing efferent signals through neurons that communicate with these vascular beds. Since gas exchange can occur across small diameter arterioles and venules as

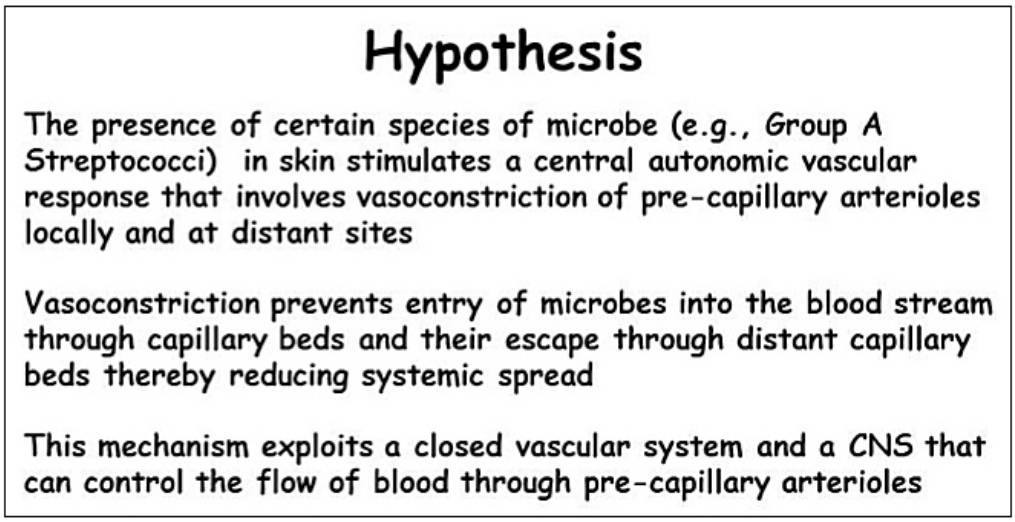

Figure 3: Hypothesis on how the central nervous system can restrict the spread of microbes throughout the body from an infected site by control of the flow of blood through capillary beds.

effectively as across capillary beds (*Vovenko*, 1999), the closure of the capillary beds would restrict nutrient passage, lymphatic flow, and cellular (and microbial) traffic, but would not necessarily reduce gas exchange.

These observations lead us to the hypothesis that the central nervous system can restrict the spread of microbes throughout the body from an infected site by control of the flow of blood through capillary beds. The hypothesis, which is presented in Figure 3, can explain one of the more extraordinary and mysterious stories in the history of medicine. In the late 1800's, William Coley, an orthopaedic surgeon, reported that patients with aggressive soft tissue sarcomas would experience complete regression should they also develop erysipelas (*Nauts* et al., 1946). Coley's observation was based on the co-incidental infections that occurred in his cancer patients, and then on the many positive responses of patients "therapeutically" infected with streptococcus to cause erysipelas. Unfortunately, streptococcal infection was itself rather dangerous with a significant independent mortality. Coley attempted to refine the method by extracting non-infectious microbial components. As the preparations became better characterized, they lost efficacy. Coley believed that sarcoma was caused by a bacterial infection, and his use of the bacterial preparations somehow stimulated an immune response.

It is interesting to review Coley's description of the response of the sarcoma in the setting of an induced erysipelas in a patient with a soft tissue sarcoma (*Coley*, 1910):

"...Finally in October 1891, with 5 decigrams of a bouillon culture of Streptococcus of erysipelas just brought me from Koch's laboratory in Germany by Dr. Frank Ferguson, the pathologist of the New York Hospital, a most severe attack of erysipelas developed, nearly causing the death of the patient. Within an hour after the injection a severe chill occurred followed by a temperature of 105°F. After an interval of 12 hours a typical attack of erysipelas developed starting at the point of injection and extending over the neck and face. It ran its usual course.

The tumour of the neck began to break down on the second day, and a discharge of broken down tumour tissue continued until the end of the attack. At the end of two weeks the neck tumour had disappeared and the tonsil tumour had decreased in size. The patient remained well for 8 years."

Coley carefully documented the usual changes that he observed in the tumour following the induction of erysipelas:

"...First, the tumour becomes much paler owing to decreased vascularity; second, it becomes much more movable and less fixed to the surrounding tissues; third it soon begins to show areas of softening, due to caseous degeneration or necrobiosis of the tumour elements; fourth, gradual disappearance, either by absorption..or in other cases by breaking down and liquefaction."

I would suggest that what Coley was observing was a specific neurally mediated immune vasocontrictive response to presence of *Streptococcus* that interfered with blood flow to the sarcoma (Figure 3).

The role of the nervous system in the immunity of the skin as highlighted here involves the capacity of the nervous system to spatially identify the anatomical site of injury or infection, its ability to direct neuropetides that promote wound healing and provide antimicrobial defense to that site, and its capacity to prevent metastatic spread of infection through the vascular space. I propose that this system of immunity represents the basic outline of the immune function of the nervous system of all vertebrates.

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THE DISCOVERY OF HUMAN EPITHELIAL ANTIMICROBIAL PEPTIDES

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SUMMARY

All higher organisms are living in an environment, which is laden with incredibly large numbers of microorganisms that potentially can harm them. Further, each organism has at its surface a more or less specific commensal microflora, which is characteristic for each species and its anatomical sites. Despite the enormous numbers of microorganisms at body surfaces, infections are a rather rare event. In humans we had explained this unexpected phenomenon until ecently by the presence of humoral and cellular components of the adaptive immune system. This, however, cannot explain, why organisms lacking an adaptive immune system naturally resist infections and therefore this indicates the presence of another, more ancient innate defence system. By addressing the question, why invertebrates resist infections and why cattle epithelia, which are permanently in contact with microbes, are not infected upon wounding, a number of antimicrobial peptides have been discovered using biochemical approaches. These discoveries led to the hypothesis that also human epithelia (skin, gut, lung, genitourinary tract) should produce such antimicrobial compounds for innate defence against infection. This was one of the key questions in the author's team, for which we found some answers by the use of classical biochemical analyses with modern analytical equipment and reasonable antimicrobial read-out systems. It is not the aim of this review to give an overview and discussion of the role of the human peptide antibiotics, which were isolated in our laboratory. Instead, I will give some information how we came to these discoveries, giving more an overview about the stories behind the discovery stories.

INTRODUCTION

Although all higher organisms are living in an environment laden with potentially pathogenic microbes, infections of the skin are rather rare and mostly occurring upon disruption of the epidermal barrier or upon wounding. At a first glance it seems to be clear why this is the case: we are equipped with a very effective adaptive immune system with its phagocytes and selective antigen-recognizing and antibody-producing immune cells. When thinking about this explanation in more detail, we will recognize several discrepancies: healthy skin does not contain any professional phagocytes, which permanently eliminate microbes from the skin surface and animals (including invertebrates) and plants, which both have no adaptive immune system and

mostly no phagocytes, have to solve the same problem to protect their body surface from infection. How is this possible? A plausible explanation for this, in principle unexpected, phenomenon is the presence of factors which limit the growth of microbes and/or the presence of antibiotic factors, in particular antimicrobial peptides (AMPs). When such factors are located at the body surface, these should effectively control invasion of microbes and inhibit infection. The important question, how an adaptive immunesystem-lacking organisms (like invertebrates) protect themselves from microbial infection has been addressed by pioneering work of the group of Hans Boman in silkworm larvae (*Steiner* et al., 1981) and the group of Jules Hoffmann in the fruitfly (*Fehlbaum* et al., 1994). Both groups identified insect antimicrobial peptides like cecropins or drosomycin and others. These findings prompted search for the existence of AMPs in vertebrates, where textbook knowledge told us that antimicrobial defence was achieved, apart from the lytic complex of activated complement, by phagocytes as professional effector cells of the adaptive immune system. As bactericidal effector molecules, reactive oxygen species, generated from the myeloperoxidase-hydrogen peroxide-halogenide system, as well as nitric oxide seemed to be solely relevant. Not before Robert Lehrer and co-workers observed that apart from these short-living inorganic compounds also protein-like bactericidal and fungicidal activity is associated with phagocytes, it became clear that also vertebrates are preventing microbial infection via peptides called "defensins" (*Ganz* et al., 1985).

Utilizing the *Xenopus laevis* oocyte system to study RNA expression in eukaryotes, Michael Zasloff wondered why incisions made in the frog's skin did not cause any infections despite the fact that freshly surgically treated animals were put into a microbially contaminated laboratory tank. This surprising observation led to the hypothesis that frog skin contains an antibiotic principle, which protects it from microbial infection. As antibiotic compound, a peptide has been characterized which was termed "Magainin" (*Zasloff*, 1987). Magainin is the first discovered vertebrate AMP produced by epithelial cells. With the finding that frog skin contains antibiotic peptides it was suggested that also epithelia of other vertebrates, such as cattle, could have the capacity to produce AMPs. Subsequently a structurally unrelated epithelial antimicrobial peptide (AMP) has been discovered in cattle trachea ("tracheal AMP, TAP") (*Diamond* et al., 1991). The hypothesis that grazing led to many small wounds on the tongue epithelium without infection, led to the discovery of another AMP belonging to the so-called beta-defensin family, the "lingual antimicrobial peptide, LAP" (*Schonwetter* et al., 1995). Subsequently another structurally related epithelial beta-defensins has been discovered in cattle gut epithelia ("enteric beta-defensin, EBD") (*Tarver* et al., 1998). These previous observations suggested that also human epithelia, in particular the skin, should also have the capacity to produce similar AMPs. Interestingly, in the midst of the 90's all efforts failed to identify human orthologs of LAP, TAP and EBD in human epithelia, including the skin by using a cloning strategy.

Recent microbiome studies revealed that healthy skin is colonized by a huge number of different genera and species of bacteria and fungi (*Findley* et al., 2013). Further, different skin habitats are hosting different microbial species, e.g. in the moist, rather mucosal areas of the aero-digestive tract and urogeni-

tal tract other species are living than in rather dry and/or lipid-rich areas like the skin of the face, scalp or lower legs.

So the questions come up: what shapes the composition of the cutaneous microbiome at the different skin habitats, why is the number of microbes at the skin surface relatively constant, and why are some microbes present and others not although in principle the growth conditions should be optimal.

THE DISCOVERY OF THE FIRST HUMAN INDUCIBLE PEPTIDE ANTIBIOTIC, HUMAN BETA-DEFENSIN-2 (HBD-2): A HISTORICAL SUMMARY

The major scientific focus of our research in the 90's has been the role of neutrophils and eosinophils in cutaneous inflammation, in particular psoriasis (PS) and atopic dermatitis (AD). In particular, the mediators and cytokines which cause skin infiltration by neutrophils in PS or eosinophils in AD, were in our focus. After we had discovered that the major neutrophil attractants in psoriatic lesions (which we had isolated and purified from lesional psoriatic scale material) are chemotactic cytokines (*Schröder* et al., 1992) [originally termed MONAP or ANAP and today termed as the chemokines interleukin 8 (CXCL8) and Gro-α (CXCL1)], we were interested to know whether, apart from these chemokines, additional neutrophil chemo-attractants are of relevance in psoriasis lesions. With the use of a monoclonal antibody, which recognized an epitope common in both, IL-8 and Gro-α, an affinity-column was generated and lesional psoriatic scale extracts were analysed for remaining PMN-chemotactic activity in the through flow. These findings indicated, that apart from the chemokines IL-8 and Gro-α no other PMN-chemoattractant is present in lesional PS-skin extracts. To recover the affinity-bound IL-8 and Gro-α we stripped the affinity column with acidic glycine-buffer and performed a reversed phase (RP) high performance liquid chromatographic (HPLC) analysis with the stripped material. To our surprise, the major UV-absorbing peaks came not from both expected chemokines, but from unknown proteins. The major UV-absorbing peak gave upon SDS-PAGE analysis a single, silver-stained 15k-band, which stained in the upper area rather brownish and in the lower area rather dark grey (*Schröder*, 2010). Further analyses indicated that the material was not pure and consisted of two components. We had one component identified as lysozyme. The other was unknown. A special HPLC-column allowed separation of lysozyme from the unknown protein, which, after performing SDS-PAGE analysis in urea-containing buffer, now gave a single band at 4k and not 15k, which suggested that this protein forms a tetramer. Although this protein lacked PMN-chemotactic activity, we performed N-terminal sequencing by Edman-degradation. We had not found the proposed amino acid (AA) sequence in a 1992 data bank and there was no similarity to any protein. By chance, I had read a paper about epithelial antibiotics (*Schonwetter* et al., 1995), where the frog skin-derived AMP Magainin showed exactly the same N-terminal three amino acid (AA)-containing peptide motif Gly-Ile-Gly as the 4k-peptide. To check whether we possibly found a human ortholog of Magainin, we re-analysed the Edman degradation raw data and found at two positions a blank (instead

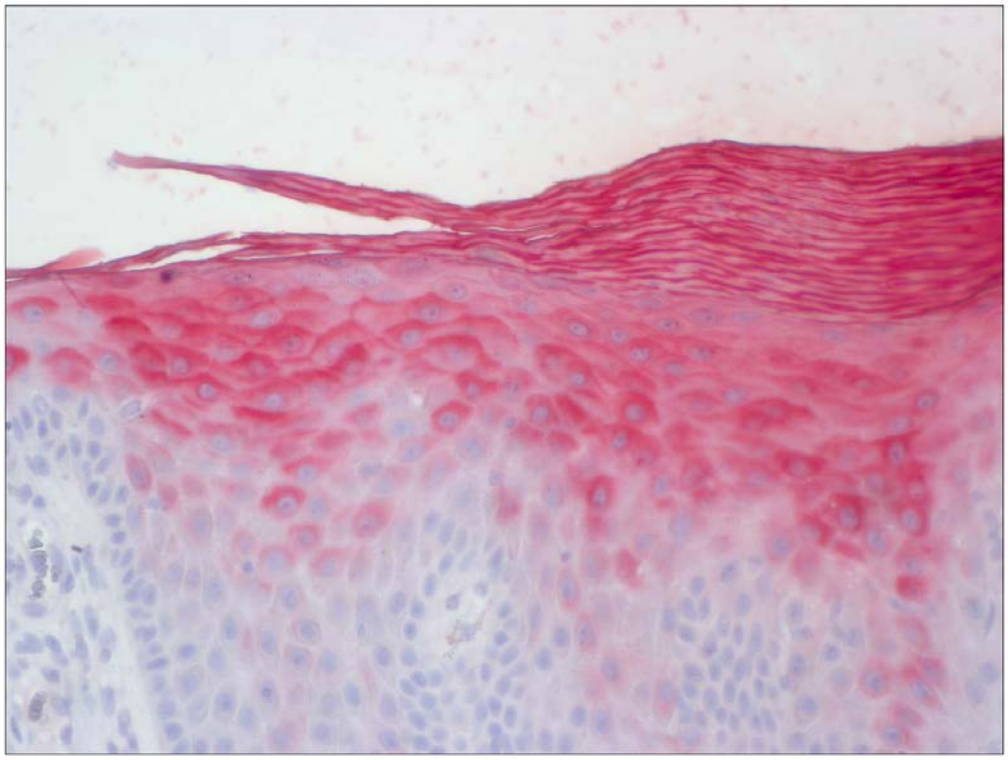

Figure 1: Human beta-defensin-2 is strongly expressed in lesional psoriatic skin. Note the presence of immunoreactive (i)hBD-2 mainly in the upper, granular layers of lesional psoriatic skin, where it is located within granules. In the uppermost granular layer intracellular staining seems to be missing, suggesting release. In the stratum corneum, released ihBD-2 seems to stick to the corneocyte surface.

of the computer-proposed AA). Cysteines in an AA sequence give a blank upon Edman degradation. We therefore inserted Cys residues in the proposed AA sequence at these positions and now found similarity to bovine beta-defensins, suggesting that this peptide is a human beta-defensin. We created the name "human beta-defensin-2 (hBD-2), because another, in blood filtrates found peptide, was termed hBD-1. We then established a radial-diffusion antimicrobial assay (RDA) system as it has been optimized by Bob Lehrer's group (*Steinberg* and *Lehrer*, 1997) to check whether hBD-2 is an AMP. The use of agarose instead of agar (as used for testing of antibiotics in medical microbiology) was essential for detecting antimicrobial activity of hBD-2 (*Harder* et al., 1997) and other cationic peptide antibiotics, confirming recommendations repeatedly reported by Bob Lehrer's group.

INITIAL STUDIES ON THE ROLE OF HBD-2 IN SKIN PHYSIOLOGY AND INFLAMMATION

hBD-2 has been discovered in extracts of lesional psoriatic scale material (*Harder* et al., 1997). Stratum corneum extracts obtained from the heel did not

or nearly not contain hBD-2. This suggests that hBD-2 needs to be induced. Numerous studies reported that hBD-2 is exclusively produced by epithelial cells, like keratinocytes and others (*Schröder* and *Harder*, 2006). Immunohistochemistry analyses show a characteristic staining pattern with strong intracellular expression of hBD-2 in the uppermost stratum granulosum cells, where it is located within the lamellar bodies (Figure 1). The next apical stratum granulosum cell layer now lacks immunoreactive hBD-2, which suggests a release (as lamellar bodies are depleted in that cell layer) of hBD-2 and now localized at the surface of corneocytes.

Although hBD-2 was originally found to be an AMP for *E. coli* (*Harder* et al., 1997), later on various Gram-negative bacteria and the yeast *Candida albicans* were seen to be killed, whereas for *S. aureus* only bacteriostatic activity was seen at high hBD-2-concentrations (*Harder* et al., 2000). Thus, we hypothesized that hBD-2 might be a rather Gram-negative bacteria-directed peptide antibiotic of the skin. The observation that hBD-2 is absent in healthy skin suggested that it needs to be induced. Because it has been isolated from the scales obtained from psoriasis skin lesions and because psoriasis is an inflammatory skin disease where pro-inflammatory cytokines play an important role, TNF-α and IL-1β were tested and found to be powerful hBD-2-inducers in keratinocytes.

Previous studies on induction of beta-defensins in cattle have shown that also heat-inactivated bacteria are able to induce AMPs in tracheal epithelial cells (*Diamond* et al., 1991). This observation prompted us to test the hypothesis of hBD-2-induction in keratinocytes by heat-killed bacteria. Because our research focus was on the role of neutrophils and eosinophils in inflammatory skin diseases and not in microbiology, we had to look for bacteria. Fortunately, the department of dermatology has had that time a microbiology laboratory for diagnostic investigations. When asking for any bacterium we could use in bacterial stimulation experiment, we got a laboratory strain of *Pseudomonas aeruginosa*, which had been isolated from a leg ulcus.

Indeed, this heat-inactivated *P. aeruginosa* strain was found to be able to induce hBD-2 in keratinocytes (*Harder* et al., 1997), suggesting that generally heat-inactivated bacteria are inducing hBD-2 in keratinocytes and, as seen later on, in other epithelial cells. We became aware, however, that this seems not to be a general fact: after we had published our results, someone let us know that they could not reproduce the bacterial stimulation of hBD-2. The strain they had used was a laboratory strain of *Pseudomonas aeruginosa*, PA01. This strain in our hands also failed to induce hBD-2 suggesting that the clinical *Pseudomonas aeruginosa* strain we had accidentally used, should bear some unique characteristics. When re-culturing this ulcus-derived strain, we saw a slimy growth, which was not seen with the laboratory *Pseudomonas* strain PA01. In order to characterize the factor(s) responsible for hBD-2 induction, we also analysed bacteria-free culture filtrates and found strong activity, which apparently was released from the bacteria. A likely candidate bacterial "microbe-associated molecule, MAM" was lipopolysaccharide, which would activate keratinocytes via TLR-4. For keratinocytes there are contradictory studies about the TLR-4 expression in normal keratinocytes, and a recent study suggests that TLR-4 is expressed only in pro-inflammatory cytokine-activated keratinocytes (*Terhorst* et al., 2010). In

addition, studies claiming that LPS is an inducer of hBD-2 in keratinocytes showed that concentrations of 10-100 µg/ml of a LPS-preparation were necessary. These concentrations are nearly 10,000-100,000-fold higher than those able to activate macrophages via TLR-4 for IL-1-production, suggesting that the active principle is a contaminant in the LPS-preparation. This hypothesis is supported by the fact that synthetic LPS is inactive as hBD-2 inducer.

FLAGELLIN IS THE PRINCIPAL *PSEUDOMONAS AERUGINOSA* DERIVED HBD-2-INDUCER

We first followed the hypothesis that *Pseudomonas aeruginosa* laboratory strains should be able to produce and release the hBD-2-inducer. Numerous experiments with different culture conditions eventually revealed that *P. aeruginosa* culture filtrates contain maximum hBD-2-inducing activity, when the bacteria were grown: a) at low nutrient availability conditions, and b) at stationary growth conditions. At these culture conditions the bacteria grow as slimy colonies, similar as we had seen with our clinical *P. aeruginosa* isolate. All attempts to purify the active principle failed. We often ended up, however, with rhamnolipids, which are biosurfactants released from biofilm-forming *P. aeruginosa*. To further study the role of rhamnolipids, which are by themselves not able to induce hBD-2 (*Gerstel* et al., 2009), various *P. aeruginosa* strains were treated with rhamnolipids and supernatants analysed for hBD-2-content. Surprisingly, all *P. aeruginosa* strains released hBD-2-inducing activity, except a flagellin (Fln)-knock out-strain, suggesting that flagellin is the hBD-2 inducer. This could be confirmed and it was seen that Fln induces hBD-2 at a half maximum effective dose of 5 ng/ml (*Gerstel* et al., 2009), a finding that implicates attention for Fln as possible hBD-2-inducing contaminant when using partially purified bacterial MAM-preparations for cell culture experiments.

HBD-2 IS A CHEMOKINE AND CHEMOKINES ARE ANTIMICROBIAL PEPTIDES

The major scientific focus at the time that we had discovered hBD-2 were still the chemotactic cytokines, which since 1992 are termed chemokines. At a Keystone conference on chemokines in the winter of 1996-1997 I had a private scientific conversation with J.J. Oppenheim, a well-known chemokine scientist, on the role of alpha-defensins as leukocyte chemotactic factors. When I told him that we had discovered in psoriasis scale material a new defensin which was a beta-defensin, he speculated that this could be, like the alpha-defensin HNP-1, also a leukocyte chemo-attractant acting as a chemokine. Although initial tests with chemokine-receptor transfected HEK293 cells failed to support his hypothesis, a CCR6-HEK293 transfectant responded by chemotaxis towards psoriatic scale-derived hBD-2 in a dose-dependent manner. This finding indicates that beta-defensins use a chemokine receptor although there is no sequence homology with the only chemokine lig-

and for CCR6, LARC (CCL20) (*Yang* et al., 1999). Although the concentration of hBD-2 needed for half maximum chemotaxis is much higher than that of CCL20, it is likely to be relevant *in vivo* because the amounts of hBD-2 produced within the skin for direct antimicrobial defence are within the µ *M*-range whereas tissue CCL20 concentrations are in the n*M*-range. Due to the limited availability of natural hBD-2 (which was purified from psoriatic scale extracts), hBD-2 has been chemically synthesized. Initially synthetic hBD-2, however, was far less potent than the psoriasis-derived hBD-2 in chemotactic activity, although antimicrobial properties of natural and synthetic hBD-2 did not differ. The reason for this discrepancy are connections of the three disulphide-bridges, which have a defined connectivity in the natural hBD-2 and which represent a mixture of all theoretically possible variants in synthetic hBD-2. In the case of the beta-defensin hBD-3 it was shown that only the natural isomer is chemotactically active and binds to the chemokine receptor. CCR6 is highly expressed in dendritic cells (DC) and memory T cells. So hBD-2 *in vivo* may, through CCR6, recruit immature DCs and memory T cells to sites of microbial invasion in the skin and mucosa (*Yang* et al., 1999).

The observation that the chemokine receptor CCR6 is a target of a beta-defensin led to the hypothesis that also chemokines could be antimicrobial peptides. This was proven for CCL20 and several others including the IFN-γ-inducible chemokines MIG (CXCL9), IP-10 (CXCL10), as well as SDF-1 (CXCL12). Interestingly neither IL-8 (CXCL8) nor RANTES (CCL5) were antimicrobially active (*Icard* et al., 1986). The high concentrations (10 µg/ml) necessary to elicit antimicrobial activity would explain that an unbiased biochemical approach so far failed to identify chemokines as AMPs and makes it unlikely that chemokines represent AMPs *in vivo*.

DISCOVERY OF PSORIASIN/S100A7 AS THE PRINCIPLE SKIN FACTOR PREVENTING *E. COLI* INFECTION

More than 30 years ago medical students had to perform an experiment in a medical microbiology course where they dipped fingers of one hand in a *S. aureus* suspension and fingers of the other hand in an *E. coli* suspension. After incubation in a moist atmosphere they made a fingerprint on agar plates and investigated bacterial growth after some time. They found, to their surprise, that clear fingerprints were seen with *S. aureus*, but nothing was seen with *E. coli*. The conclusion drawn from this observation was that finger surfaces contain factors that kill *E. coli*, but not *S. aureus*. Surprisingly, this experiment has been done by generations of medical students but never the question was addressed why and how solely *E. coli* is killed. It was therefore our aim to understand the molecular basis of these findings. Because originally these experiments have been done with fingers, the whole hand was incubated in buffer-containing gloves and thereafter the washing fluid was analysed for *E. coli*-cidal activity. As principal *E. coli*-cidal component the S100 protein psoriasin (S100A7) was identified (*Gläser* et al., 2005). *In vitro* dose-response studies revealed an LD90, which is well in the range found at the skin surface (*Gläser* et al., 2005). To investigate whether psoriasin is an

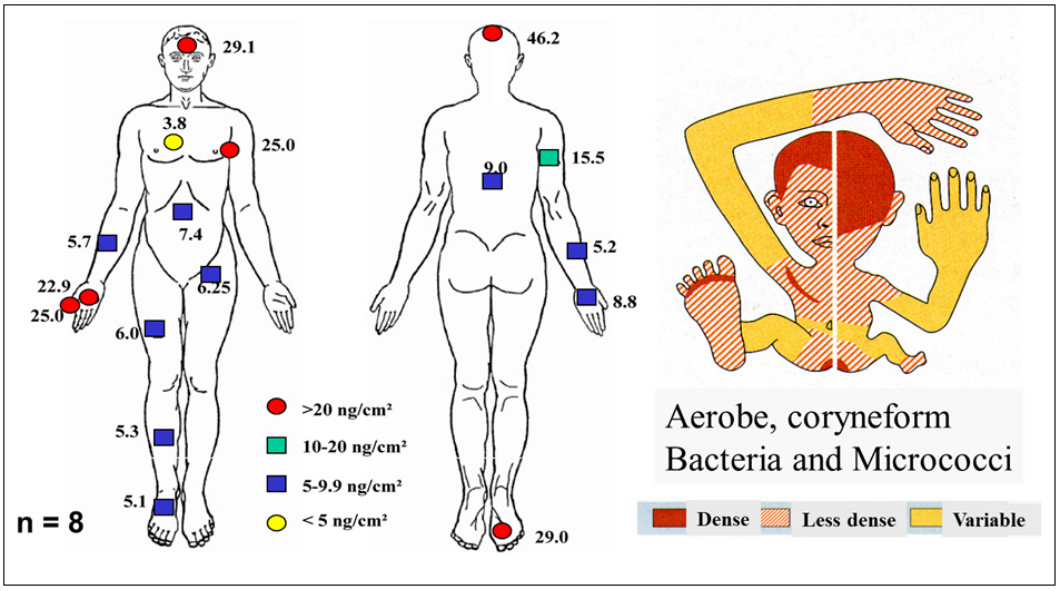

Figure 2: Psoriasin is secreted *in vivo* on the body surface. Standardized areas of various body locations on healthy volunteers were rinsed with 10 mM sodium phosphate buffer, pH 7.4, to determine the concentration of psoriasin present on the skin by ELISA. Note the presence of highest amounts of psoriasin in areas, where bacterial densities are highest (adapted from: Gläser et al, 2005).

AMP *in vivo* capable of killing *E. coli* on the skin surface, we applied *E. coli* psoriasin antibody or an irrelevant antibody. As result it was shown that psoriasin antibodies increased survival of *E. coli* on the skin surface (*Gläser* et al., 2005). Thus, psoriasin contributes to the skin's marked resistance towards infection by the gut bacterium *E. coli* although additional, yet not identified factors at the skin surface, play an additional role in defence. We were interested to see where psoriasin is located in healthy skin. Immunohistochemistry revealed unexpectedly a patchy staining pattern only in the uppermost keratinocyte layers (stratum granulosum) of healthy skin. Sometimes patches within the stratum corneum layer showed marked psoriasin staining, but not living epidermis areas beneath. This could be interpreted as local, temporary induction of psoriasin, maybe two weeks before taking the on defined forearm skin areas in the absence or presence of a neutralizing biopsies. In other words, psoriasin seems to be induced locally at the skin surface. This suggestion was confirmed by analysis of the local psoriasin amounts present at different anatomical skin sites (Figure 2). A markedly increased psoriasin amount was seen at some anatomical locations such as the scalp, hands, feet, axilla and face. On the other side, at rather dry anatomical sites such as forearm or lower leg, psoriasin amounts were rather low (*Gläser* et al., 2005). Anatomical areas with high amounts of psoriasin are highly colonized by microbes. Therefore, it was tempting to speculate that the local skin microflora, or in general bacteria, induce psoriasin in the ecological niches where they are colonizing. Immunohistochemical analyses supported this suggestion: here we saw strong psoriasin staining in the lower

parts of hair follicles, exactly where high microbe densities are present. To further test the hypothesis that bacteria induce psoriasin in skin keratinocytes, *E. coli* culture media were applied to healthy donor's skin, which increased psoriasin levels in washing fluid (*Gläser* et al., 2005). This suggests that bacterial culture supernatants contain a psoriasin-inducing component (which was identified as flagellin) and not, as commonly suggested, bacterial LPS (*Abtin* et al., 2008). It is important to note that ng/ml-amounts of flagellin are sufficient to induce psoriasin or hBD-2. Therefore, the high amounts of LPS preparations (10-100 µg/ml) necessary to elicit AMP-induction in keratinocytes (or other epithelial cells) and the absence of the LPS-receptor TLR4 suggests that commercially available LPS-preparations (phenol-extracts) commonly used could be contaminated by ng-amounts of flagellin.

The rather preferential killing of *E. coli* by psoriasin suggests that it could represent an important defence effector molecule of the genito-urinary system at locations where *E. coli* contamination is a principal risk. Most commonly, urinary tract infections are due to uropathogenic *Escherichia coli*, which are classically thought to migrate from the gut to the bladder and then subsequently undergo highly specialized adaptations to increase their pathogenicity within the bladder (*Schaeffer*, 2013). Since *E. coli* permanently challenges the healthy female reproductive tract it should have an effective epithelial defence system to inhibit *E. coli* survival at these locations and its migration to the bladder. A recent study analysed *E. coli*-cidal factors in vaginal secretions of healthy women and identified psoriasin as the principal *E. coli* killing antimicrobial (*Mildner* et al., 2010), which is constitutively expressed in vulva, vaginal, and ectocervical epithelium but not in endocervical epithelium.

The human mouth is an area colonized by a high variety of microbes and challenged by microbes contaminating food. Despite these permanent threats, the human tongue is highly resistant against microbial colonization and infection by oral intake. *E. coli* acts as an indicator organism for the microbiological quality of food and beverages. Despite daily exposure of *E. coli* strains from animal reservoirs through the mouth, lingual infections with *E. coli* (which is not a member of the oral microflora) are rather rare. This suggests the presence of *E. coli*-cidal factors in the mouth. Biochemical analyses of tongue tissue-extracts identified again psoriasin (S100A7) as a dominating antimicrobial component of the healthy human tongue (*Meyer* et al., 2008). The highest psoriasin expression was found in the anterior part of the tongue with decreasing expression posteriorly. Since the anterior part of the tongue has first contact with microbes and is more vulnerable to surface trauma, it is plausible that this lingual region requires additional protection by high expression of antimicrobial proteins such as psoriasin. Psoriasin seems to be stored and rapidly released to the surface with minimal adherence to the most superficial epithelial cells, as suggested by high psoriasin levels found in the rinsing fluids of human tongues. Interestingly repeatedly rinsed standardized areas of the human tongue of healthy volunteers could not reduce the psoriasin concentration, which might indicate a high psoriasin production in the upper lingual epithelium, possibly as effective defence response towards to permanent microbial and/or inflammatory stress.

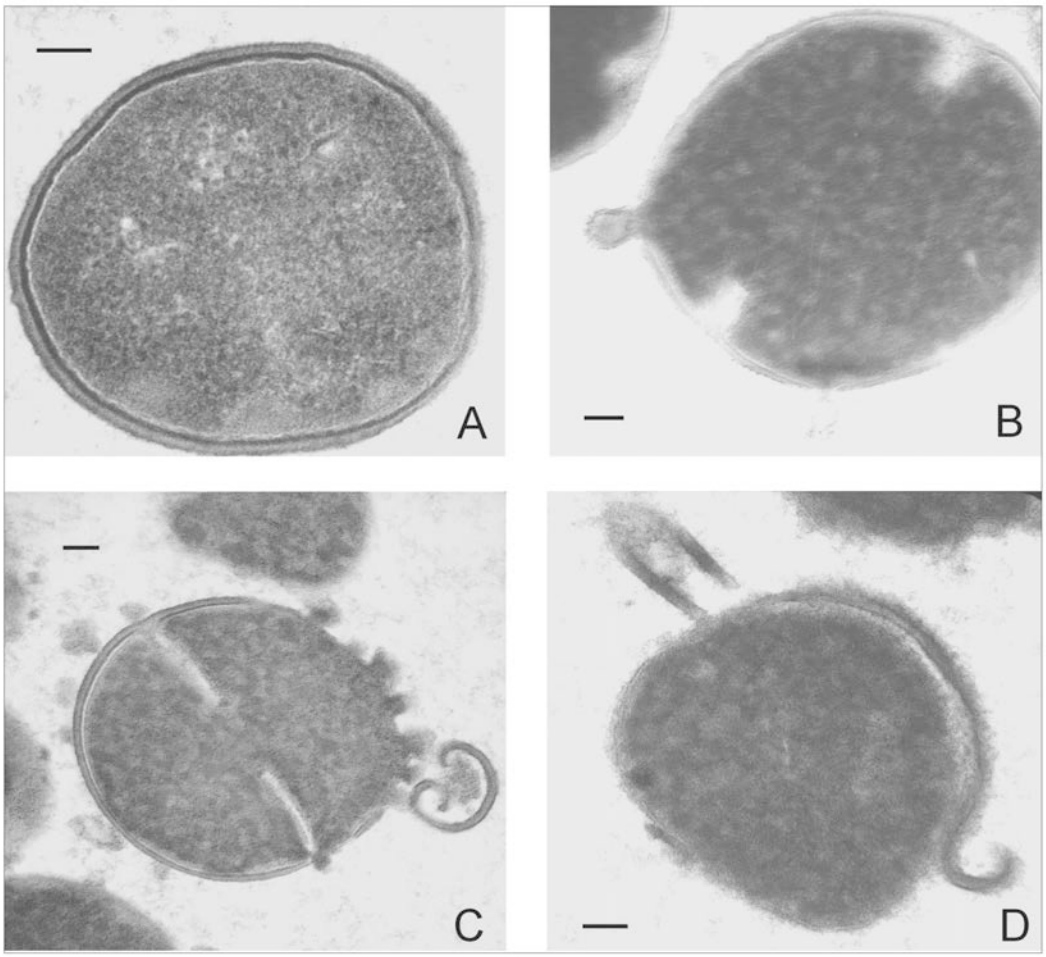

Figure 3: Morphology of hBD-3-treated *S. aureus*. Transmission electron micrographs of *S. aureus* (10^8 cells/ml) incubated in 10 mM phosphate buffer for 2h (A) or treated with synthetic hBD-3 (500µg/ml) for 30 min (B) or 2h (C and D) are shown. Bars represent 0.1µm (from: Harder et al, 2001).

THE DISCOVERY OF HUMAN EPITHELIAL ANTIMICROBIAL PEPTIDES CAPABLE OF KILLING *STAPHYLOCOCCUS AUREUS*

The observation that hBD-2 is selectively killing Gram-negative bacteria and *Candida albicans* but not *S. aureus* (*Harder* et al., 2000) prompted us to search for human *S. aureus*-killing AMPs. With the idea that a *S. aureus*-killing AMP should bind first to *S. aureus*, we generated affinity columns where *S. aureus* has been covalently coupled. These columns indeed bound *S. aureus*-killing peptides, which we suspected in lesional psoriatic scale extracts, the source of hBD-2. Purification and subsequent structural analyses gave a new, 5,054 Da peptide, which was termed "human beta-defensin-3, hBD-3" due to its structural similarity to beta-defensins (*Harder* et al., 2001). Unlike hBD-2, hBD-3 is a broad-spectrum AMP. Although it has been dis-

covered as *S. aureus*-killing factor, it showed activity against various Gram-positive and Gram-negative bacteria as well as *Candida albicans* with minimal bactericidal concentrations at sub-micromolar concentrations (*Harder* et al., 2001). It is killing *S. aureus* by interacting with the lipid II-system, similar as penicillin, resulting in very similar ultrastructural changes (Figure 3; *Harder* et al., 2000) Although hBD-3 is inducible by mucoid *P. aeruginosa* strains (*Harder* et al., 2001), its major induction pathway is EGF-receptor-dependent (*Sørensen* et al., 2005), making this AMP important in wound healing processes where the missing physical skin barrier makes the wound highly vulnerable towards infection. Main cellular sources of hBD-3 are epithelial cells, although also muscles contained hBD-3-transcripts (*García* et al., 2001). Apart from being a broad-spectrum antibacterial peptide, hBD-3 has antiviral activity: several studies report expression of hBD-3 in common warts, molluscum contagiosum and human papilloma virus infections of the skin and mucosa (*Meyer-Hoffert* et al., 2008, 2010). This induction, which seems to be independent of NFκB, possibly occurs TLR-3-dependently, as it has been seen by other antiviral peptides like HD-5.

SYSTEMATIC SEARCH FOR OTHER SKIN-DERIVED AMPS

hBD-2 and hBD-3 have been originally discovered and purified by following the hypothesis that these should bind to targeted bacteria with the use of bacteria-coated affinity columns. To perform the analyses in a more general biochemical systematic way, we optimized conditions for AMP-extraction, separation and purification. Various frustrating pitfalls to obtain some AMPs from skin specimens in the last twenty years prompted me to publish a successful way to purify natural human skin-derived AMPs (*Schröder*, 2010). Several aspects needed special consideration: the read-out system should allow the detection of cationic antimicrobial peptides. Therefore instead of agar, always low electro endosmotic agarose had to be used because agar acts as cation exchanger, binding all cationic AMPs, which leads to false-negative results (*Schröder*, 2010). Biochemical techniques had to be established, which allowed sufficient separation as well as structural characterization of AMPs. We used the strategy to first extract AMPs from stratum corneum (SC), because preliminary experiments revealed SC as rich source of antimicrobially active factors. Extraction conditions needed optimization with ethanol-containing acidic buffers with volatile acids (to perform ESI-MS-analyses without problems). Enrichment of cationic AMPs was possible by heparin-affinity chromatography (because heparin is a weak cation-exchanger). The low antimicrobial activity in the effluent confirmed that the majority of antimicrobial activity came from cationic AMPs and not from anionic compounds such as fatty acids.

By reversed-phase (RP) high performance liquid chromatography (HPLC), a procedure, which allowed separation according to hydrophobicity, we separated heparin-bound material. Then aliquots of HPLC fractions were analysed for antimicrobial activity. Using different bacterial species (which could have been cultured at different conditions) and different radial-diffusion-test conditions (aerobic, anaerobic, neutral pH, low pH, low or high ionic strength, presence or absence of nutrients) a

wide variety of broad-spectrum AMPs or bacteria-specific AMPs could be identified.

Final purification to homogeneity needs methods which allow separation of contaminants by utilizing the different physicochemical properties of AMPs and contaminants, e.g. by using cation-exchange-HPLC followed by narrow pore RP-HPLC (*Schröder*, 2010).

THE DISCOVERY OF RNASE 7, HEALTHY SKIN'S PRINCIPAL AND BROAD-SPECTRUM AMP

The discovery of high amounts of AMPs like hBD-2 and hBD-3 in lesional psoriatic scale material supports the hypothesis of psoriasis skin lesions being infected less frequently by bacteria and fungi than one would have expected. Thus, induction of AMPs at inflammatory conditions, such as psoriasis lesions, was observed which might help to better understand the resistance of psoriatic lesions towards infection.

It, however, does not give a reasonable explanation why healthy skin shows rare infections, because these inducible AMPs are absent. With the hypothesis that also healthy skin should contain antimicrobial factors, we have chosen the stratum corneum from a healthy person as substrate to test our hypothesis. Indeed SC-extracts contained high titre antimicrobial activity against several Gram-negative and Gram-positive bacteria.

Using *E. coli* and *S. aureus* as test organisms in our antimicrobial readout-system, we could purify the 14.6 kDa protein RNase-7 (R7) as principal AMP of healthy donor's SC (*Harder* and *Schröder*, 2002). R7, by keratinocytes and various other epithelial cells, is produced constitutively (*Harder* and *Schröder*, 2002). It is active against many Gram-negative and Gram-positive bacteria as well as fungi. By yet unknown reasons enterococci are extremely sensitive towards R7, suggesting that it serves as special AMP killing gut-derived Gram-positive bacteria.

R7 possesses RNase-activity and represents the principal RNase of human skin, which let molecular biologists require bearing of gloves for molecular biology studies to protect RNA from degradation. Unlike one would have expected, antimicrobial activity of R7 does not depend on its RNase-activity, as site-directed mutagenesis has revealed (*Huang* et al., 2007). Artificial membrane studies with R7 and some truncated R7-peptides indicated that membrane disruption, as it is seen with diverse AMPs like defensins, is not R7's mode of bactericidal action (*Huang* et al., 2007).

The high amounts of R7 present in SC extracts and the nearly complete inhibition of *S. aureus* antimicrobial activity by R7 antibodies (*Simanski* et al., 2010) suggests that R7 is an important component of the skin's antimicrobial defence system against *S. aureus*. Indeed, determination of R7 transcripts in travellers returning with *S. aureus* positive skin infections relative to levels in controls revealed higher transcript levels in unaffected control subjects, compared with unaffected skin of case patients. No such association was present for hBD-2 or hBD-3 (*Zanger* et al., 2009).

Recent studies reveal an important role of R7 in protecting the urinary tract from infection (*Spencer* et al., 2011). The urothelium of the lower urinary tract and kidney cells produce

RNase 7. Regulation of its antimicrobial activity depends, similarly as seen in the skin (*Abtin* et al., 2009), on an endogenous inhibitor, ribonuclease inhibitor (RI) which forms in healthy epithelium a stable complex with R7, inhibiting its antimicrobial activity. Upon infection, RI is destroyed by proteolysis, liberating now antimicrobially active R7 and thus defines a unique regulatory pathway that may affect how RNase 7 maintains urinary tract sterility (*Spencer* et al., 2014).

STRATUM CORNEUM: AN UNIQUE SOURCE OF HUMAN PEPTIDE ANTIBIOTICS

With the discovery of several human antimicrobial peptides to be more or less specifically targeting different microbes, it seemed to be worth to test the hypothesis that skin (and stratum corneum) contains a huge number of AMPs, which act against different bacteria with differences in its preference to kill them. There are a number of bacteria and fungi, which are found to have the skin as habitat. Some of them are growing in an environment which is rather humid, others are growing in lipid-rich or in rather dry skin areas. Further, some are growing at rather aerobic conditions; others have their habitats in the deep, rather anaerobic, areas of the stratum corneum.

Therefore, it would be interesting to test a standard stratum corneum extract for antimicrobial activity against different bacteria. Such an analysis, which we had performed with psoriasis scale extracts in previous studies (*Harder* and *Schröder*, 2005), revealed that each bacterium shows a rather microbe-characteristic antimicrobial activity profile of HPLC fractions of a healthy person's stratum corneum extract. It was seen that in these unbiased studies that stratum corneum contains a high number of factors (characterized by its different retention times upon RP-HPLC analyses), which kill either *E. coli* or *P. aeruginosa* (different factors). With this observation one is tempting to speculate that this might be one reason why the skin is well protected from infection by the soil- and water-born bacterium *P. aeruginosa* (as well as the gut bacterium *E. coli*).

On the other side, there have been seen only very few fractions containing *S. aureus*-killing activity. This might be a reason why *S. aureus* skin-infections are not so rare. Using different microbes (skin commensals and skin pathogens) as targets in the antimicrobial read-out system, one should be able to identify with this strategy all major skin-derived antimicrobial compounds, which are produced by either healthy or inflamed skin and thus giving a profile of the antimicrobial potential of healthy and inflamed skin.

OUTLOOK

The skin is a habitat of numerous microbes, which shows an anatomical site-dependent colonization at the skin surface as well as in deeper stratum corneum layers (*Zeeuwen* et al., 2012). This is difficult to be explained solely by the presence of, until now discovered, skin-derived AMPs because their activity profile does not cover all of them. In addition, we have conditions,

which could dramatically affect activity of AMPs, such as local salt concentration, the pH and the redox potential (which would affect the presence or absence of di-sulphide bridges in e.g. defensins). Furthermore, it is possible that AMPs are generated *in situ* by cleavage of bigger skin proteins by specific enzymes which were released by bacteria. This would represent a versatile, rather microbe (enzyme)-specific defence system of the skin. Testing this hypothesis with identification of peptide fragment size and cleavage site will only be possible by analysing skin-derived material.

There is no reasonable explanation why healthy skin's microbiota, is as it has been identified (*Grice* et al., 2009), and why so many differences in its composition have been found when different anatomical sites were analysed. One would postulate that in addition to host-derived AMPs, which should be present in the stratum corneum, also microbe-derived AMPs and other, rather specifically acting antimicrobial compounds of microbial origin, contribute to the composition of the microflora. Challenging these questions by detailed analyses of host-derived protein fragments as antimicrobials and skin bacteria-derived antibiotic compounds could be a new way not only to understand what shapes the skin microflora, it could also be an innovative way for the discovery of novel antibiotics which are permanently present at our skin surface and thus are optimized for the human skin surface during evolution.

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THE EPIDERMIS OF MAN: CO-EXISTING WITH COMMENSALS (SUMMARY OF THE 28TH OLD HERBORN UNIVERSITY SEMINAR)

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"A BODY WITHOUT SKIN PROVES THAT WE ARE ALL THE SAME, BUT WITHOUT THE SKIN THERE, THERE CAN BE NO US"
(*Jablonski*, 2006).

The statement reproduced above may be a colloquial way to express the importance of skin. From a biologic perspective, the story of human skin has also been considered to represent a multitude of critical functions integral to the survival of many species. Skin has been instrumental in the long history of human racial and cultural evolution and conflicts to date. The human skin has represented the beauty and the beast in its colours, shine, wrinkles, pimples, sweat and odour, and a reflection of emotions in its sensitivity to touch. The skin has also been viewed as an assemblage of the "wounds of knowledge, the scars of truth and the limits of power" (*Emberley*, 2008). Human skin has been an object of art from times immemorial with use of cosmetics, tattoos, insertion of jewellery and other pierced decorations (*Mayell*, 2002).

The skin constitutes the biggest and the single most visible organ of human body, estimated to weigh about 40 pounds and measuring about 21 square feet in a fully-grown man. The outer layer is sloughed off and regenerated continuously. Normal human skin is an organ with multiple folds, invaginations and creases, hair follicles, sebaceous and sweat glands and characterized by varying degrees of melanin pigmentation. Although infections of the skin, associated with neurologic and or immunologic imbalance with or without manifestations of systemic disease have been well known for thousands of years, it is only recently that careful investigations have been undertaken to explore in detail the interaction between the skin, and the diverse spectrum of environmental microorganisms including bacteria, viruses, fungi, mites and other parasitic organisms which colonize the skin under different environmental and cultural settings (*Sanford* and *Gallo*, 2013). The information available about the skin, at the time this seminar was being planned, is briefly summarized.

SKIN STRUCTURE

The epidermal surface of the skin is formed by a network of cross-linked cornified cell envelopes (CCE) embedded in a matrix of water repellent lipid containing extracellular material rich in ceramide, cholesterol and free fatty

acids. The skin is richly endowed with eccrine and apocrine sweat glands, hair follicles, sebaceous glands (often as pilo-sebaceous units) and secretes sebum, which lubricate the hair and the skin surface. The breakdown-products of sebum include free fatty acids, cathelicidin, β-defensins, and other antimicrobial histones (*Gallo* and *Hooper*, 2012*)*. The anatomic differences of texture between different parts of the skin strongly impact on the qualitative and quantitative nature of microbial colonization in different sites. Although the outmost layers of the skin are critical components of the barrier function, several layers of skin below the epidermis as well profoundly impact on the barrier function. An aqueous layer above the epidermis also contributes significantly to the ecologic balance of epidermis (*Segre*, 2006). The intercellular tight junctions play an important role in the barrier function of the skin, although its role in antimicrobial barrier has not been fully explored. The epidermis represents a robust physical barrier and its cool, acidic nature is generally not a welcome environment for microbial growth.

SKIN IMMUNE SYSTEM AND MICROBIOME

It is estimated that over one billion bacteria reside in a square centimetre of skin surface and its associated appendages and glandular tissues (*Hentges*, 1993; *Sanford* and *Gallo*, 2013). At the same time, the functional components of both innate and adaptive mechanisms of immunity exist within the skin (*Kupper* and *Fuhlbrigge*, 2004). The keratinocytes as epithelial cells express several Pattern Recognition Receptors (PRR) designed for interaction with specific conserved Microbial associated Molecular Patterns (MAMP) such as lipoproteins, nucleic acids, microbial cell wall components and other microbial determinants. Keratinocytes express several antimicrobial peptides, chemokines and cytokines. Langerhans cells function as a subset of dendritic cells as antigen presenting cells and participate in regulation of immune response, including induction of tolerance via activation of regulatory T (Treg) cells. In addition, macrophages, mast cells, NK cells, CD8+, CD4+ Th1, Th2, or Th17, γδ-T cells and Treg are present within and below the epidermis. This information has been reviewed extensively in several recent publications (*Nestle* et al., 2009; *Afshar* and *Gallo*, 2013). It has been suggested that skin has the greatest diversity of variables that influence its surface characteristics, and may be better viewed as a "melting pot" of different microenvironments shifting constantly in response to the external environment and host's internal milieu (*Sanford* and *Gallo*, 2013). Thus, the microbiome in different sites of the skin must be considered as a dynamic entity, involving constantly changing quality and the quantity of its content.

The skin microbiome is in general considered either as resident or transient. The majority of normal resident inhabitants belong to the following phyla, based on 16S ribosomal RNA gene sequencing: Actinobacteria, Bacteroidetes, Firmicutes, or Proteobacteria. However, their distribution is site-dependent and exhibit significant differences between moist, sebaceous or dry areas. Microbiome in moist sites is dominated by *Staphylococcus* and *Corynebacterium* species, in sebaceous sites by *Propionibacterium* species of Actinobacteria phyla (the least diverse population of microbes), and in dry

areas by the most diverse of the microbes with varying representation from all four phyla identified above (*Eckburg* et al., 2005; *Dewhirst* et al., 2010). The resident microbes are a relatively fixed group of organisms found routinely in normal skin and they re-establish themselves after being dislodged by a variety of environmental insults. The transient microbes do not generally reside constantly in a defined skin site. However under several pathologic conditions, many organisms exhibit abnormal colonization patterns, proliferate locally, and may result in clinical disease (*Bik* et al., 2006; *Turnbaugh* et al., 2006). Differences in skin microbiome have also been observed as a function of temporal patterns of testing and interpersonal variations secondary to culture and geographical differences (*Grice* and *Segre*, 2011). The skin is initially colonized at the time of birth with a microbiome of very low diversity and is largely shaped by the method of delivery. Subsequently, by 2-3 years of age the microbiota at various body sites acquire more diversity and specificity harbouring over 150 species of microbial phylotypes (*Grice* et al., 2009).

During the past few years, a number of investigators have begun to explore the functional association of resident commensal skin microbiota with skin-derived immunologic responses, the impact of competition between commensals and pathogens via induction of innate immunity and specific priming for adaptive immune response, and the clinical consequences of altered microbiome in atopic dermatitis, psoriasis, opportunistic infections, and other disease states of host-microbial interactions (*Fukao* and *Koyasu*, 2003; *Strober*, 2004; *Sanford* and *Gallo*, 2013; *Oh* et al., 2013).

SKIN NERVOUS SYSTEM AND MICROBIOME

Contact with the skin and the sense of touch have been crucial elements in the evolution of emotional bonding between the mother and her new-born baby, and between other individuals in the social group in virtually all mammalian species. A high degree of correlation exists between lack of physical contact in childhood and higher rates of aggression later in life. Additionally hospitalized premature infants exposed to frequent physical contact, appear to have better weight gain, earlier hospital discharges and reduced rates of depression (*Feldman* et al, 2014). There is now increasing evidence to suggest that the skin establishes contact with external environment via sensory neuron end-organs. A complex network of sensory nerve fibres has been identified in the skin, which terminate as free nerve endings or specialized end-organs such as Meissner corpuscles or Touch domes. These nerve fibres are responsible for the perception of touch, temperature and local pain. In addition, the Schwann cells, and the nerve bundles that they ensheath as Remak Bundles, have been shown to function in a manner similar to the Glial cells of the central nervous system. Recent immunohistologic studies have demonstrated the presence of other sub-epidermal nerve plexus *in situ*, and the existence of other markers of neurologic function on the epidermal and dermal sheets (*Tschacheler* et al., 2004). These include, cytokeratin-20, protein gene product 9S, and neurofilament nerve growth factor (*Johansson* et al., 1990; *Griffin* et al., 2001). Furthermore, cutaneous nerves have been shown to release neuropeptides which can activate target cells such as keratinocytes,

Langerhans cells, mast cells, and endothelial cells in the skin. Finally, peptides such as substance P, IL-1, calcitonin gene-related peptide (CGRP), and vascular cell adhesion molecule VCAM-1 have also been identified in the skin after appropriate stimuli. These observations clearly support the role of nervous system in the skin in mediating biologic functions in health and disease (*Ansel* et al., 1997).

The information summarized above provides a bird's eye view of the skin surface, its acquired microbiome, and their possible interactions with the immune system and the nervous system of the skin related sites. More detailed information is available in several recent publications (*Baker*, 2006; *Grice* et al., 2009).

Several dominant organisms, especially *Staphylococcus* and *Propionibacterium* constitute the major portion of resident microbial flora. However, the mechanisms underlying the development of transient and pathogenic flora and their association with disease remain to be defined. Furthermore, factors that affect the balance between different bacterial species in different skin sites are poorly understood. The extent and the nature of potential benefits offered by the commensal and resident organisms to the host remain to be fully explored. Finally, the precise nature of cellular interactions between the skin microbiome, skin immune system, and their impact on the host's health or outcome of disease remains to be elucidated (*Kong* and *Segre*, 2011).

28TH OLD HERBORN UNIVERSITY SEMINAR

This seminar was planned to explore some of the questions raised above and other, still to be resolved, issues. It was also hoped to update the current state of knowledge about the epidermis and its microbial endowments, especially as they relate to the development of several disorders of the skin.

The seminar began with an introductory overview of the skin and its appendages as a dynamic barrier by Prof. Peter Elias. He provided a holistic view of epidermal defences based on the structural aspects of the epidermis including events associated with intra-epidermal metabolism regulated by barrier requirements and the homeostatic signalling mechanisms responsible for such metabolic events (*Elias* et al., 2008, 2014). He reviewed in some detail the structure of stratum corneum, and stratum granulosum in the inner epidermal surface, and the functions of the defensive gradient in the outer epidermis relative to permeability barrier and antimicrobial barrier, antioxidant activity, cellular cohesion, cytokine activation, neurosensory mechanisms and their possible functions, and the impact of hydration on epidermal homeostasis. The principal message conveyed by Prof. Elias in his overview was that stratum corneum is metabolically highly active and the epidermal defence functions are inter-related, co-regulated and highly interdependent. Although antimicrobial and permeability barriers function independently, they share many common features and functions. Prof. Elias also discussed the role of hydration of the skin and the biochemical and structural basis for stratum corneum hydrolysis, and the possible role of glycerol, NMF-Fil-aggrin derived products, lactic acid, glucose, salts, and urea. An interesting observation highlighted by Prof. Elias was the development of mast cell hypertrophy and degranulation after prolonged exposure of the skin to dry environment.

Prof. Paul Forsythe provided a systematic review of the role of mast cells in linking microbiome to the development of allergy. He pointed out that the induction phase of most allergic disorders are characterized by events associated with alterations in the phenotype and function of APC, induction of Treg and shift in Th1/Th2 balance. Paul reviewed recent information which suggests that microbiota significantly determines the outcome of the effector phase of the disease. He discussed the role of mast cells in microbiota-allergy axis, in the context of regulation of mast cell function by commensal bacteria employing *Lactobacillus rhamnosus* strain JB-1 as an experimental model (*Forsythe* et al., 2012). He also discussed potential mechanisms of mast cell stabilization by bacteria, employing quorum sensing molecules, and evaluation of mast cell immuno-regulation and microbial interaction in other areas such as neural, immune and endocrine systems (*Kendall* and *Sperandio*, 2006; *deKivit* et al., 2012; *Forsythe* and *Bienenstock*, 2012).

These studies have demonstrated that mast cells play an important role in host-microbiome communications and influence the development, outcome and severity of allergic manifestation at a clinical level. Cutaneous microbiome appears to have a major role in modulating mast cell function, based on the observations that non-pathogenic *E. coli* and other commensals function via induction of mast cell degranulation. However, other microorganisms utilize other distinct and very different functional mechanisms to stabilize mast cell function (*Forsythe* et al., 2012).

Prof. Richard Gallo examined in his comprehensive review the essential immune functions for commensal bacteria of the skin. He discussed the role of resident commensals in maintaining skin homeostasis, specifically via the generation of many antimicrobial peptides (AMP) (*Lai* et al., 2009, 2010). He suggested that normal resident skin microbiome plays a critical role in the suppression of any overwhelming inflammatory tissue response following mucosal injury (*Naik* et al., 2012). Based on information generated in an experimental mouse model developed in his laboratory with *Staphylococcus epidermidis*, some AMPs such as Firmocidin, appear to exhibit antibacterial as well as anti-neoplastic functions. In addition, such commensals also induce defensins and other antimicrobials to provide protection against pathogenic organisms. In particular AMP from *Staphylococcus epidermidis* have been shown to activate TLR2/CD36-P38, strengthen the cellular tight junctions in keratinocyte culture system, and enhance antiviral activity in murine skin by enhancing mast cell derived AMP, especially cathelicidin

Prof. Gallo also suggested that epidermis might be more appropriately considered a modest antimicrobial filter rather than an absolute antimicrobial barrier, based on several recent studies (*Amann* et al., 1990; *Horz* et al., 2005). He also discussed a fascinating pilot study on atopic dermatitis (AD), using lesional and non-lesional skin from the fore-arm from patients with AD. His group has demonstrated that the microflora in non-lesional skin of patients with AD consisted mainly of Proteobacteria and/or Actinobacteria. On the other hand, lesional skin contained higher proportion of Firmicutes and increased *Staphylococcus aureus* (*Sanford* and *Gallo*, 2013). He concluded that human body is truly a collection of non-human and human cellular structures, hoping to work together towards a common good. As part of this balance, non-human structures such as bacteria and other microbial antigenic

determinants may enter deep into dermis in order to maintain homeostasis via the induction of specific immune responses.

Following the discussion of the basic cellular structures and the characteristics of skin microbiome and immune system, subsequent presentations during the seminar focused on major clinical disease states, namely Staphylococcal colonization of the skin and nose, atopic dermatitis and, psoriasis. These presentations were followed by a comprehensive review on vitamin D and sunlight, participation of cutaneous nervous system in immunologic functions in the skin, and a comprehensive review of available antimicrobial peptides (AMP).

Prof. Andres Peschel reviewed the colonization with *Staphylococcus aureus* as a paradigm for the ecology of endogenous pathogens. Epidemiologic studies have shown that 80% of all severe bacterial infections are caused by only few endogenous pathogens. These include *Enterococcus faecalis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter spp.*, *Pseudomonas spp.*, *E. coli* and *Enterobacter spp*. An individual is at a significantly higher risk of invasive infection after nasal colonization by *Staphylococcus aureus*, and for increased eczema relapses after skin colonization in patients with atopic dermatitis. Colonizing pathogenic organisms appear to exhibit a complex interplay with the resident microbiome and host defence mechanisms. These include alterations in the content of nutrients, overgrowth of the endogenous pathogens and factors involving the qualitative and quantitative nature of the resident commensal microbiome. Other studies have demonstrated the impact of microbiome generated Bacteriocins and host defence immune mechanism on the endogenous pathogens and the impact of microbiome induced immune conditioning on pathogen-induced inflammation in the host. Prof. Peschel reviewed studies on nasal colonization with *Staphylococcus aureus* which indicate that interactions between Wall Teichoic acid (WTA) and scavenger receptors (SREC-1) are essential for colonization. It appears that WTA structures also govern horizontal gene transfer among major bacterial antigens. Understanding the ecology of endogenous pathogens will be the key to the effective control and prevention of diseases with such organisms.

Prof. Adrian Gombart provided an extensive review of the vitamin D and its diverse spectrum of biologic functions in man. He began his presentation with a discussion of the cleavage of B-ring of 7-dehydrocholesterol in the skin to its conversion to calcitriol, the bioactive form of vitamin D which binds to vitamin D receptor (VDR). VDR is a ubiquitous receptor expressed on a variety of cells, including T and B cells, monocytes-macrophages, dendritic cells, and neutrophils. Prof. Gombart reviewed available information about the role of vitamin D in modulating immune response via its effects on TLR, inhibition of Th17, and Th1 T cells function, promotion of tolerance in DC, and expansion of Th2 helper and regulatory T cells. He also discussed the epidermal barrier function and the role of vitamin D, antimicrobial peptides, and the skin microbiota. Vitamin D has been shown to increase expression of cathelicidin gene via activation of TLR 2/1 signalling. Considerable information is now available regarding the impact of microbiota, AMP and epithelial homeostasis in the gut (*Hooper* and *Macpherson*, 2010; *Kellermayer* et al., 2011). It appears that loss of

cathelicidin may affect the composition of gut microbiota and thus alter gut metabolism (*Gombart*, 2009). Similar to the gut, the skin has dynamic microbial ecosystems in different parts. Changes in microbial composition are associated with many skin disorders, and in certain situations the decreased bacterial diversity correlates with disease severity (*Kong* et al., 2012). However, the precise relationship between abnormal AMP expression and possible shift in microbial composition and pathology of the skin disease remains to be determined. Of particular interest was the observation that the vitamin D-CAMP pathway is primarily human and primate specific (*Gombart* et al., 2009). Based on the observation reported here, Prof. Gombart concluded that cutaneous synthesis of vitamin D and induction of cathelicidin and other antimicrobial peptides offers a unique mechanism for modulation of skin microbiota by natural sunlight.

Prof. Thomas Bieber reviewed the current state of knowledge about atopic dermatitis (AD) and the associated skin microbiome. AD is a disease of complex phenotypes and severity. He discussed the natural history of the disease in the man and in the dogs, which exhibit similar phenotypes and precipitating factors for the expression of the disease. Available information suggests that the clinical expression of AD is characterized by loss of diversity of microbiome during flare ups, stray colonization with *Staphylococcus aureus*, lack of tolerance to allergens, altered DC phenotype (FcεR++), and abnormal expression of TLR in skin cells. It has been suggested that the nature of interaction between TLR2 and FcεR1 in the dendritic cells (DC), modulate their phenotypes and function, associated with induction of Th17 and reduced expression of TLR2 in the keratinocytes and Langerhans cells in the skin. However, it remains to be shown if the microbial microenvironment is solely responsible for the modification of LC phenotypes and function. It is also not known why TLR2 expression is reduced in LC and if the altered microbiome has any lasting epigenetic impact.

Prof. Michael Gilliet discussed the immunologic and microbiologic aspects of psoriasis, a chronic relapsing, inflammatory skin disorder, affecting 2-3% of the human population worldwide. Psoriasis is an autoimmune disorder characterized by increased epidermal proliferation, significant immune activation of auto reactive T cells, and increased production of IL-22, IL-17, and IL-23 cytokines. Studies carried out in his laboratory have shown that Plasmacytoid (pDC) dendritic cells play a critical role in the development and pathogenesis of psoriasis. The pDC bearing a unique phenotype (BOCA2+, CD123+, CD4+, HCADR+) represent about 0.2% of peripheral blood mononuclear cells. It has been proposed that skin pDC are activated following injury or damage to the skin as an initial insult, which results in expression of IFN-α by the pDC. Expression of IFN-α is followed by expression of TNF-α and subsequent activation of IL-22, IL-17A/F and IL-23 cytokine and associated genetic phenotypes. These events are responsible for the maintenance phase of the clinical disease. The pDC are activated by specific antimicrobial peptides, especially the highly cationic amphipathic human β-defensins (hBD2, hBD2), Iyz, LL-37. These peptides are produced by the keratinocytes and induce the breakdown of tolerance to self-DNA. These peptides also disrupt the bacterial membranes, form complexes with native DNA and

thereby promote their immunogenicity. Autoimmunity against native DNA also appears to be promoted by IL-26 with direct antimicrobial activity, and by IL-17 that appears to be bactericidal via the induction of IL-26. Based on these studies, Prof. Gilliet proposed that the pathogenesis of psoriasis is mediated in part by abnormally increased production of some AMPs, followed by increased pDC and IFN-α production resulting in persistent chronic inflammation and auto-reactive immune responses and specific increase in epidermal proliferation (*Reizis* et al., 2011).

Prof. Michael Zasloff provided an integrated perspective of the basic biology of the microbiome, nervous system and immunity in the skin as discussed by other speakers as it relates to infection-induced tissue damage in clinical situations: tissue injury in diabetes mellitus and anti-tumour effects of bacterial infections of skin. He reviewed the historical aspects of severe experimentally-induced erysipelas on the regression of sarcoma lesions in man (*Linder* et al., 2010). Based on available limited evidence, Prof. Zasloff proposed that the process of tissue damage in the ulcer in a diabetic foot begins with loss of sensory nerve fibres which results in:

a) impaired homeostasis of AMP expression and significant breakdown of antimicrobial barrier,
b) impaired release of neuropeptides such as substance P (SP) and CGRP, and loss of neuronal mediated pro-inflammatory responses, and
c) failure to communicate effectively with other functional elements of central nervous system.

The loss of sensory nerve fibre function is related to impairment of innate immune responses in the epidermis in such patients.

In an effort to explain the historical observation on the regression of sarcoma tissue following experimentally induced erysipelas, Prof. Zasloff proposed that invading microorganisms or their toxins stimulate central autonomic vascular responses, associated with vasoconstriction of pre-capillary arterioles surrounding the sarcoma mass, resulting in anoxia, and breakdown and liquefaction of the tumour mass (*Chiu* et al., 2013).

One of the principle reasons for organizing this seminar was the recent surge of academic interest in mucosal microbiome and in the identification of many antimicrobial peptides, In particular, the Magainin antimicrobial peptides in the Xenopus skin (*Zasloff*, 1987).

This seminar concluded with a special lecture delivered by Prof. Jens Schröder on the discovery of human epithelial antimicrobial peptides. He began with lessons learned about the AMPs identified in patients with psoriasis. As pointed out earlier, it is a disease with widespread and extensive breakdown in epithelial barrier, and yet, overt microbial infections are surprisingly rare in psoriasis. It is now evident that uppermost layers of epidermis generate neutrophil attracting chemotactic factors and a large number of other antimicrobial peptides. These include many defensins, psoriasins, calprotectin and others peptides. Human β-defensin-2 (hBD-2) has been shown to link innate and adaptive immunity through dendritic cells and T cell receptor CCR6 and specially target Gram-negative bacteria and Candida. Human β-defensin-3 (hBD-3) is a broad spectrum AMP directed against many Gram-positive organisms. Resistance to skin specific infections by *E. coli* is largely mediated via Psoriasin (s100A7) and calprotectin (s100A8/9),

which are expressed in site-specific patterns in different skin sites. RNase7 another important AMP specifically targets enterococcus for its antimicrobial effects (*Schröder* et al., 1998; *Schröder* and Harder, 2006; *Simanski* et al., 2010).

CONCLUDING REMARKS

Human skin has often served as a mirror of many systemic infections and autoimmune disease processes. These include, systemic infections associated with cutaneous exanthema, enteric diseases characterized by cutaneous manifestations, and allergic or autoimmune disorders in which different parts of the skin may serve as the as primary target of disease manifestations. It is also of interest to note that intra-cutaneous and sub-cutaneous sites have been employed with varying degrees of success for delivery of vaccines, induction of allergic desensitization and delivery of pharmaceuticals for prophylactic or therapeutic intervention against many systemic disorders. Recent evidence has suggested that immune system of skin is well integrated into the common mucosal immune system with significant circulation of APC, antigen sensitized T and B cells and other mediators between the mesenteric lymphoid tissue, gut mucosa and the skin (*Glenn* et al., 2007; *Lawson* et al., 2010).

Because of limited availability of time, it was not possible to discuss these areas in much detail during this seminar. However, during the open discussion next day, Prof. Richard Walker introduced recent studies supported by the program for appropriate technology in health (PATH), involving successful intradermal immunization with enterotoxigenic *E. coli* (ETEC) against an oral challenge with virulent coliform organisms. Other phase I trials with transcutaneous patches impregnated with vaccine antigens have also demonstrated effective development of mucosal immune responses in the gut. These observations provide further support for a common immune system involving the skin and the mucosal sites.

Several other important aspects of the skin microbiome and its interaction with its neural and immunoreactive cellular components were highlighted during the discussion. The spectrum of such interactions range on one hand, from the development of clinical disease with *Staphylococcus aureus* in atopic dermatitis and the diabetic foot, and on the other, to relative protection against bacterial infections in psoriasis. The role of the interactive skin associated innate and adaptive immune responses, cutaneous nervous system, and of antimicrobial peptides in the regulation of homeostasis of the skin under normal or pathological studies remains to be precisely defined. There is no clear explanation for the mechanisms underlying the existence and qualitative nature of human skin microbiome as it has been identified to date. It is not known if the skin microbiome has undergone significant alteration during cultural evolution of man to date. The extent to which antimicrobial peptides observed in the skin are host or microbiome derived is also not known. Hopefully, this seminar will stimulate sufficient interest to pursue these and many other important questions regarding the skin and its microbiome. Nevertheless, it is now abundantly clear that skin is a remarkable organ and its biologic complexity far exceeds its cultural and cosmetic perceptions. It represents a major habitat

of a multitude of organisms in a site-dependent colonization pattern at the surface, deeper stratum corneum and its associated appendages.

It was in the context of this information that Prof. Thomas Bosch provided a final thought provoking evolutionary perspective of the early life forms and their interaction with the native microbiome in the marine ecosystem, He elegantly explained the complex communications linking the rudimentary nervous system in *Hydra* as a "holobiont" to its marine microbial composition and the bidirectional communication between the microbiota and the components of host epithelium. Holobiont is a host organism (plant or animal), which is in constant interaction with all its associated microorganisms, as an entity for selection of evolution. Based on the lessons learnt from *Hydra* and the observations in human and other mammalian species, it is clear that humans represent the best example of a holobiont to date (*Matyssek* and *Luttge*, 2013). However, the course of their future evolutionary outcome will be ultimately determined by the nature and the critical balance, between its human elements and all associated non-human component microorganisms.

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