Epidermal tight junctions in health and disease

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Keywords: claudins, skin barrier, skin innate barrier, skin immune system, tight junction

Abbreviations: AD, atopic dermatitis; AMP, antimicrobial peptides; Cldn, claudin; DC, dendritic cells; FLG, filaggrin; JAM, junctional adhesion molecule; LC, Langerhans cells; MM, malignant melanoma; PS, psoriasis; SCC, squamous cell carcinoma; SC, stratum corneum; SG, stratum granulosum; SNP, single nucleotide polymorphism; PRR, pattern recognition receptor; Th, T helper; TJ, tight junction; TER, TransEpithelial Electrical Resistance; TLR, Toll-like receptor; ZO-1, zonula occludens 1

The skin, the largest organ of the body, is an essential barrier that under homeostatic conditions efficiently protects and/or minimizes damage from both environmental (e.g. microorganisms, physical trauma, ultraviolet radiation) and endogenous (e.g., cancers, inflammation) factors. This formidable barrier function resides mainly in the epidermis, a dynamic, highly-stratified epithelium. The epidermis has 2 major barrier structures: stratum corneum, the outmost layer and tight junctions, intercellular junctions that seal adjacent keratinocytes in the stratum granulosum, found below the stratum corneum.

In recent years there have been significant advances in our understanding of tight junction function, composition and regulation. Herein we review what is known about tight junctions in healthy skin and keratinocyte culture systems and highlight the dynamic crosstalk observed between tight junctions and the cutaneous immune system. Finally we discuss the preliminary observations suggesting that tight junction function or protein expression may be relevant for the pathogenesis of a number of common cutaneous inflammatory and neoplastic conditions.

Overview of the Skin Barrier

All animal and plants are armed with “physical barriers” that separate the internal space from their sometimes hostile and ever changing environment. In mammals, the major epithelial barriers are found in the cutaneous, respiratory, urogenital and gastrointestinal systems. In addition to physically separating the internal compartment from the external environment, these epithelial structures control the highly selective and specialized directional transfer of gases or solutes from outside-in (e.g. O₂ and metabolites) and from inside-out (e.g., CO₂ and waste).

The skin by virtue of its unique anatomical position is the epithelial surface at the frontline of host defense. A healthy skin

barrier protects and/or minimizes damage from both routine and extreme environmental factors (outside-in barrier; i.e., microorganisms, physical insults and UV radiation). In addition, the skin barrier regulates the loss of fluid, electrolytes and proteins (inside-out barrier). This formidable barrier function resides mainly in the epidermis, the outermost layer of the skin; a dynamic, continually renewing and highly stratified epithelium. Skin epithelial cells or keratinocytes are by far the most abundant cell type in the epidermis. As keratinocytes differentiate and move away from the basement membrane they undergo a complex terminal differentiation process that culminates with the formation of the stratum corneum (SC; For a review see ¹). The different stages of keratinocyte differentiation can be visually depicted by 4 histopathologically recognizable layers by H&E staining. The basal layer consists of undifferentiated, mitotically active keratinocytes, which are attached to the epidermal basement membrane and generate cells for the more superficial layers. The spinous layer is characterized by prominent desmosomes, which are intercellular structures with a spine-like appearance, synthesis of differentiation-specific keratins (i.e., keratins 1 and 10) and early differentiation markers (e.g. involucrin). The granulosum layer is recognized by the keratohyalin granules, which are cytoplasmic organelles packed with electron dense proteins, such as pro-filaggrin and keratin intermediate filaments. Lastly, the stratum corneum, the outmost layer, is made up of protein-enriched enucleated corneocytes surrounded by a complex extracellular lipid matrix. This extracellular lipid matrix is largely formed by the contents of lamellar bodies which are secretory organelles packed with precursor lipids found in SC. These lamellar bodies are first evident in the upper spinous layer, but it is at the interface between the granulosum and corneum layer that they cluster together, fuse with cell membranes and ultimately release their contents into the extracellular space. Corneocytes are large, flattened, and terminally differentiated keratinocytes, which have typically lost their nuclei and other cell organelles. Although keratinocytes make up at least 80% of the cellular composition of the epidermis, other cells are present including melanocytes, Merkel cells, and immune cells such as antigen presenting cells (Langerhans cells and dendritic cells) and leukocytes.¹ Epithelial and immune cells form an integrated system of barriers (i.e., physical, chemical, immunologic). The number and type of immune cells generally increases under pathological situations.

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Submitted: 08/27/2014; Revised: 10/03/2014; Accepted: 10/04/2014
http://dx.doi.org/10.4161/21688370.2014.974451

Tight Junctions – The Under-Appreciated Epidermal Barrier Structure

Tight junctions (TJs) seal the intercellular spaces between epithelial cells and the ‘tightness’ of this structure is dynamically regulated by environmental factors and addresses physiologic needs.² This dynamic regulation is important as it determines the trafficking patterns of ions, proteins and even the penetration of the dendrites of Langerhans or dendritic cells.^{3,4}

Not so long ago, the mere existence of epidermal TJs was debated but now the controversy is their relative importance for epidermal barrier function. For a long time, the dominant concept was that the paracellular flux of ions, water and solute in the epidermis was exclusively regulated by the intercellular lipid present within the SC (for a review see ⁵). In 1944, Drs. Rothman and Flesch⁶ suggested that another barrier structure in the skin might control diffusion of water and solutes through the paracellular pathway. They speculated that: “the absorption-barrier is to be placed in the transitional layers between cornified and non-cornified epithelium, i.e., in the stratum granulosum and stratum lucidum, which represent an electric double layer with positive hydrogen ions on the outside and negative hydroxyl ions on the inside” (quoted from⁶). In 1971, K. Hashimoto using high resolution electron microscopy (EM) described intercellular

junctions morphologically compatible with TJs and the exclusion of the electron dense lanthanum tracer at the granular cell layer.⁷ However, more conclusive evidence of epidermal TJs in human adult skin was not established until the beginning of the millennium.⁸⁻¹⁰ Almost at the same time, the notion of a functional TJ barrier in mammalian epidermis was revived by the observation that epidermal permeability barrier dysfunction developed in claudin (cldn)-1 knockout and cldn-6 transgenic mice.^{11,12}

Unlike the simple monolayer epithelium present in the gastrointestinal tract and bronchi where each cell expresses the full range of cellular junctions (e.g., adherens junctions, desmosomes, gap junctions, TJs), the epidermis is a highly stratified structure where cells from different layers express unique combinations of junctional complexes.¹³ For example, hemidesmosomes are only expressed in the basal layer. Desmosomes, which are composed of desmoglein/DSG and desmocollin/DSC proteins and adherens junctions typically identified by expression of E-cadherin and β -catenin, are present in all viable layers. However, desmosomes differ in their specific composition based on the epidermal layer (e.g. DSG3 in basal and spinosum; DSG1 above spinosum).¹³ Modified desmosomes called corneodesmosomes are present in the SC, and play an important role in corneocyte cohesion/disadhesion and in so doing regulate desquamation. Typical

Figure 1. Representative staining of claudin-1, occludin and CD1a (LCs marker) in healthy human epidermis (whole-mount). Occludin (red), (A) staining showed a characteristic chicken wire pattern in the upper layers of human epidermis, while claudin-1 (green), (B) staining appeared less prominent (Top view). As noticeable in the side view of 3D reconstructed z-stacks, occludin expression is restricted to top stratum granulosum layers, as it can be identified with DAPI (blue-nuclear staining). Claudin-1 staining is visible from the basal layer up to stratum granulosum. Under homeostatic condition, Langerhans cells (CD1a-red); (C) bodies are located in the basal layer with the dendrites extending toward the SG (side view), (D). This can be appreciated better in the side view of merged claudin-1 and CD1a image (D) and with DAPI (E). Importantly, majority of CD1a-positive dendrites did not extend above the SG and stop at the top of claudin-1 staining. Bars: 40 μ m.

TJ complexes have been identified by EM studies in the SG.^{3,8,10,11,14-16} Intriguingly, in the epidermis, as well as in cultured primary keratinocytes, some TJ proteins (e.g., cldn-1, cldn-4 and cldn-7) can be observed in layers that are not thought to have functional TJs (Fig. 1). Accordingly, TJ-related structures have been identified by EM below and above the SG, especially in the interdesmosomal region of various stratified epithelia.^{9,17} Over the past 2 decades outstanding progress has been made in this field, but much remains to be done to fully appreciate the role epidermal TJs play in human health and disease. In this paper we will review what is known about the epidermal TJ barrier function and composition, its regulation by the immune system as well as the relevance of these observations for dermatologic diseases.

TJ Composition and Function in Healthy Skin (and Cultured Keratinocytes)

A number of TJ proteins have been identified in human (and/or murine) epidermis and their cultured keratinocytes. They include claudins, TJ associated MARVEL protein (TAMP) and junctional adhesion molecule (JAM) transmembrane families as well as several TJ plaque proteins (e.g. Zonula occludens/ZO and cingulin).¹⁸ Interestingly, most of the TJ proteins, identified by immunostaining of the epidermis, localized to the cell-cell borders of the SG (e.g., cldns-1, -4, -6, -7, -11, -12, -18, occludin, ZO-1, ZO-2, multi-PDZ domain protein/MUPP-1,

cingulin; see Table 1), where the functional TJ barrier has been found (representative staining of human epidermis for occludin, ZO-1 and cldn-1 is shown in Figs. 1 and 2). In addition to their localization in the SG, some TJ proteins have been observed in the upper spinous cell layer (ZO-1, ZO-2, cldns-4, -6, -18), or in all layers with variable degrees of staining intensity in the basal cell layer (cldn-1, -7, -12, JAM-A, MUPP-1), suggesting functions unrelated to typical TJ structures.¹⁹ In addition to the already large number of TJ proteins identified in human or mouse epidermis, some TJ proteins have been identified only in cultured keratinocytes (Table 2). Further studies are needed to

Table 1. TJ- related Proteins localized to the stratum granulosum in human and mouse. Note that several of these proteins are also found in other epidermal layers. (Only the first description is cited.)

Protein with Localization in the Stratum Granulosum	Human	Mouse	Citation(s)
Claudin-1	+	+	10,11
Claudin-2	Only cytoplasmic	Not investigated	117
Claudin-3	+ (?)	-	86,113,118
Claudin-4	+	+	108,119
Claudin-5	Only cytoplasmic	Not investigated	84
Claudin-6	-	+*	120
Claudin-7	+	Not investigated	83
Claudin-11	Not investigated	+	120
Claudin-12	Not investigated	+	120
Claudin-18	Not investigated	+	120
Claudin-23	Not investigated	Cytoplasmic	121
Occludin	+	+	11,85, 122
Tricellulin	Not investigated	+	3
ZO-1	+	+	11,122
ZO-2	Not investigated	+	122
MUPP-1	+	Not investigated	83
Cingulin	+	Not investigated	83

(?): was only reported in one manuscript but not confirmed by others;
(*)Claudin-6 is only expressed in utero in mice. Claudin-6 is down regulated after birth, and if ectopically expressed in the skin after birth, severe barrier defect was induced.
Claudin-10¹²⁰ and claudin-3¹¹⁸ were also found in the SC of mice.

Figure 2. Representative staining of claudin-1 and ZO-1 in tumors and healthy skin sections. Staining of claudin-1 (A, C, E, G), ZO-1 (B, D, F, H) and claudin-5 (C2) in malignant melanoma (A, B), Merkel cell carcinoma (C, C2, D), squamous cell carcinoma (E, F) and healthy skin (G, H). Note the presence of ZO-1 in all 3 tumor entities (B, D, F), while Claudin-1 is absent/faintly cytoplasmic in melanoma (A), and Merkel cell carcinoma (C) and downregulated in squamous cell carcinoma (E) as compared to healthy skin (G). e: epidermis, t: tumor, Bar: 50 μ m.

Table 2. TJ molecules identified in cultured keratinocytes in addition to those mentioned in **Table 1**

TJ molecules identified in cultured human keratinocytes	mRNA	Protein	Immuno-localization	Citation(s)
Claudin-8	+	Not investigated	Not investigated	10
Claudin-9	+	+	Cytoplasmic	56
Claudin-12	+	Not investigated	Not investigated	10
Claudin-14	+	+	Cell-cell borders	58
Claudin-17	+	Not investigated	Not investigated	10
Claudin-23	+	+	Cytoplasmic	25,55,58

determine their expression patterns in a fully developed mature epidermis. On first glance, there seem to be differences between human and mouse TJs (**Tables 1 and 2**), but since several TJ proteins have only been investigated in human or mouse one should not conclude compositional (and functional) differences yet.

When investigating epidermal TJ protein expression patterns there are important methodological issues to consider. First, the epidermal thickness (and hence the number of keratinocyte layers) varies in samples from different anatomical locations (e.g. thickest in palms and soles and thinnest around the eyes). Additionally, diminished expression of *cldn-1* below the upper spinous layer and a broader localization of *cldn-4*, occludin and ZO-1 has been reported in chronic sun-exposed skin as compared to non-sun-exposed samples.²⁰ This is important to consider when studying the expression pattern of TJ proteins in “normal” skin and even more when comparing it to disease samples. Methodologic issues need to be considered as we have observed variability in the penetration of primary and secondary antibodies based on the thickness of the epidermis and/or SC when a whole-mount epidermis is stained (Yoshida unpublished observation). We have also observed nonspecific SG cytoplasmic staining for several TJ proteins, which was also seen in matched Ig isotype negative controls suggesting that one needs to exercise caution as the specificity and sensitivity of TJ protein antibodies which remains a problem in this field (Brandner unpublished observation). Lastly, it is believed that tissue fixation and staining procedures may also introduce artifact. To ensure reproducibility and accuracy of the findings the source of samples and the methods used should always be carefully and critically evaluated.

Epidermal TJs were shown to form an inside-out barrier for intermediate sized molecules (557 Da, first description mouse,¹¹ first description human¹⁶) and larger molecules (32 kDa²¹). Although several publications allude to an inside-out barrier to the electron dense ion tracer lanthanum in adult skin,^{3,7,15,22} it is still challenging to distinguish whether this barrier property is from TJ or SC.²³ Studies investigating the outside-in barrier function of epidermal TJs are complicated by the close anatomical proximity of the SC, which prevents potential tracers from reaching the TJ barrier. However, the permeability/barrier function of TJs is bidirectional with the direction dependent from the molecules' gradient, thus if inside-out TJ barrier properties are shown the same should apply for their outside-in barrier

property. Concerning the exact localization of the functional TJ barrier, it has been suggested that it is localized in human and murine skin in the mid layers of the SG^{3,11,21}; although other studies have found TJ barrier to be at the uppermost layer of the SG.¹⁵

Using cultured keratinocytes, it was shown that, TJs form a barrier to water²⁴ and molecules of different size (²⁴⁻²⁸) as well as ions (e.g. Na⁺, Cl⁻, Ca²⁺).²⁴ Knockdown experiments in cultured keratinocytes have shown that *cldn-1*, *cldn-4*, occludin and ZO-1 contribute to the TJ barrier for Na⁺, Cl⁻, Ca²⁺ and 4 kDa FITC Dextran.²⁴ While, only *cldn-1* and ZO-1 were indispensable for the barrier for larger molecules (40 kDa).²⁴ In skin explants, treatment with ochratoxin A resulted in a loss of the TJ barrier for a 557 Da tracer and was associated with reductions in *cldn-4* expression.²⁹ However, it is not clear whether ochratoxin A is specific for *cldn-4*, or whether it may also affect expression/localization of other barrier related proteins. It is puzzling that reduced expression of *cldn-1* in keratinocytes did not show an effect on water barrier as measured by a modified Ussing chamber,³⁰ neither in human primary keratinocytes (siRNA mediated K_D; 83 +/- 8% knockdown) nor in mouse keratinocytes isolated from *Cldn-1* homozygous knock-out (KO) mice.²⁴ This was highly surprising, because *Cldn-1* KO mice die within the first day of birth due to high transepidermal water loss.¹¹ Interestingly, no abnormal water loss was observed in heterozygous *Cldn-1* KO mice.¹¹ We propose a few possible explanations for these observations. First, the abnormal water loss observed in the homozygous mice may be a downstream effect of *Cldn-1* KO on SC proteins and lipids which leads to the impaired water barrier.^{24,31,32} Indeed, Sugawara et al.³² recently demonstrated that *Cldn-1* KO mice have an abnormal SC based on alterations in ceramide composition and flaggrin processing as well as enhanced water evaporation. Second, we can speculate that residual amount of *cldn-1* (at least in the human siRNA knock-down experiments and in the *Cldn-1* heterozygous mice) may be sufficient to control water flux. Additionally, since cultured keratinocytes were not fully differentiated (i.e., no SC barrier), it is possible that other compensatory mechanisms are in place in this model to control water flux and compensate for the reduced *cldn-1* expression.

Besides regulating paracellular diffusion barrier, studies in simple epithelial models strongly suggest that TJs contribute to cell polarity, regulation of gene expression patterns, proliferation and differentiation.³³ Validation of these suspicions and the mechanisms that underlie these biological actions need further investigation.¹⁹

Advantages and Limitations of Methodologies to Investigate Epidermal TJ Function

A measurement often used to investigate TJs ion barrier function is transepithelial resistance (TER). However, one has to be aware that TER reflects the barrier strength of the whole cell layer or epidermal tissue to ions. In monolayers, TER does not distinguish between transcellular and paracellular passage of ions³⁴; however, it was shown that the paracellular barrier formed by TJs is the main contributor to TER in keratinocytes.²⁴ Nevertheless,

it is important to highlight that TER does not exclusively reflect the function of the TJ barrier in keratinocytes. This is particularly important to consider when investigating the influence of drugs and cosmetics on the TJ barrier. Macromolecular tracer flux primarily measures TJ-dependent paracellular passage of molecules. When both TER and tracer flux change following a challenge (e.g., drugs, cytokines) it strongly indicates an effect on TJ function, with high TER and low permeability indicating increased and low TER and high permeability flux suggesting reduced TJ integrity. However, it is possible to observe changes in either the TJ ion or macromolecular permeability as an isolated event, independent of each other,³⁵ because ions and (larger) molecules are likely to use different pathways through the TJ.³⁶ Therefore, even isolated changes in TER without a change in tracer flux or vice versa may reflect TJ changes. To clearly distinguish between paracellular and transcellular ion permeability, 2-path-impedance can be used (For a review see³⁴). This approach combines impedance spectroscopy with flux measurements of small (molecular weight < 500 Da) paracellular tracers. However, this is still not in widespread use as there is not a commercially available “ready to use” system. Using this method, it was shown that knock-down of *cldn-1*, *cldn-4*, *occludin* and *ZO-1* in primary keratinocytes, induces a strong increase in paracellular ion flux.²⁴ Interestingly, reductions in *cldn-1* and *ZO-1* also induced a slight, but significant, increase in transcellular ion permeability suggesting a more global effect on epidermal function when reducing the expression of these TJ proteins.²⁴

In the fully mature, multilayered epidermis and models thereof TER measurement likely reflects the contribution of both, the TJ and the SC, to transepithelial resistance. To the best of our knowledge, no device or method exists that can selectively distinguish the relative contribution of these 2 barrier structures to measures of ion permeability. A similar criticism can be levied for tracer flux experiments. When the tracer is applied to the surface of the skin, it will have to go through the SC before reaching the TJ barrier. When employing physical (e.g. tape stripping) or chemical (e.g., acetone) strategies to remove the SC, it is important to consider the possible effect of the procedure on TJs, independent of any other variables being tested. Measurement of a residual TER after removal of the SC, by tape stripping, can result in quite high values ($\leq 5000 \Omega \times \text{cm}^2$) supporting the hypothesis that epidermal TJs have an ion barrier function.¹⁸ However, this barrier property might actually be underestimated if TJs are damaged or overestimated if TJs becomes tighter in a compensatory effort following tape stripping.

An alternative approach to investigate epidermal TJ paracellular barrier is to use Sulfo-NHS-LC-Biotin. This tracer can be visualized in the epidermis, while still allowing for concomitant staining for TJ proteins. When applying this tracer to the dermis its movement out is limited by TJs, while it is largely restricted by the SC when applied on the skin surface.^{3,11,15,16,21,29,37} This suggests that epidermal barrier function is quite complex, and the relative importance of the 2 major barrier structures (SC and TJ) may be in part determined by the route (from outside-in or from inside-out) of the challenges (ions and/or solutes, antigens, or microbes) and from the integrity of each barrier.

Epidermal TJs and Immunological Barriers

The cutaneous response to environmental (e.g. pathogens, allergens) or endogenous (e.g., cancer, tissue inflammation) stimuli is tightly regulated by the physical and immune (innate and adaptive) barrier systems, which are intimately linked. Keratinocytes are thought to be the key cellular constituent of this complex barrier system. Besides their role in forming the physical skin barrier, keratinocytes express many pattern recognition receptors (PRRs; e.g. Toll-like receptors [TRL], NOD [nucleotide-binding oligomerization domain]-like receptors), produce a wide range of antimicrobial peptides (e.g., LL-37, β -defensins, Ribonucleases [RNases], and S100 proteins), chemoattractant cytokines and adjuvants (e.g., mediators that promote and direct the adaptive immune response) in response to a range of stimuli.³⁸⁻⁴⁰ Chemo-kines and cytokines are involved in recruiting innate effector cells and T cells to the skin in addition to regulating keratinocyte functions such as proliferation and differentiation.^{41,42}

In addition to keratinocytes, the skin has a vast collection of cell types that contribute to innate and adaptive immune functions: tissue-resident phagocytes, antigen-presenting cells (LC and dendritic cells/DC), mast cells, T cells (intraepithelial lymphocytes [IEL] and circulating), and the newly identified innate lymphoid cells (For review see^{38,43}). We will discuss the putative roles of the epidermal TJ network in regulating the skin immune system under homeostatic and pathologic conditions.

TJ and Innate Immune Barrier

The first barrier that potential pathogens will encounter (outside-in direction) on the epidermis is the SC (or mucus in airway and intestine) and the second physical barrier is the TJ. Penetration of pathogens behind the physical barriers will trigger an immune response and potential tissue damage. Considering the impressively high amount of microbes constantly in contact with our bodies, one of the functions of the physical barriers is to segregate the immune receptors (and cells) from skin surface ligands to avoid activation of the receptors under non-pathologic conditions.

In the case of epithelial monolayers such as the colon, several “physiologic” mechanisms have been described that efficiently segregate the PRRs from their ligands present on luminal microbes that populate the mucosal surfaces, including the TJ barrier.^{44,45} TLR2 and TLR4 are largely expressed on crypt epithelial cells (protected from luminal flora), but the expression is lost as the cells mature and move toward the gut lumen. In contrast, TLR3, a viral sensor, is only expressed in mature epithelial cells.⁴⁴ This selective pattern of expression suggested that intact mature columnar epithelium could tolerate continuous exposure to bacteria, while still responding to viral pathogens (via TLR3), which are not thought to be part of the normal gut commensal microbiota. Similarly, activation of TLR5 occurred only when flagellin, (a TLR5-ligand) was added to the basolateral (below apical junction complex) but not the apical surface of colonic epithelial cells.⁴⁵ In a murine model flagellin exposure to injured, but not intact colonic epithelium significantly enhanced inflammation and histopathological changes in the colonic mucosa.⁴⁵

Recently, using an immunostaining approach of full thickness human epidermis, Kuo et al.⁴⁰ revealed that TLR2 and 3 localized on the cell membrane and in the cytoplasm, respectively, in the epidermal layers below the TJ network (as identified by staining for the TJ component, occludin).

Besides blocking PRRs from binding to exogenous ligands, TJ integrity might prevent viral entry by limiting access of the viruses to their entry receptors (for a review see ⁴⁶). Interestingly, several junctional components can serve as viral entry receptors (e.g., claudin-1-hepatitis C virus, nectins-herpes simplex virus, CAR-coxsackie virus and adenovirus receptor). However, when a virus approaches an intact epithelial surface (from the apical side), its receptor is likely hidden within (i.e., not accessible) or below the TJ structure. The evolutionary forces that have evolved to enable viruses to establish a productive infection are beyond the scope of this review article (for review see ^{47,48}).

It is well established that susceptibility of human keratinocytes to herpes simplex virus (HSV)-1 infection is inversely related to confluency and specifically the degree of cell-cell contact.^{49,50} This suggests that healthy junctional complexes may deter the spread of HSV-1 from one keratinocyte to another. Furthermore, disruption of TJ integrity, by incubation in low calcium media or by selectively silencing *cldn-1* mRNA, leads to increased HSV-1 infectivity in differentiated primary human keratinocytes (De Benedetto & Beck unpublished observation and ⁵¹).

Clinically, Kaposi's varicelliform eruption (or eczema herpeticum) is a cutaneous widespread eruption caused by HSV and observed in a subset of subjects with atopic dermatitis (or eczema) and possibly less commonly even in an immunologically distinct inflammatory skin condition called psoriasis.^{52,53} Subjects with atopic dermatitis can also develop similar clinical manifestations with several other viruses (e.g., Vaccinia, Coxsackie). It is noteworthy that both of these inflammatory skin disorders have been shown to have alterations in the expression of TJ proteins and abnormal keratinocyte differentiation although they are otherwise immunologically and genetically different. Therefore it is reasonable to postulate that the epithelial barrier alterations are responsible for the cutaneous viral susceptibility of these two diseases.

Importantly, the cutaneous innate immune system seems to be well equipped with mechanisms that can restore the TJ barrier integrity following physical injury or microbial challenge. For example, stimulation of primary human keratinocytes with TLR2 ligands enhances the barrier function of TJs.^{54,55} An increase in *cldn-1*, *cldn-23*, occludin, and ZO-1 expression was observed commensurate with changes in barrier function⁵⁵ while, Yuki et al.⁵⁴ demonstrated occludin-phosphorylation by atypical protein kinase C (aPKC). Similarly, treatment of human epidermis wounded by repeated tape stripping (e.g., model of mechanical injury) with TLR2 agonists enhanced skin barrier recovery (as measured by transepidermal water loss [TEWL]).⁵⁵ In keeping with this observation, Tlr2 knockout mice had a delayed and incomplete barrier recovery following tape stripping.⁵⁵ Recent studies in cultured keratinocytes have also shown that other innate mediators, such as antimicrobial peptides (AMPs) including S100A7, LL-37 and HBD3 enhance TJ protein expression

and TJ function (TER and macromolecule tracer permeability) thus contributing to repair and possibly even maintain homeostatic barrier function.⁵⁶⁻⁵⁸

It is important to note that the aforementioned studies utilized bacterial-derived peptidoglycan or synthetic TLR2-ligands, in an otherwise "sterile" environment and these stimulations were done during TJ assembly (e.g., wound repair model or differentiation of keratinocytes). The situation can be quite different and more complex when using whole bacteria or when evaluating the effect in full thickness epithelium with fully mature TJs. Ohnemus et al.⁵⁹ evaluated the effect of infection with different exfoliative toxin-negative *Staphylococcus aureus* (*S. aureus*) strains as well as *S. epidermidis* on TJ function and composition in the human keratinocyte cell line, HaCat, and in porcine skin. Interestingly, *S. aureus* strains induced a rapid (within 3 to 5 hours) redistribution (reduced cell membrane localization) of key TJ components (e.g., *cldn-1*, *cldn-4*, ZO-1 and aPKC) which was associated with reduced TER. In contrast, infection with *S. epidermidis*, a non-pathogenic normal skin inhabitant, enhanced TER, strongly suggesting that the microbial flora can significantly impact skin barrier function.⁵⁹ In the skin infection model, both pathogens induced an upregulation of TJ proteins at early time points, followed by reductions in protein expression, which were predominantly observed with *S. aureus*.⁵⁹ Consistent with these observations, primary human keratinocyte cultured so that they develop mature TJs responded to *S. aureus* exposure with an initial increase followed by a decrease in TER (Brandner unpublished observation).

In summary, a growing body of research suggests that the epidermal TJ network can be viewed as an immunologic boundary controlling the exposure of innate immune receptors (below TJs) with their ligands/antigens (above TJs and/or SC). Considering the remarkable biomass of the cutaneous microbiome (For a review see ⁶⁰), the physical separation between innate receptors and microbes could be considered an example of innate tolerance. Perturbations of this fine balance, that might be mediated by loss of TJ integrity or cutaneous pathogen load, would lead to activation of the innate immune system and in so doing the initiation of an adaptive immune response.

TJ and Adaptive Immune Barrier

A critical step in the initiation of an adaptive immune response is the encounter between the antigen and the Langerhans Cell (LC) or dendritic cell (DC). Under homeostatic conditions LC/DC reside below the TJ network, and are therefore physically separated from the myriad of antigens bombarding the skin surface. In the 1990s, Proksch and et al.⁶¹ demonstrated a strong correlation between epidermal LC density and the magnitude of the barrier disruption induced by tape stripping (measured by trans epidermal water loss) in healthy human subjects. Furthermore, they demonstrated that the increase in LC density was correlated with the inflammatory response observed following an allergen patch testing, suggesting that LC also respond after barrier disruption.⁶² One hypothesis is that tape stripping not only removes the SC, but also perturbs the integrity of TJ complex, which would increase permeability of allergens.

Recently, Kubo et al.³ elegantly demonstrated that tape stripping of murine skin resulted in the rapid (within 15 minutes after barrier disruption) upward movement of LC dendrites with some extending above TJ and taking up antigens applied on the skin surface. During this process new TJs were formed between keratinocytes and LC dendrites that arguably serve to maintain the integrity of the epidermal barrier. This is also observed in the gut and airways where DCs can establish transient TJs with the neighboring epithelial cells while penetrating the epithelial barrier to uptake antigens.^{4,63} Importantly, in the gut, *cldn-1* and occludin are expressed on DC cell membranes at baseline and ZO-1 expression can be rapidly induced following stimulation.⁶³ In the epidermis, LCs express *cldn-1*⁶⁴ and JAM-A⁶⁵ at baseline (Representative staining of *cldn-1* and CD1a [LCs marker] in human epidermis is shown in Fig. 1).

These studies highlight a dynamic interaction between LC/DC and epithelial TJs that are likely critical for the proper initiation of an adaptive immune response. One can speculate that in skin conditions characterized by epidermal barrier disruption (e.g., atopic dermatitis or psoriasis), an alteration in the TJ integrity could favor an encounter between LC/DC and antigens which may at least in part explain the nature of the inflammation observed in the dermal compartment in these disorders. This is supported by a recent study by Takano K. et al. that noted DCs expressing *cldn-1* penetrated beyond occludin-identified TJs in nasal epithelium from patients with allergic rhinitis, and this was not observed in healthy controls.⁴

After LC/DC have taken up a specific antigen/allergen, they will present it to naïve T cells to elicit an adaptive immune response. The character and magnitude of the adaptive T helper (Th) lymphocyte immune response (e.g., Th1, Th2, Th17, Th22, Treg) is determined by the microenvironment where the LC/DC activation occurred as well as where the naïve T cells are primed (lymph nodes and possibly skin⁶⁶). Tape-stripping of human (and murine) skin induces the production of a number of inflammatory mediators (e.g., ATP, CXCL8, IL10, IFN γ and TGF α), as well as pro-Th2 (TSLP), pro-Th22 (TNF α , IL6) and pro-Th17 (IL1 β , IL6, TGF β) cytokines.⁶⁷⁻⁷⁰ Brandner et al. have recently observed that the knockdown of ZO-1 induces the release of IL1 β from primary keratinocytes.⁷¹ We are not aware of any additional studies that have investigated whether perturbations of TJ complexes indirectly or directly induce the production of specific inflammatory mediators. It is our hypothesis that a specific epidermal barrier defect (genetic and/or acquired) could lead to a signature microenvironment and in so doing modulate the character of the adaptive immune response.

The crosstalk between TJs and the adaptive immune response is bidirectional. Numerous studies have demonstrated that several inflammatory mediators (e.g., IL4, IL13, IL25, IL17A and histamine) affect keratinocyte differentiation, most often leading to impaired barrier formation.⁷²⁻⁷⁵ Studies on noncutaneous epithelium have established that inflammatory mediators are involved in TJ assembly and disassembly during inflammatory conditions,⁷⁶ and also in keratinocytes an influence of several cytokines on TJ proteins/function was described (see below). Whether these mediators directly affect epidermal TJs or whether

it is an indirect effect via altered differentiation is an active area of research. Under homeostatic conditions a proper differentiation process is required to form competent TJ structures; consequently, abnormal TJ formation should be expected in the context of processes that impair differentiation. On the other hand, abnormal epidermal TJ could lead to impaired terminal differentiation process, as has been shown in the *cldn-1* knockout mice (e.g., abnormal lipids profile, defective processing of pro-filaggrin to filaggrin)³² and reconstructed human skin with occludin knockdown²⁰ (see also¹⁸ for the correlation of differentiation and TJs).

IL1 β , which is highly expressed in plaques of Psoriasis and to a lesser degree in Atopic Dermatitis lesions, alters TER and TJ protein expression when applied to differentiating epidermal cultures in a biphasic way, by first increasing TJ protein expression and TER followed by a decrease in both.⁶⁵ TNF α , another prominent cytokine observed in psoriasis lesions, results in a similar biphasic alteration of TER in developing TJs.⁶⁵ Interestingly, TNF α has also been shown to affect SC by reducing the expression of filaggrin and loricrin.⁷⁷ When these same cytokines were applied to mature, well-developed TJs, IL1 β had no effect while TNF α enhanced TER.⁶⁵ This clearly shows that these cytokine influence TJs, but that their effect depends on the differentiation status of the keratinocytes and the duration of exposure.

Recently, we have investigated the effect of IL17A on epidermal barrier function. IL17A is a cytokine involved in host defense against various pathogens and it is thought to play a central role in psoriasis while its function in atopic dermatitis remains unclear.^{78,79} In agreement with previous studies in intestinal epithelial cells,⁸⁰ we observed that IL17A enhanced TJs assembly (e.g., earlier TER peak as compared to media alone) in cultured primary keratinocytes.⁸¹ This was associated with an increase in *cldn-4* expression. However, co-treatment with IL4, a cytokine critical in atopic dermatitis pathogenesis, blocked the IL17A-mediated effects on TJ.⁸¹ At longer time points IL17A reduced the expression of several markers of terminal differentiation as well as intercellular junctions, including TJ proteins (e.g., filaggrin, loricrin, *cldn-7* and E-cadherin) (⁷² and Beck & De Benedetto unpublished). Table 3 summarizes a list of references of mediators currently tested on keratinocytes TJ.

Collectively, these studies suggest that epidermal TJs are dynamically modulated by the inflammatory milieu. It is tempting to speculate that under pathologic situations where skin barrier is compromised (e.g., infection, wound, trauma) the enhancement of epidermal TJ integrity through the use of inflammatory mediators might be a rapid and cost-effective way to quickly improve the epidermal barrier function as compared to SC repair. These preliminary observations also highlight the importance of understanding the differentiation status of ones epidermal model when determining the effect of cytokine stimulation or infection. Since KC differentiation affects the expression of TJ and other intercellular junction proteins, as well as cytokine/innate receptors it is imperative that investigators characterize their epidermal model carefully in order to more precisely interpret observations made with the model.

Table 3. This table provides a list of recent references that investigated the effect of inflammatory mediators on TJ protein expression and/or function in tissue, primary or immortalized keratinocytes

Inflammatory mediators tested on Keratinocytes TJ (references)	
Cathelicidin LL37	56
EGF	123
Estradiol	124
FGFs	125
Histamine	74
Human β -Defensin 1	126
Human β -Defensin 3	58
IL1 β	65,86
IL4	25
IL13	25
IL17A	72
Progesterone	124
S100/A7Psoriasis	57
Somatostatin	127
TLR2 agonists	54,55
TNF α	65

TJ Abnormalities in Human Skin Disorders

Common human inflammatory skin disorders, such as atopic dermatitis (AD) and psoriasis (PS), as well as skin tumors, have been associated with the abnormal expression of epidermal TJ components. The functional relevance of these abnormalities needs further study.

Psoriasis (PS) vulgaris is a chronic, inflammatory skin disease (Th1/Th17) driven by a combination of genetic, environmental, and immunological factors, with an overall prevalence of 2% to 3% of the worlds population.⁸² Clinically, PS is characterized by well-demarcated, erythematous, plaques covered with silvery scales. Histologic hallmarks include thickened epithelium, retention of nuclei within the cells of the SC (parakeratosis), and loss of the granular layer (hypogranulosis) due to premature keratinocyte maturation and incomplete differentiation. In addition, infiltrating immune cells can be found within the upper papillary dermis (DCs and CD4⁺ Th cells) and within the epidermis (CD8⁺ T cells and neutrophils).

Altered expression of several TJ proteins have been observed in lesional PS skin. A wider (e.g., more positively stained layers) expression pattern for occludin, ZO-1 and cldn-4 with both cell membrane and intracellular localization was observed in PS.^{8,65,83-85} Kirschner et al.⁶⁵ reported reduced expression of cldn-7 at the cell-cell borders. There is currently no consensus about cldn-1 patterns as some report no differences and others show reduced expression at different strata of the epidermis.^{65,84,86-88} The reason for such variability is unclear to us. Unfortunately, the published studies do not allow for correlation of the staining results with any particular feature, such as disease severity, age of the PS plaques (e.g., acute vs chronic), systemic or local treatment, anatomical localization (e.g., sun-exposed) or even staining procedures (e.g., reagents, skin processing). Clearly more rigorous studies are needed to clarify the relative expression of cldn-1 in PS. Nevertheless, the altered expression of a number of TJ components (cldn-1, cldn-4, ocln and ZO-1) is observed

in early PS plaques,⁶⁵ strongly implicating their involvement in disease pathogenesis. However, no changes were observed in normal appearing skin from PS subjects,^{25,84} thus arguing against a primary TJ defect in this disease. This is also supported by results from in vitro studies described above where cytokines present in PS lesions (e.g., IL1 β , TNF α , IL17A) have been shown to affect the epidermal TJ complex.

The biologic relevance of altered expression of TJ proteins in PS pathogenesis is still unclear. Kirschner et al.¹⁶ used the Sulfo-NHS-LC-Biotin assay to investigate the TJ barrier property in PS skin samples. In both, healthy skin and psoriasis, a stop of the tracer is found at sites positive for occludin and claudin-1 colocalization, demonstrating that in PS a functional TJ barrier still exists. In healthy skin, the stop of the tracer (applied from the dermal side) is in the SG layer bordering to the SC. In PS skin, this tracer is stopped earlier, with still several keratinocytes layers between the tracer stop and the SC. This suggested us that not the TJ barrier function per se, but its localization might play a role in PS pathogenesis. Further, alterations of TJ proteins result in alteration of differentiation (for review see¹⁹), which might also contribute to the PS pathogenesis.

Atopic Dermatitis (AD) is a chronic, relapsing inflammatory skin disease, which affects up to 20% of school-aged children.⁸⁹ It is characterized by an overactive Th2 ($\pm$ T22/Th17)^{79,90} dermal immune response to a host of environmental allergens and the entire skin surface appears dry or xerotic. Recent evidence suggests that both features are due in part to an epidermal barrier defect.^{91,92} The theory is that a leaky barrier promotes immunologic responsiveness to allergens.⁹³ A critical aspect of the barrier theory is that even nonlesional or clinically “unaffected” AD skin is compromised, thus suggesting that the epidermal barrier defect precedes the development of the clinical manifestations. The SC is dysfunctional in AD as the result of one or more of the following defects: reduced levels of SC lipids,⁹⁴⁻⁹⁶ acquired or genetic defects in structural proteins such as filaggrin (FLG)⁹⁷⁻⁹⁹ and/or scratching that is a cardinal feature of the disease. De Benedetto et al. have identified reduced expression of cldn-1 and -23,²⁵ and more recently cldn-4 in clinically uninvolved, nonlesional AD skin of a Northern American cohort (De Benedetto & Beck unpublished). Importantly, the reduced expression of TJ components was associated to a significant alteration in the bioelectric characteristics of AD epidermis (non-lesional, non-sun-exposed) with markedly lower TER, higher albumin permeability was associated with the loss of ion permeability selectivity. Although a contribution of SC barrier alteration is likely (as discussed above), these observations suggest that there is also a TJ defect in these AD patients. In addition, there was an inverse relationship between epidermal cldn-1 expression and markers of Th2 polarity (e.g., blood eosinophilia [eosinophils/mm³] and serum total IgE) when evaluating all study phenotypes (AD, nonatopic and psoriasis). This suggests that inflammation, particularly Th2 inflammation, may inhibit the expression of key claudin family members (e.g., cldn-1, -4 and -23) or vice versa. To investigate whether single nucleotide polymorphisms (SNPs) in the claudin-1 (CLDN1) gene may associate with AD, AD severity or the AD subphenotype, eczema herpeticum in two North American

populations (African American/AA and European American/EA) we employed a haplotype-tagging approach. The most robust association was in the AA population, with an intronic SNP associated with reduced (rs17501010; $P = 0.003$) and an adjacent SNP associated with enhanced (rs9290927; $P = 0.004$) risk of AD. SNP-rs17501010 also showed a modest association with early onset AD (<5 yrs of age; $P = 0.04$) in AA. In addition, 2 SNPs (rs893051 in intron 1 and rs9290929 in the promoter region) were associated with greater disease severity in AA ($P = 0.010$ and $p = 0.007$, respectively). More modest associations were observed in the EA population and include a promoter SNP (rs16865373) associated with lower risk of AD ($P = 0.034$) and lower risk of early onset AD (<5 yrs of age; $P = 0.034$). Interestingly, several of these same CLDN1 SNPs (rs893051, rs9290927, rs9290929 and rs17501010) were associated with sensitization to contact allergens in a Northern European population.¹⁰⁰ Nothing is known about epidermal cldn-1 expression in subjects with contact dermatitis and yet these preliminary observations suggest that this would be worth investigating.

There is a rare human syndrome called neonatal ichthyosis with sclerosing cholangitis (NISCH), caused by a null mutation in CLDN1. The skin phenotype of the few reported NISCH cases is notable for ichthyotic, xerotic and erythematous lesions.¹⁰¹ Cldn1 is also highly expressed in the liver (e.g., hepatocytes and bile duct epithelia) and this explains the liver pathology (sclerosing cholangitis which can lead to liver failure) observed in these patients. While reductions of cldn1 in the keratinocytes affects the appearance of skin, it leads to a dry appearance much like what is observed in the nonlesional skin of AD subjects.

Ichthyosis vulgaris (IV) is the most common form of inherited ichthyosis with autosomal semi-dominant transmission pattern. Most cases are caused by a loss of function mutation in the filaggrin gene (FLG). Homozygous patients show a more severe phenotype compared to heterozygous patients, suggesting a gene-dose effect. It is clinically characterized by fine white to gray scales, which are more prominent on the abdomen and outer surfaces of the extremities and typically spare the flexures and face. Palms and soles are often hyperlinear. About 1/3 of these subjects will also develop AD. Subjects with IV have reduced epidermal expression of occludin and ZO-1, which is more pronounced in homozygous than in heterozygous patients. In addition, barrier function for lanthanum is abolished.¹⁰² Interestingly, this study was performed in IV subjects without any clinical signs of inflammation, arguing that the TJ dysfunction is a direct effect of reduced/absent FLG. In the flaky tail mouse, which has a spontaneous mutation in FLG (similar to the mutations found in subjects with IV) and a matted tail mutation (Tmem79/matt) investigators have observed a reduction in occludin (at both mRNA and protein level).¹⁰³ These investigators also observed reductions in occludin (mRNA and protein levels) in HaCaT cells transfected with FLG-siRNA and highlighted that lorixin and the silent mating type information regulation 2 homolog 1 (SIRT1) might be part of the signaling pathway that links FLG and occludin.¹⁰⁴ However, it cannot be ruled out that the Tmem79/matt mutation may contribute to changes of occludin

in the flaky tail mouse. In our studies, we observed disrupted ZO-1 membranous staining in the epidermis from FLG-deficient mice (backcrossed onto BALB/c strain and to remove the matted mutation) as compared to wild type (BALB/c) control mice.⁸⁷ Altogether these studies suggest that a primary defect in FLG, a SG-SC barrier protein, can secondarily affect the expression and possibly function of TJs.

Growing body of evidence suggests that the TJ proteins are involved in important steps of tumorigenesis, such as apoptosis, alteration of cell-cell adhesion and tumor invasion (for a review see¹⁷). Malignant melanoma (MM) is the most lethal skin cancer. MM originates from melanocytes in the skin, but it can also arise on mucosal surfaces (e.g., oral, vaginal), in the uveal tract of the eye and leptomeninges. The incidence of MM has increased dramatically worldwide, with the highest incidence reported in Australia with more than 30 cases per 100,000 subjects per year (Western Australian Cancer Statistics; <https://www.cancerwa.asn.au/resources/statistics/>). Several studies have investigated the expression and function of TJ proteins in MM. The use of different MM cell lines and evaluation of a number of TJ proteins have resulted in confusing and sometimes discrepant observations, making it difficult to summarize the role of TJ proteins in MM. An increase in ZO-1 expression (mRNA and protein level) was observed in melanoma cells compared to melanocytes. Even though ZO-1 was found in the tumor cells at the cell-cell borders (see Fig. 2), it was demonstrated to be associated with N-cadherin, thus suggesting a non-TJ related function.¹⁰⁵ When ZO-1 expression was reduced in a melanoma cell line, this was associated with diminished invasiveness, suggesting a contribution of ZO-1 to melanoma invasiveness.¹⁰⁵ Cohn et al.¹⁰⁶ demonstrated by immunolabelling intracellular localization of cldn-1 in primary MM (25%) as well as metastatic MM (3.2%). Interestingly, in several melanoma cell lines (e.g., M93-047, UACC903, M6012B), cldn-1 was also observed primarily in the cytoplasm and these cell lines had very low TER values indicating an absence of mature TJ complexes.¹⁰⁷ Overall, the intracellular localization of cldn-1 in MM suggests that this protein may have some functions independent of those linked to TJ barrier function. Indeed, overexpression and knockdown of cldn-1 in melanoma cell lines resulted in increase or decrease, respectively, of MMP-2 expression and activity.¹⁰⁷ The overexpression of cldn-1 was associated with an increase in motility of melanoma cells as assessed by scratch wound assay.¹⁰⁷ Morita et al.¹⁰⁸ identified cldn-12 by mRNA and membranous staining in melanoma cell lines but no TJ functional assays were performed to address whether this resulted in an alteration in barrier function.¹⁰⁹ In summary, most of the TJ proteins identified so far in MM are primarily expressed intracellularly or are associated with adherens junctions, and preliminary observations suggest that they may be important for motility and invasiveness.^{105,107}

Merkel cell carcinoma is a rare but aggressive skin tumor, associated with an increased expression of several TJ proteins at the cell-cell borders. The most highly expressed TJ proteins are cldn-3 and -5 as well as occludin and ZO proteins, with only occasional ($\approx 20\%$; see Fig. 2) expression of Cldn-1.^{110,111} TJ structures can be visualized by EM in Merkel cell carcinoma cell

lines,¹¹² however, virtually nothing is known about their function. One might suggest, that the TJs present in the tumor can isolate certain tumor areas and reduce their accessibility for immune cells and chemotherapeutics, therefore resulting in difficulties to clear and target the tumor.

For keratinocyte-derived tumors, such as squamous cell carcinomas (SCC), a broader expression pattern of ZO-1 and occludin and reduced expression of cln-1 in deeper epidermal layers (e.g., lower SS and basal) has been shown.^{20,113} However, this was also found in SCC precursors and in sun-exposed skin²⁰ and seems therefore mainly due to chronic sun exposure. Downregulation of cln-1 in the uppermost layers was found in SCC and its precursors, but not in sun-exposed skin and thus seems to play a role in tumorigenesis.²⁰ A striking difference between SCC and actinic keratosis or Bowen's disease was found for occludin. Although the protein expression of occludin was completely lost in most SCC cases, it was still detectable in actinic keratosis and Bowen's disease.²⁰ Occludin knockdown experiments reconfirmed its influence on TJ barrier function²⁴ as well as highlighted its role in differentiation, cell-cell adhesion and keratinocyte susceptibility to apoptosis in response to UVB exposure and TNF-related apoptosis-inducing ligand (TRAIL) stimulation.²⁰ Reduced expression of occludin was also found in other squamous cell tumors originating in the lung and oral cavity,^{114–116} thus suggesting it might be a common feature for these types of tumors. Hintsala et al.,¹¹³ described an upregulation of cln-2, 3 and 5 immunostaining in some SCC, but no images were shown to assess if these proteins were expressed in typical sites. In conclusion, TJ protein alterations have been described in various skin tumors (Fig. 2) including melanoma, Merkel cell carcinoma and squamous cell carcinoma and its precursors. Evidence from studies in melanoma suggest that TJ protein contribute to tumor progression which is largely TJ barrier independent, while in other tumors TJ barrier dependent and independent influences may play a role.

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Concluding Remarks

Over the past few decades, we have gained extensive knowledge about TJ complexes localization, composition and function in human epidermis as well as in cultured keratinocytes. Novel methods have been developed or adapted from other epithelial models to study epidermal TJs. Moreover, we have started to recognize the abnormal expression of TJ proteins in several common skin inflammatory diseases and cancers.

The challenges for the upcoming years in this field are to develop methods to quantify the TJ barrier in multilayered epithelia, and to clarify non-barrier related TJ protein functions within the epidermis in healthy and disease states. Understanding the molecular pathways that regulate epidermal TJs (function and composition) will be critical since they will likely have immediate therapeutic relevance. Hopefully, in the near future we will be able to test novel therapeutics to restore TJ integrity as a novel approach to treat common skin conditions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

The authors recognized the following funding sources: Deutsche Forschungsgemeinschaft (FOR 721/2, BR 1982-4-1 JMB); Atopic Dermatitis Research Network (NIAID subcontract HHSN272201000020C and HHSN272201000017C; L.A.B.), NIH/NIAMS R21 (AR062357; L.A.B.); Dermatology Foundation (A.D.B.) and University of Rochester Medical Center (A.D.B.).

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