PATHOGENICITY AND EPITOPE SPECIFICITY OF IgG4 AUTOANTIBODIES IN ENDEMIC PEMPHIGUS FOLIACEUS

Flor de María Evangelista Montoya

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Approved by:

Stephen H. Clarke, Ph.D.

Luis A. Diaz, M.D.

Zhi Liu, Ph.D.

Stefanie Sarantopoulos, M.D., Ph.D.

Barbara J. Vilen, Ph.D.

ABSTRACT

FLOR DE MARIA EVANGELISTA MONTOYA: Pathogenicity and Epitope Specificity of IgG4 autoantibodies in Endemic Pemphigus Foliaceus
(Under the direction of Dr. Zhi Liu)

Endemic pemphigus foliaceus, also known as Fogo Selvagem (FS), is an autoimmune blistering skin disease that exhibits geographic clustering and 100% mortality without treatment. An active focus of FS is located in the rural reservation of Limao Verde (LV), Mato Grosso do Sul, Brazil. It is widely accepted that the autoantibodies that mediate FS are predominantly of the IgG4 subclass against Desmoglein 1 (Dsg1) and that healthy individuals living in LV posses a mixture of circulating IgG anti-Dsg1 autoantibodies. It has been suggested that an unknown environmental trigger present in LV may induce an anti-Dsg1 autoimmune response, which in certain genetically predisposed individuals leads to a pathogenic IgG4 response and clinical disease. The transition from preclinical to clinical disease in FS has been associated with subclass switching and epitope spreading within the extracellular domain of Dsg1.

The N-terminal region of Dsg1 has been reported to be the target for pathogenic FS IgG autoantibodies. However, the fine epitope specificity for FS IgG4 autoantibodies remains unknown. The work in this dissertation refines the identification of epitopes in Dsg1 that are recognized specifically by IgG4 autoantibodies from FS patients. Two dominant conformational epitopes were identified using epitope excision and MALDI-MS/MS. One epitope was located within the EC1 domain of Dsg1 (A129-R144) and the second epitope in

the EC2 domain (Q201-R213). Moreover, residues M133 and Q135 are required to achieve the proper conformation of the epitope recognized by pathogenic IgG4 autoantibodies in FS.

This study also reports that IgG4 FS antibodies recognize LJM11, a protein found in the saliva of *Lutzomya longipalpis*. In addition, mice immunized with LJM11 generate anti-Dsg1 antibodies. Thus, insect bites may deliver salivary antigens that initiate a cross-reactive IgG4 autoimmune response in genetically susceptible individuals, which subsequently leads to development of FS.

The findings of this dissertation may uncover targets such as the epitope in EC1 for the development of epitope-specific preventative and therapeutic strategies. Additionally, this work identifies an environmental, non-infectious antigen, which may trigger development of potentially pathogenic autoantibodies in autoimmune disease.

This dissertation is dedicated to my husband Kevin, for his unconditional love, support and for being a blessing in my life. To my mother Pilar and to the memory of my father Félix, for all their love and for providing me with the support to get to this moment. To my brother Félix for all his love, for being my inspiration to pursue an intellectual career, and for taking care of our mother during my "adventure" away from home.

"How will I pay the Lord for all the good He has done for me?"

(Psalm 116,12)

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LIST OF ABREVIATIONS

AFM	atomic force microscopy
Anti-HA	anti-influenza hemagglutinin antibody
Anti-His	anti-histidine antibody
A129	alanine at position 129
A130	alanine at position 130
Bcl-2	B-cell lymphoma 2
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CAR	cell-adhesion recognition site
cDNA	complementary Deoxyribonucleic acid
CH1	constant domain 1
CNBr	cyanogen bromide
DEAE	diethylaminoethyl cellusose
DP	desmoplakin
Dsc	desmocollin
Dsg	desmoglein
EC	extracellular domain
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EM	electron microscopy
F(ab)	fragment (antigen binding)

FAT	phenylalanine, alanine, threonine
FBS	fetal bovine serum
Fc	fragment crystallizable region
FS	Fogo Selvagem (endemic pemphigus foliaceus)
GST tag	glutathione S-transferase tag
HAV	histidine, alanine, valine
HC	human control
hDsg1	human desmoglein 1
hIgG4	human immunoglobulin class G subclass 4
His-tag	histidine-tag
HLA	human leukocyte antigen
HRP	horseradish peroxidase
HSP	heat shock protein
IB	immunoblot
IEN	intraepidermal neutrophilic
IgA	immunoglobulin class A
IgG	immunoglubulin class G
IgG4	immunoglobulin class G subclass 4
IP	immunoprecipitation
IRB	Institutional Review Boards
kD	kilodalton
LV	Limao Verde (rural reservation in Brazil)
MALDI-TOF/TOF MS/MS	matrix-assisted laser desorption ionization time of flight mass spectrometry

MALDI-MS/MS	short version of MALDI-TOF/TOF MS/MS
MAPK	mitogen-activated protein kinase
MEC	mutated extracellular domain
mPV	mucosal Pemphigus Vulgaris
mcPV	mucocutaneous Pemphigus Vulgaris
μg	microgram
μL	microliter
mM	millimolar
M133	methionine at position 133
NCBI	National Center for Biotechnology Information
ng	nanogram
NHS	normal human serum
NIAID	National Institute of Allergy and Infectious Diseases
Ni-NTA	nickel-nitriloacetic acid
O.D.	optical density
PBS	phosphate buffered saline
PCR	polymerase Chain Reaction
PF	pemphigus foliaceus
PG	plakoglobin
PKP	plakophilin
PNP	paraneoplastic pemphigus
PV	pemphigus vulgaris
Q135	glutamine at position 135

Q201	glutamine at position 201
RAL	arginine, alanine, leucine
ROC	receiver-operating-characteristic analysis
RT	room temperature
R144	arginine at position 144
R213	arginine at position 213
scFv	single-chain variable fragment
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SFM	serum-free medium
SGLL	salivary gland from *Lutzomyia longipalpis*
SPD	subcorneal pustular dermatosis
TBS	tris buffered saline
TFA	trifluoroacetic acid
TPCK	L-1-tosylamido-2-phenylethyl chloromethyl ketone
UNC	University of North Carolina at Chapel Hill
US	United States
VHH	heavy-chain single variable domain antibody
WT	wild type
W51	tryptophan at position 51
YAG	yttrium-aluminum garnet
YAS	tyrosine, alanine, serine
YAT	tyrosine, alanine, threonine

CHAPTER I

INTRODUCTION

1.1. PEMPHIGUS

Pemphigus is a group of rare, autoimmune blistering diseases of the skin and mucous membranes mediated by autoantibodies against desmosomal members of the cadherin superfamily. There are several variants of pemphigus, each with unique clinical, histologic, and immunologic features. Interestingly, when different desmosomal proteins are targeted by the autoimmune response, different clinical and histologic features are seen [1, 2].

There are two major intercellular junctions that mediate cell-cell adhesion in squamous epithelia: desmosomes and adherens junctions. Keratinocytes express transmembrane glycoproteins such as desmogleins and desmocollins to form desmosomes, whereas E-cadherin forms adherens junctions. It is widely accepted that the antibodies that mediate pemphigus are of the IgG isotype, predominantly of the IgG4 subclass, and are directed against the extracellular domain of desmosomal cadherins [3, 4]. Binding of these autoantibodies causes loss of epithelial cell adhesion, via a process known as acantholysis, which leads to blister formation in the epidermis. Pemphigus autoantibodies are pathogenic when passively transferred into neonatal Balb/c mice, generating blisters at the injection site, thus resembling the human disease [5]. The immunological targets for pemphigus autoantibodies have been characterized by immunochemical methods such as immunoprecipitation and immunoblotting since the 1980s [6, 7]. With the introduction of

cloning techniques and cDNA libraries, the isolation of cDNA for pemphigus antigens demonstrated that desmogleins are indeed the target antigens in pemphigus.

1.2. INTERCELLULAR JUNCTIONS AND THE CADHERINS:

Intercellular junctions in the epidermis allow keratinocytes to adhere to one another and maintain the integrity of the epithelium. Morphological and biochemical studies have defined two major types of intercellular junctions in epithelial cells: adherens junctions and desmosomes [8, 9]. These intercellular junctions are composed of cadherins, a superfamily of calcium-dependent adherent proteins, which play an important role in the dynamic regulation of intercellular adhesion [10]. The cadherin superfamily is characterized by multiple "cadherin repeat" sequences of about 110 aminoacids in their extracellular domains (EC) and comprised of two major groups: *Classical cadherins* (E-, P-, N-cadherin) and *desmosomal cadherins* (desmogleins and desmocollins).

Classical cadherins are expressed in the adherens junctions and share high structural and sequence homology with the extracellular domains of *desmosomal cadherins* that are expressed in the desmosomes. All cadherins are type 1 transmembrane glycoproteins presenting 5 tandem cadherin repeats (EC1 to EC5) of about 110 aminoacids containing calcium–binding sites and a cell-adhesion recognition (CAR) site in their extracellular domains [11-13]. The CAR site is a tripeptide motif located within EC1 (the most amino-terminal repeat). In *classical cadherins*, this motif is HAV (histidine, alanine, valine), whereas in *desmosomal cadherins* the motif may be RAL (arginine, alanine, leucine), YAT (tyrosine, alanine, threonine), FAT (phenylalanine, alanine, threonine) or YAS (tyrosine, alanine, serine) [14]. *Classical cadherins* are ultimately linked to the actin cytoskeleton via

α- and β-catenin [15, 16], and *desmosomal cadherins* are linked to the intermediate keratin filaments through plakoglobin and desmoplakins I and II [9, 17].

E-cadherin and P-cadherin are classical cadherins expressed in the epidermis. E-cadherin is expressed in all layers of the epidermis, whereas P-cadherin is limited to the basal cell layer [18].

THE DESMOSOME:

The first observation of the *desmosome* was made by the Italian pathologist Giulio Bizzozero (1864). He observed them as small, dense nodules and called them "nodes of Bizzozero". The term *desmosome* emerged later with Joseph Shaffer in 1920, from the Greek words "desmo" (bond) and "soma" (body) [19]. In the subsequent years, techniques such as electron microscopy (EM), have revealed the complex structure and organization of the desmosomes. The desmosome consists of two morphologically identifiable zones: the extracellular core region (desmosomal core) and the intracellular dense plaque region (desmosomal plaque). With the introduction of techniques such as genetic cloning and immunohistochemistry, the components of the desmosome have been cloned, their organization within the organelle has been mapped, and their ability to interact with one another has been defined [20]. As shown in **Fig. 1**, there are three major components in the desmosome : a) *desmosomal cadherins* (desmogleins and desmocollins), b) *the armadillo family members* (plakoglobin and plakophilins) and c) the *plakin family* (desmoplakins). All 3 components link to each other, and ultimately to the intermediate keratin filaments, in order to maintain cell-cell adhesion.

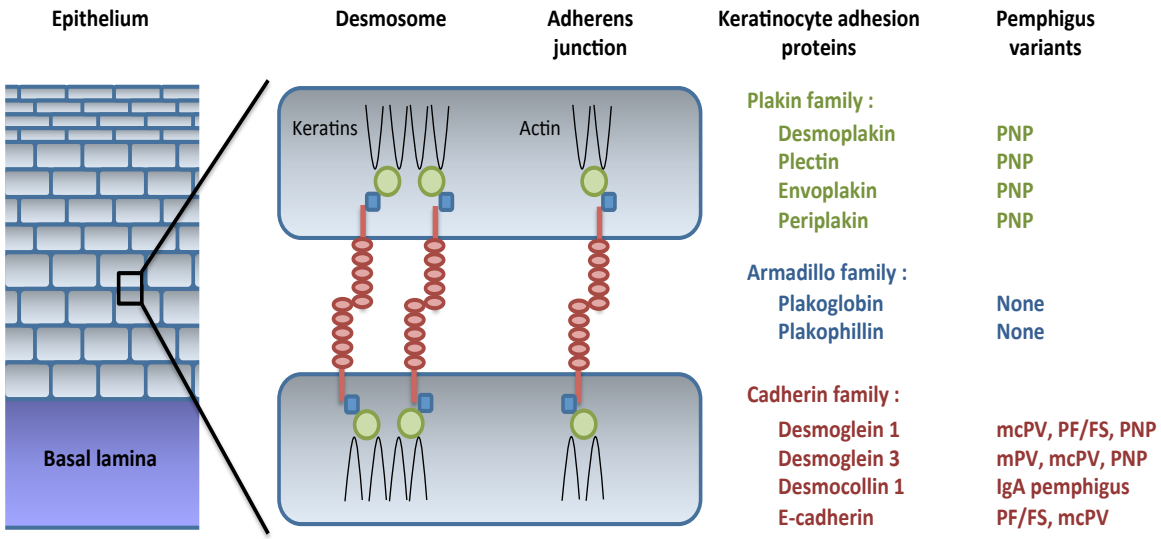

Figure 1. ***Molecular components of intercellular junctions and pemphigus variants.*** Adhesion proteins expressed in the intercellular junctions as targets for pemphigus variants are shown. PF=pemphigus foliaceus, FS=Fogo Selvagem, mPV=mucosal pemphigus vulgaris, mcPV= mucocutaneous pemphigus vulgaris, PNP=paraneoplastic pemphigus.

a) Desmosomal cadherins:

Desmosomes were originally isolated from cow nose epidermis [21] and chemically characterized [22]. High levels of glycosylated proteins were found to be the main component of the desmosomes, and they were postulated to mediate cell-cell adhesion [23, 24]. Antibodies against a particular desmosomal glycoprotein were developed and used to screen a cDNA library. In this manner, the first desmosomal cadherin was cloned, sequenced, and named desmoglein derived from the greek "glein" (glue) [25]. Since then, the *desmosomal cadherins* family has been found to consist of seven different proteins: four

desmoglein isoforms (Dsg1, Dsg2, Dsg3 and of Dsg4) and three desmocollin isoforms (Dsc1, Dsc2 and Dsc3), all encoded by separate genes located on chromosome 18 in humans [26, 27].

Dsg1 and Dsc1 are the major desmosomal cadherins in the skin where they are expressed throughout the epidermis, but most prominently in the upper layers. Dsg3 and Dsc3 expression is predominant in the lower epidermis and decreased toward the upper layers. Dsg1/Dsg3 and Dsc1/Dsc3 are mostly restricted to stratified epithelial tissues, such as the skin. Dsg2 is expressed in a wide range of other simple epithelial tissues and myocardia in the heart, whereas Dsg4 is mostly expressed in hair follicles [28]. Desmocollins and desmogleins are always found as a pair in the desmosomes; however, the precise nature of their interaction is still under debate. Several studies indicate that *desmosomal cadherins* contain a CAR site within their N-terminal EC1 extracellular domain and that this site is critical for maintenance of desmosomal adhesion [11]. The CAR site sequence for Dsg1 and Dsc1 are RAL and YAT, respectively [13]. Interestingly, peptides derived from these sequences were able to block the homophilic adhesion mediated by Dsg1 and Dsc1 upon incubation [12, 13]. Based on predictions from the C-cadherin crystal structure, recent cryo-electron microscopy studies in human epidermis showed *cis*- and *trans*-interactions of the EC1 domains, possibly via insertion of the tryptophan in position 51 (W51) into the hydrophobic pocket of the CAR site [29]. However, they also found some *cis*-interactions of the EC 4-5 domains and it has been suggested that *desmosomal cadherins* may show periodically zipper-like arrangements similar to classical cadherins [30].

It is well known that *classical cadherins* form homo-dimers in order to mediate adhesion. In contrast, several studies indicate that *desmosomal cadherins* undergo both

homophilic and heterophilic trans-interactions. Using EC1-2 fragments of Dsg2, Dsc2 or Dsg3, it was shown that homophilic interactions occur in vitro [31, 32]. In addition, Waschke et al. have utilized atomic force microscopy (AFM) to estimate the strength of interaction between purified Dsg1 ectodomains [33] and showed that, in fact, Dsg1 could form homo-dimers and that the molecular binding properties of this homophilic adhesion may be comparable to other cadherins. Thus, mutation of key amino acid residues such as tryptophan in position 51(W51) and alanine at position 130(A130) that are involved in adhesion mediated by classical cadherins, abolished homophilic adhesion of desmosomal cadherins [34]. Recent studies using a similar AFM approach have shown Dsc3 homophilic binding and heterophilic interaction with Dsg1, but not with Dsg3 [35]. Heterophilic binding of Dsg2 with Dsc1 or Dsc2 have also been demonstrated [32, 36]. Furthermore, these heterophilic interactions also form in a calcium-dependent manner.

b) The armadillo proteins in the desmosome:

In the desmosomal plaque, the armadillo proteins plakoglobin and plakophilin associate with the cytoplasmic domains of desmosomal cadherins.

-*Plakoglobin (PG).* Originally defined in the Drosophila homolog Armadillo, PG is also called γ-catenin and is encoded by a gene in chromosome 17 [37]. Its counterpart is β-catenin, expressed in adherens junctions. Similar to β-catenin, PG is characterized by 12 armadillo (arm) repeats, which are ~ 42 amino acid domains flanked by distinct N- and C-terminal domains, which are less structured than the central region and have been suggested to affect PG function [38, 39]. The central arm region of PG shares 65% amino acid identity

with β-catenin. PG can substitute for β-catenin in adherens junctions because both bind E-cadherin with similar affinity. However, PG has higher affinity for desmogleins, which explains the exclusion of β-catenin from desmosomes [40]. The arm domain of PG associates with both the intracellular domain of desmosomal cadherins and the N-terminus of desmoplakin [41-43]. Thus, PG is a critical linker in desmosomal adhesion.

-*Plakophilins (PKP).* PKP are members of a subfamily of armadillo proteins related to p120ctn. Three desmosomal PKPs (1, 2 and 3) have been described, and the genes encoding them are located on chromosomes 1, 12 and 11 respectively [44]. PKP1 and PKP2 have 2 isoforms ("a" and "b") that result from alternative splicing [45, 46]. There is a fourth plakophilin : PKP4, also known as p0071, which is highly related to p120ctn and δ-catenin [47]. PKPs (1-4) have been shown to bind directly to the intracellular domain of desmosomal cadherins. This interaction is mediated by their amino-terminal head domain and the functions for their armadillo repeats remain unknown. PKPs can also bind PG in order to facilitate clustering of desmosomal cadherins through lateral stabilizing interactions, which increases desmosome strength [41].

c) The plakins: Another family of proteins responsible for cell-cell adhesion is the plakins, which are linkers between the cytoskeleton and cell-cell or cell-matrix interactions [48]. Desmoplakin (DP) is an essential component of the desmosomal plaque, and is recruited by the armadillo proteins in order to link the intermediate filament network with the desmosomal junctional complex. DP is encoded on chromosome 6 and has 2 variants (DP-1 and DP-2) [49, 50]. The amino-terminal domain of DP binds the other desmosomal plaque

proteins (PG and PKP), its central coiled-coil rod domain mediates dimerization, and its carboxy-terminal tail is the linker for intermediate filaments [41, 51]. Other members such as plectin, envoplakin and periplakin are also found in desmosomes; however, their roles are still unclear. Genetic studies have shown that desmoplakin is the only indispensable plakin in intercellular junctions. Thus, inactivating plectin, which is present in desmosomes and hemidesmosomes, did not affect cell-cell adhesion in mice or humans [52, 53]. In addition, when using DP-deficient mice, skin blistering was observed and the desmosomes were not anchored to the intermediate filaments [54].

1.3. THE PEMPHIGUS GROUP: Clinical Manifestations and Desmosomal Targets

The pemphigus group consists of many disease variants, each with unique clinical, histologic, and immunologic features [55]. These disease variants include pemphigus vulgaris (PV), pemphigus foliaceus (PF), endemic pemphigus foliaceus (also known as Fogo Selvagem: FS), paraneoplastic pemphigus (PNP), and IgA pemphigus.

PV is the most common form of the pemphigus group [2], and typically presents with erosions limited to the mucosal (oropharyngeal and/or genital) tissue, classified as mucosal PV (mPV). In most patients the disease progresses to involve the cornified cutaneous surface as well with blisters and/or erosions over the trunk and extremities (classified as mucocutaneous PV or mcPV). Histologically, PV exhibits a suprabasilar split with acantholysis. While mPV patients classically harbor autoantibodies to desmoglein 3 (Dsg3) alone, mcPV patients show autoantibodies to both Dsg3 and Dsg1 [2, 55-57]. Dsg3 and Dsg1 affinity purified autoantibodies from mcPV patients are sufficient to induce suprabasilar

acantholysis upon passive transfer in a neonatal mouse model of disease (discussed in Section 1.6), thereby establishing pathogenicity [58-60].

PF presents clinically with superficial blisters or erosions over the trunk and extremities. In many patients, the blisters rupture spontaneously prior to presentation and the clinical exam reveals only superficial crusting and erosions. There is no mucosal involvement in PF. Histologically, PF shows a subcorneal split with acantholysis. Patients with PF classically harbor autoantibodies to Dsg1 alone [2, 55, 56]. Similar to PV, the establishment of Dsg1 as the target autoantigen was defined by affinity purified anti-Dsg1 autoantibodies in the neonatal passive transfer model [61].

FS represents an endemic form of PF with identical clinical, histologic, and immunologic features. The endemic nature of this disease makes it an ideal population to study the fine aspects of genetic susceptibility and environmental triggers of autoimmune disease [62]. Autoantibodies to Dsg1, particularly those of the IgG4 subclass, are a serologic indicator of disease in this patient population [63]. E-cadherin has also been shown to be a potential antigenic target in FS [64]. In addition, a recent serological analysis reveals that FS patients and many healthy controls living in endemic areas also harbor autoantibodies to other Dsg and Dsc family members, in addition to the anti-Dsg1 antibodies previously reported [65].

PNP is a very unique pemphigus variant that presents with extensive mucositis and polymorphic cutaneous lesions in the clinical setting of malignancy. Histology of these lesions reveals intraepidermal clefting with acantholysis, but also shows a dense lichenoid infiltrate with interface dermatitis and necrotic keratinocytes [66, 67]. Patients with PNP harbor autoantibodies to Dsg1 and Dsg3, which are thought to be responsible for the

acantholysis, as removal of the Dsg3 specific antibodies from PNP sera abrogates the pathogenicity in the neonatal passive transfer model [68]. These patients also have autoantibodies to the plakin family of proteins, which are thought to be responsible for the dyskeratosis/necrotic keratinocytes [66, 67]. Interestingly, patients with erythema multiforme, a condition classically felt to be a non-immunologically mediated reaction pattern, can have a falsely positive indirect immunofluorescence on the classic PNP rat bladder substrate. There are recent reports of patients with erythema multiforme harboring anti-desmoplakin antibodies, though the pathogenicity of these autoantibodies has not yet been established [69, 70].

Finally, IgA pemphigus is distinguished by the presence of autoantibodies of the IgA subclass present on direct immunofluorescence in a classic intercellular space staining pattern. Histologically, IgA pemphigus can be of the intraepidermal neutrophilic (IEN) variant or can be of the subcorneal pustular dermatosis (SPD) variant [71]. Dsc1 has been shown to be the target antigen in the IEN variant of IgA pemphigus, whereas the target antigen in the SPD variant of IgA pemphigus is likely a non-desmosomal antigen as shown by immuno-electron microscopy studies [72]. Pathogenicity studies are currently lacking.

To date, there are no descriptions of isolated anti-desmoplakin (outside of what is seen in PNP and erythema multiforme) or anti-plakoglobin mediated cutaneous disease.

1.4. IMMUNOPATHOGENESIS OF AUTOANTIBODY-INDUCED BLISTERING IN PEMPHIGUS

PF and PV are the most studied variants of pemphigus. Researchers around the world are making their best efforts to explain how pemphigus autoantibodies cause disease. An

association of susceptibility to pemphigus with certain HLA-DR and -DQ alleles has been suggested [73]. However, the exact mechanism and whether pemphigus autoantibodies induce loss of epidermal cell adhesion directly or indirectly is controversial. Different mechanisms have been proposed such as: direct interference (steric hindrance), activation of transmembrane signaling that downregulates cell-cell adhesion, proteinase activation (plasminogen activator), and desmoglein internalization. There are two major theories that are the most accepted but still under debate: a) direct interference of desmogleins transinteraction by pemphigus autoantibodies, and b) activation of transmembrane signaling pathways and/or apoptosis by pemphigus autoantibodies that indirectly results in acantholysis.

a) ***Direct interference of desmogleins transinteraction by pemphigus autoantibodies (steric hindrance theory)****:* Accumulated evidence from several epitope mapping studies indicates that pathogenic pemphigus autoantibodies target the amino-terminal end of Dsg1 and/or Dsg3 ectodomains [74-76]. Data based on the crystal structure of classical cadherins suggest that this N-terminal region harbors the adhesive interface of desmosomal cadherins [30, 77]. In addition, the pathogenicity of pemphigus IgG autoantibodies has been consistently demonstrated by passive transfer studies in neonatal mice [60, 78]. Moreover, not only the whole IgG molecule, but also the F(ab)2 and Fab fragments were found to be pathogenic, independently of complement or plasminogen activator [79-82], suggesting that due to their inability to cross-link cell-surface molecules, it is possible that they interfere directly with adhesion. Furthermore, monoclonal antibodies (AK23) derived from a PV mouse model that binds the functionally N-terminal adhesive interface of Dsg3 induced pemphigus vulgaris

lesions in mice, whereas monoclonal antibodies recognizing other regions of Dsg3 ectodomain did not cause lesions in mice [83]. Using single-molecule atomic force microscopy (AFM), it has been shown that PV-IgG and AK23 monoclonal antibody directly inhibit Dsg3 homophilic binding under cell-free conditions, suggesting that direct inhibition of Dsg3 binding occurs in PV [84, 85]. Direct blocking of Dsg1 binding wasn't observed by AFM, however, keratinocyte dissociation and loss of Dsg1- and Dsg3- coated microspheres to cultured keratinocytes were observed when using laser tweezer trapping [86], suggesting that acantholysis may not be solely dependent on direct interference of Dsg1-Dsg1 binding by pemphigus autoantibodies. Recent studies using peptides against the desmoglein adhesive interface as well as tandem peptides (obtained by dimerization of two of the initial peptides) added evidence that direct inhibition contributes to acantholysis when the tandem peptide prevented acantholysis induced by PV-IgG, yet this was not the case when using PF-IgG. Thus, some investigators proposed that PV and PF acantholysis may involve different mechanism [87].

b) Activation of transmembrane signaling pathways and/or apoptosis by pemphigus autoantibodies. The direct interference of desmoglein transinteractions by pemphigus autoantibodies has been suggested to be insufficient to disrupt keratinocyte adhesion [33]. Therefore, additional cellular events may be needed to cause blistering. Previous in vitro studies show that PV IgG induces cellular signaling events in cultured keratinocytes including a transient increase in intracellular calcium and inositol 1,4,5-triphosphate [88], activation of protein kinase C, and phosphorylation of Dsg3. These events may lead to internalization of Dsg3 from cell surface, therefore depleting Dsg3 from desmosomes [89-

94]. Thus, activation of intracellular signaling within the target keratinocyte induced by binding of pemphigus IgG has been proposed to contribute to the loss of cell-cell adhesion.

Previous studies have shown that phosphorylation of p38MAPK and HSP25 (the murine homolog of human HSP27) occurs rapidly after exposure of cultured keratinocytes to pemphigus IgG and in the skin of mice injected with pathogenic IgG [95]. Also, phosphorylation of both p38MAPK and HSP27 has been observed in perilesional epidermis of pemphigus patients [96]. Furthermore, p38MAPK inhibitors block both histological and gross blister formation in the PF passive transfer model [95], suggesting that activation of p38MAPK is an early and key step in PF IgG-induced acantholysis. p38MAPK signaling has been implicated in other cellular responses such as desmosome assembly, cytoskeleton reorganization, changes of the cell cycle and apoptosis [97, 98]. Moreover, there is evidence that p38MAPK is involved in keratinocyte apoptosis [99, 100] and that DNA fragmentation and caspase activation are induced in the epidermis of PF IgG-treated mice [101]. Keratinocyte-derived and local production of apoptotic inducers such as nitric oxide synthase, Fas and Bcl-2, have also been detected in lesional skin of PF patients [102, 103] along with increased levels of Fas ligand in serum of pemphigus patients [104]. Futhermore, a biphasic activation of p38MAPK after the binding of pemphigus IgG has been recently demonstrated where the first activation peak is linked to acantholysis and the second peak coincided with apoptosis, suggesting that apoptosis occurs downstream to acantholysis in pemphigus [99]. However, other studies suggests that apoptosis occurs before acantholysis develops [101]. There is also the hypothesis that apoptotic signaling could precede acantholysis in the absence of apoptotic cell death [98]. More importantly, caspase inhibitors have been shown to block pemphigus serum-induced keratinocyte apoptosis. Thus, at

present, it is not clear which process precedes the other, but there is evidence that both are involved in pemphigus pathogenesis.

Another signaling pathway suggested to be involved is mediated through plakoglobin [105]. The observation was made that keratinocytes from plakoglobin-deficient mice were resistant to keratinocyte dissociation induced by PV IgG, suggesting that direct inhibition of Dsg binding may not be sufficient to cause acantholysis, and that plakoglobin could be part of a complex responsible for transferring the signal upon autoantibody binding from outside into the keratinocyte: "outside-in" signaling [106]. In addition, it has been shown that plakoglobin is involved in c-Myc repression, and c-Myc was also shown to be elevated in keratinocytes exposed to pemphigus autoantibodies [107, 108]. Nonetheless, the role of c-Myc signaling in pemphigus acantholysis remains unclear.

1.5. ENVIRONMENTAL FACTORS IN ENDEMIC PEMPHIGUS FOLIACEUS

The endemic form of pemphigus foliaceus, known as Fogo Selvagem (FS) which translates to "Wild Fire" in English because of the burning sensation that the disease causes, was first described in the early 1900s in certain areas of Brazil [109]. FS is highly endemic in the rural reservation of Limao Verde, in Mato Grosso do Sul, Brazil, where the prevalence is 3.4% and an incidence of 1-4 new cases per year. Other less-characterized forms have been also reported in Colombia, Peru and Tunisia [62, 110, 111].

A unique characteristic of FS is it epidemiology. Autoantibody response against Dsg1 in healthy individuals and disease prevalence are directly related to proximity to the reservation [112]. Several observations indicate that FS is triggered by exposure to an environmental agent(s) [113]. The geographical and temporal clustering of the cases as well

as the decreasing prevalence of FS upon improvement of living conditions, suggest the presence of an environmental antigen that may exhibit molecular mimicry with Dsg1. Circulating anti-Dsg1 autoantibodies in the serum of 42% of healthy individuals living in endemic Brazilian areas has been reported [65]. Moreover, anti-Dsg1 antibodies are also detected in FS patients before onset of disease as a mixture of IgG1 and IgG4 autoantibodies that recognize the EC5 portion of Dsg1 [75, 114]. An additional report showed that patients with disease transmitted by hematophagus vectors (black flies, sand flies and kissing bugs) possess antibodies against the extracellular EC5 domain of Dsg1 [115]. These findings suggest that saliva of these vectors may contain Dsg1 homologs that induce a non-pathogenic response to Dsg1.

Another feature of FS is the increased frequency of familial cases and the association to certain HLA-DR alleles [116]. These alleles are HLA-DRB1-0102, 0404, 1402 and 1406. The hypervariable region of the DRB1 gene of these alleles share the sequence LLEQRRA at positions 67-74, suggesting that this sequence may be critical for susceptibility to FS. It is possible that an environmental antigen induces an initial cross-reactive anti-Dsg1 non-pathogenic response and only in individuals sharing these alleles is FS later developed.

IgG, IgM and IgE antibodies against Dsg1 are present not only in FS patients but also in healthy individuals living in Brazilian endemic areas [63, 117, 118]. Analysis of the hypervariable V genes of FS autoantibodies showed that they are antigen selected and this selection occurs before the onset of clinical FS [119].

Taken together, these observations suggest that recurrent and persistent exposure to an environmental cross-reactive antigen leads to the development of FS in genetically

predisposed individuals living in FS endemic areas. Furthermore, insect bites may deliver antigens/allergens that drive the production of cross-reactive anti-Dsg1 autoantibodies.

1.6. ANIMAL MODELS IN FS

A passive transfer mouse model was developed to test *in vivo* pathogenicity of autoantibodies from FS patients [78]. Serum or IgG fraction from FS patient is given to neonatal mice by intradermal injection. The animals develop skin blisters and typical histological and ultrastructural features of the human disease, such as subcorneal vesicles [5]. The extent of disease correlates well with the indirect IF titers of human autoantibodies detected in the mouse skin. FS was also induced in these animals by injecting monovalent Fab fractions from FS autoantibodies [82], thus, the blistering in this animals is independent of complement activation. Despite the availability of recombinant human and mouse Dsg1, development of an active model for FS remains unsuccessful.

1.7. CONCLUDING REMARKS

Although the mechanisms linking pemphigus IgG binding to acantholysis are not completely understood, much progress has been made in the characterization of target pathways that may help elucidate the primary event that drives pemphigus acantholysis. The interplay of direct interference and signaling mechanisms in pemphigus pathogenesis remains unclear, and this underscores the complexity and heterogeneity of pemphigus acantholysis. Studies towards the identification of the primary component(s) of the molecular mechanism of acantholysis will advance our understanding of pemphigus pathogenesis and therefore provide targets for the development of preventative and improved therapeutic strategies.

FS provides a good model to study human autoimmune disease in which the auto-antigen Dsg1 is fully characterized and the relevant autoantibodies are known to be pathogenic. In addition, the epidemiology of FS provides an opportunity to study the development of autoimmune disease in a well-defined, geographically limited population with a high prevalence of the disease. The saliva of hematophagus insects such as black flies have been suggested to harbor the cross-reactive antigen that induces a non-pathogenic anti-Dsg1 response in healthy individuals living in FS endemic areas. However, the association of an environmental trigger with the pathogenic anti-Dsg1 response in FS remains unknown.

The objective of this dissertation is to identify the principal antigenic determinant in the extracellular domain of Dsg1 that is recognized by pathogenic FS autoantibodies and their association with a potential environmental agent. Chapter 2, describes the identification of conformational epitopes for pathogenic IgG4 autoantibodies from FS patients living in LV, Brazil. Chapter 3 reports a non-infectious environmental agent that induces a potentially pathogenic anti-Dsg1 response in FS patients. Finally, Chapter 4 discusses the implications of the findings of this dissertation and future directions of this research.

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CHAPTER II

DISSECTING THE CONFORMATIONAL EPITOPES RECOGNIZED BY PATHOGENIC IgG4 ANTI-DESMOGLEIN 1 AUTOANTIBODIES IN ENDEMIC PEMPHIGUS FOLIACEUS

2.1. INTRODUCTION

Fogo Selvagem (FS), the endemic form of Pemphigus Foliaceus (PF), is a cutaneous autoimmune blistering disease that exhibits geographic clustering and a mortality of 100% without treatment. FS is highly endemic in the rural reservation of Limao Verde (LV), Brazil, where the prevalence is 3.5%, over 30 times higher than the non-endemic form seen in the US [1, 2].

FS and PF are mediated by autoantibodies against the extracellular domain of the epidermal cell adhesion protein, Desmoglein 1 (Dsg1) [3]. The binding of these autoantibodies causes cell detachment, a process known as acantholysis, and leads to blister formation. The autoantibodies that mediate FS are predominantly of the IgG4 subclass [4, 5]. Serum or IgG autoantibodies from FS patients are pathogenic when passively transferred into neonatal Balb/c mice, eliciting blisters in the injection site and thus resembling the human disease [4]. Moreover, $F(ab)_2$ and monovalent Fab fragments also reproduce human disease in the passive transfer model, indicating an Fc-independent mechanism in FS pathogenesis [6].

Interestingly, 42% of healthy individuals living in LV, and thus at risk of developing FS, possess circulating IgG anti-Dsg1 autoantibodies [7] that are not pathogenic when tested

in the passive transfer model [8] Thus, it appears that an environmental antigen(s) present in LV may trigger anti-Dsg1 autoantibody production, which in certain genetically predisposed individuals subsequently leads to pathogenic IgG4 secretion and clinical disease. Our recent report (**see Chapter 3**) strongly suggests that salivary antigens from sand flies and Dsg1 contain cross-reactive epitopes that may be relevant in the progression of the pathogenic autoimmune response in FS [9].

Previous reports demonstrate that the progression from preclinical to clinical stage of disease is associated with a dramatic rise of IgG4 anti-Dsg1 autoantibodies [5]. In fact, detection of IgG4 anti-Dsg1 autoantibodies in serum of normal individuals living in LV are predictors of clinical disease in approximately 49% of the cases [10]. It has been suggested that the transition from preclinical to clinical disease in FS is associated with a switch from a mix of IgG1 and IgG4 anti-Dsg1 autoantibodies during pre-clinical stage to a predominantly IgG4-mediated anti-Dsg1 response at the onset of clinically active disease [5]. Thus, polarization of the humoral response to a predominantly IgG4 mediated response is a key step in the development of FS.

The extracellular domain of Dsg1 is the target for PF and FS autoantibodies. Like other desmosomal cadherins, the extracellular domain of Dsg1 is composed of five calcium-binding cadherin repeats (EC1 to EC5), with EC5 being proximal to the cell membrane (C-terminal end). The epitopes recognized by FS autoantibodies are conformational and calcium dependent [11, 12]. The majority of pemphigus autoantibodies recognize the N-terminal region of Dsg1 [13, 14], and intramolecular epitope spreading in Dsg1 extracellular domain occurs in FS. We have previously demonstrated that anti-Dsg1 autoantibodies from healthy individuals and individuals in the preclinical stage recognize the EC5 domain, and upon

onset of clinical FS, a pathogenic response against the EC1 and/or EC2 domains of Dsg1 arises [8].

The majority of previous epitope mapping studies in pemphigus have been performed using domain swapped molecules and whole serum or IgG autoantibodies from PF or FS patients. These studies consistently suggested that clinically active PF/FS is driven by IgG autoantibodies specific for EC1 and/or EC2 domains of Dsg1 [8, 14]. However, the fine epitope specificity of IgG4 anti-Dsg1 autoantibodies has never been defined. In this study we have identified for the first time the conformational epitopes recognized by pathogenic IgG4 autoantibodies from FS patients. Employing recombinant human Dsg1 (hDsg1) extracellular domain, highly purified FS IgG4 from 20 FS patients and the combination of Epitope Excision with Matrix Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS/MS), we identified two dominant conformational epitopes on hDsg1. One located within the EC1 domain and another within the EC2. Moreover, site-directed mutagenesis of the residues M133 and Q135 in hDsg1 (EC1), demonstrate that these residues are crucial in the binding of pathogenic IgG4 autoantibodies from FS patients.

2.2. MATERIAL AND METHODS

Sources of human FS sera:

Well-characterized sera from patients with FS (n=20) from Brazil, as well as sera from clinically healthy individuals from Limao Verde (n=1) and from the US (n= 1) were used for epitope mapping studies. The diagnosis of FS in these patients was established by clinical, histological, serological and epidemiological criteria [15, 16]. Serum samples from

other FS patients and sera from normal donors from the UNC Blood Bank were also available in our laboratory. Sera were collected following IRB policy from UNC-Chapel Hill (US) and University of Sao Paulo (Brazil).

Purification of IgG4 autoantibodies from FS sera:

IgG4 autoantibodies were purified from sera by affinity chromatography using Capture Select® human IgG4 affinity matrix (BAC BV, Leiden, The Netherlands). The Capture Select human IgG4 affinity matrix contains a 12kD llama antibody fragment that specifically recognizes human IgG4 without cross-reacting with other human IgG subclasses 1,2 or 3. Briefly, FS serum was loaded onto the matrix and later washed with PBS, pH=7.4. The matrix was eluted with 0.1M Glycine, pH=3.0. The bound fraction (elution) contained 97% IgG4 and the unbound fraction contained 92% IgG1, with small amounts of IgG2 and IgG3. Both fractions were dialyzed against PBS pH=7.4, concentrated by ultrafiltration and stored at -20°C. In addition, total IgG was purified from a normal individual living in LV by Protein G-affinity chromatography using HiTrap Protein G HP cartridge (GE Healthcare).

Levels of total IgG and IgG subclasses in sera or purification fractions were measured by a sandwich quantitative ELISA established in our lab for this study, using goat $F(ab)_2$ anti-human IgG (Fab specific) as capture antibody, and monoclonal anti-human IgG or IgGsubclass horseradish-peroxidase conjugates as detecting antibodies.

Plasmid constructs:

We have previously successfully constructed and expressed the entire extracellular domain of human Dsg1 in a baculovirus system [17, 18]. Briefly, cDNA encoding the entire

extracellular domain of Dsg1 including a COOH-terminal Histidine-tag was subcloned into the baculovirus transfer vector pVL1393 (BD Biosciences, San Jose,CA) and later sequenced to verify sequence integrity.

We generated 4 domain-swapped molecules bearing the Dsg1 extracellular domain fragments EC1, EC1-2, EC4-5 and EC5 (**Fig. 1**). Recombinant Desmocollin 1 (Dsc1) was used as a backbone. Dsc1 is a desmosomal cadherin that shares extracellular structure with Dsg1; however, it is not recognized by typical pemphigus autoantibodies[19]. cDNA encoding the extracellular domain of Dsc1 was a kind gift from Dr Takahashi Hashimoto (Kurume University, Kurume, Japan) and also contains a C-terminal His-tag. cDNA fragments encoding various EC fragments of Dsg1 and Dsc1 ectodomain were amplified by PCR from full length human Dsg1 (GenBank accession: X56654) and human Dsc1 (GenBank accession: X72925). The sequences of the PCR primers used are shown in **Table I**. The swapped-domain constructs were later ligated into the baculovirus transfer vector pEVmod and sequenced to verify the expected hybrid sequences.

In order to introduce point mutations in Dsg1 and Dsg4, we performed site directed mutagenesis using plasmid templates encoding the entire extracellular domains of hDsg1 and hDsg4 respectively. cDNA encoding the entire extracellular domain of human Dsg4 harboring an E-tag and His-tag was cloned into pQE-Trisystem vector (QIAGEN, Inc.) as previously described [20]. The pQE-hDsg4-His plasmid DNA was kindly provided by Dr Masayuki Amagai (Keio University, Tokyo, Japan). Primers containing the desired mutations **(Table I)** were synthesized and used following manufacturer instructions for QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). The mutated constructs were sequenced to verify the introduction of the desired mutations within EC1 and/or EC2

extracellular domains from hDsg1 and hDsg4, and to ensure the absence of additional mutations in the rest of the sequence.

Table I. Primers used for construction of swapped-domain recombinant proteins and site directed mutagenesis.

	Constructs	PCR product /Amino acid change	Forward Primer	Reverse primer
Swapped-domain Recombinant proteins	**Dsg1(EC1)** / Dsc1	Dsg1(EC1)	5'GCCAGATCTCCTATAAATATGGACTGGAGTTTCTTCAG	5'AGTCACTCTGTGTTCTGAAAACACTGGAGGGTTG
		Dsc1(EC2-5)	5'GAACACAGAGTGACTATCTTTACTG	5'CCGCTCGAGTCTTCCAAGTATTACATTTG
	Dsg1(EC1-2) / Dsc1	Dsg1(EC1-2)	5'GCCAGATCTCCTATAAATATGGACTGGAGTTTCTTCAG	5'TTTACATGTAAGGGATATTATCATTG
		Dsc1(EC3-5)	5'CTTCCATGGAAACAGAAACTTCTTATGTTACAG	5'CCGCTCGAGTCTTCCAAGTATTACATTTG
	Dsc1/ **Dsg1(EC4-5)**	Dsc1(EC1-3)	5'GCCAGATCTCCTATAAATATGGCTCTGGCCTCTGCTGC	5'CATTCAGGGCCCTCATCACT
		Dsg1(EC4-5)	5'ATGAGGGCCCTGAATGCCGTCCAGGTTCAAAGACATA	5'CCGCTCGAGAGGACCAAAATGTACATTGTCTG
	Dsc1 / **Dsg1(EC5)**	Dsc1(EC1-4)	5'GCCAGATCTCCTATAAATATGGCTCTGGCCTCTGCTGC	5'CGTGAACGTTGTAATCATCCAAATGAAC
		Dsg1(EC5)	5'ATTACATCGATCACGCACCTACTAAAATTACTACCAATACTGGC	5'CCGCTCGAGAGGACCAAAATGTACATTGTCTG
Site-directed mutagenesis	Dsg1-MEC1	M133R Q135E	5'CGAGCTCTGAACTCACGGGGCGAAGATTTAGAGAGGCCTCTAGAGCTC	5'GAGCTCTAGAGGCCTCTCTAAATCTTCGCCCCGTGAGTTCAGAGCTCG
	Dsg1-MEC2	D204G, S205A, I211L	5'GATTATAAGACAAGAACCTTCAGGTGCACCAATGTTTATTCTGAACAGAAATACTGGAGAAATTCGAACG	5'CGTTCGAATTTCTCCAGTATTTCTGTTCAGAATAAACATTGGTGCACCTGAAGGTTCTTGTCTTATAATC
	Dsg4-MEC1	R133M, E135Q	5'CGGGCTCTGAATTCAATGGGTCAAGATTTAGAAAGGCCTC	5'GAGGCCTTTCTAAATCTTCACCCCGTGAATTCAGAGCCCG
	Dsg4-MEC2	G204D, A205S, L211I	5'GTCTCTCAGGAGCCATCAGATTCACCCATGTTCATTATCAATAGGTACACTGGAG	5'CTCCAGTGTACCTATTGATAATGAACATGGGTGAATCTGATGGCTCCTGAGAGAC

Expression and Purification of recombinant desmosomal proteins:

Plasmids encoding whole, chimeric and mutated proteins were cotransfected and expressed in a baculovirus expression system (BD BaculoGold, BD Biosciences) using Sf9 insect cells in Sf-900™ III SFM containing 5% FBS (Invitrogen, Life Technologies Corp.) according to the manufacturer's instructions. High-Five insect cells cultured in Express-Five serum-free medium (Life Technologies Corp.) were used to produce recombinant soluble antigens by infection with the baculovirus stocks. Next, recombinant proteins were purified from the culture supernatants by affinity chromatography using Ni-NTA cartridges (QIAGEN, Chatsworth, CA). Purified recombinant proteins were later dialyzed in Tris-buffered saline buffer containing 5mM calcium (TBS-Ca2+) and stored at -80°C.

Epitope Mapping by Epitope Excision and Mass Spectrometry:

Purified IgG4 was immobilized on CNBr-activated Sepharose 4B (GE Healthcare, Sweden) in compact reaction columns (CRC, USB Corporation, Cleveland, OH) and incubated with recombinant protein encompassing the entire extracellular domain of Dsg1. The Sepharose-IgG4-Dsg1 complex was then sequentially digested with sequencing grade chymotrypsin (Promega) for 1 hr at 37°C immediately followed by digestion with sequencing grade TPCK-treated trypsin (Worthington, Lakewood, NJ) for 20 minutes at 37°C. Dsg1 peptides that remained bound to IgG4 after digestion were eluted with 0.1% Trifluoro acetic acid (ThermoScientific) and sequences were determined by mass spectrometry.

Samples were analyzed using a 4800 Plus Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry (MALDI TOF/TOF MS/MS) in conjunction

with Protein Pilot software (AB SCIEX, Foster City, CA). The samples were spotted on a stainless-steel target with α-cyano-4-32hydroxycinnamic acid matrix (Sigma). The instrument used has a YAG laser with λ=355nm. The potential difference between the source acceleration voltage and the collision cell was set at 2kV. Calibration was done internally with self-digested TPCK treated trypsin (same as above). Peak absorbances in MS spectra are not indicative of the abundance of peptide species because of the differences in individual peptides ability to ionize. All analysis was done with Protein Pilot using an NCBI Mascot search.

The two most common peptides (epitopes) identified by this method were later synthesized at the Peptide Synthesis Core Facility at UNC.

Molecular modeling of the extracellular domain of human Dsg1 and Dsg4:

The atomic structures of the extracellular domain of human Dsg1(hDsg1) and human Dsg4 (hDsg4) were predicted or modeled using 2 structural templates : 1) PDB ID 3Q2W (mouse N-cadherin); and 2) PDB ID 3Q2V (mouse E-cadherin).

Molecular modeling was performed at the Structural Bioinformatics Core Facility at UNC. The structural templates were identified using the HHpred Fold Recognition Server and the hDsg1 and hDsg4 homology models were built using the Modeller software package. The cadherin structural templates were in the homodimeric adhesive conformations. Thus, the structures of hDsg1 and hDsg4 were predicted in their homodimeric adhesive conformation.

Immunoprecipitation (IP):

All IPs were performed in TBS buffer containing 5mM $CaCl_2$ (TBS-Ca2+). 200 µL of cell culture supernatant containing baculovirus-expressed Dsg1 or Dsg4, or 100 ng of purified WT or mutated hDsg1, were incubated with 2 uL of serum or purified IgG4 fractions in an end-over-end rocker at 4°C overnight. The following day, 25 µL of Protein G-Agarose slurry was added to the mix and incubated for 90 minutes at 4°C. After washing with TBS-Ca2+ containing 0.1% Tween-20, each IP sample was subjected to SDS-PAGE and subsequent Western Blot. The probe used was anti-His-HRP conjugate (QIAGEN, Valencia, CA).

Establishment of chimeric Dsg1 ELISA for detection of IgG4 autoantibodies:

Immunomicrotiter plates were coated with purified chimeric Dsg1-EC1 extracellular domain (200 ng/well) at 4°C overnight. After washing 5 times with TBS-Ca^{2+} buffer containing 0.05% Tween-20 (TBS-Ca^{2+}/T-20), the plate was blocked with 1% BSA in TBS-Ca^{2+}/T-20 at room temperature for 1 hour. Plates were then washed and incubated with duplicate samples of diluted serum (1:100) for 1 hour at room temperature. Following the washes, plates were incubated with a 1:500 dilution of horseradish-peroxidase (HRP)–conjugated mouse anti-human IgG4 for 1 hour. The color development was achieved with the peroxidase substrate o-phenylenediamine. Results were expressed in index values calculated as previously reported [21]. A receiver-operating-characteristic (ROC) analysis was performed for the ELISA index values, generated from a set of 55 normal serum and 55 FS serum samples. An ROC curve was generated to display the sensitivity and specificity of the ELISA assay and to determine the cut-off. A sensitivity of 67% and a specificity of 98%

were provided by a cutoff of 7.54. Index values above 7.54 were considered positive for this ELISA.

Dsg1 and Dsg4 mutant ELISA for detection of IgG4 autoantibodies:

Purified WT or mutated Dsg1 and Dsg4 were used to coat immunomicrotiter plates (50 ng/well) at 4° C overnight. After 5 washes with TBS-Ca^{2+}/T-20, the plate was blocked with 1% BSA in TBS-Ca^{2+}/T-20 at room temperature for 1 hour. Plates were then washed and incubated with duplicate samples of diluted serum or purified IgG4 fractions (1:300) for 1 hour at room temperature. hIgG4 (Abcam®, Cambridge, MA) and normal human serum were used as negative controls. Following the washes, plates were incubated with a 1:1000 dilution of horseradish-peroxidase (HRP)–conjugated mouse anti-human IgG4 for 1 hour. The color development was achieved with the peroxidase substrate o-phenylenediamine.

Affinity Purification of IgG4 antigen- specific autoantibodies:

Affinity columns were prepared by immobilizing recombinant Dsg1 ectodomain in CNBr-activated Sepharose (GE Healthcare) following the manufacturer's instructions. Purified IgG4 from FS serum was loaded into the Dsg1 affinity column. The successfully purified IgG4 anti-Dsg1 antibodies were then dialyzed against 1X PBS and stored at -20°C until further use.

Passive Transfer studies:

Balb/c neonatal mice (24-36 hrs old, body weight 1.4-1.8 g) were intradermally injected as previously described [22] with different quantities (contained in a volume of 100

μL) of either IgG4 or IgG1 purified fractions from FS sera. Commercially available hIgG4 (Sigma Aldrich) was injected as a control. After 18-24 hrs post-injection, the skin of the mice was evaluated for blisters. Skin biopsies were obtained for histology and direct immunofluorescence analysis.

2.3. RESULTS

2.3.1. IgG4 anti-Dsg1 autoantibodies from FS sera are pathogenic

To investigate the Dsg1 epitopes recognized by FS IgG4 autoantibodies, we first purified IgG4 from the serum of one FS patient and tested its pathogenicity using the passive transfer model. We found that the llama anti-human IgG4-Sepharose matrix was a very effective affinity chromatography method to purify IgG4 from FS sera. The bound fraction contained 97% IgG4, and the unbound fraction contained 92% IgG1 as measured by quantitative IgG4 and IgG1 ELISAs. Both fractions showed the classic staining pattern on the epidermal intercellular spaces by indirect IF. Importantly, when these fractions were passively transferred to neonatal Balb/c mice, only the IgG4 fraction was pathogenic, inducing extensive blistering in these animals **(Fig. 2).** In contrast, the unbound IgG1 fraction was not pathogenic, even at doses 4 times higher than IgG4 dose. These findings were confirmed using a second FS serum.

To further analyze the domains of Dsg1 that are bound by these two fractions (IgG4 and IgG1), we employed 4 domain-swapped molecules bearing the EC1, EC1-2, EC4-5 and EC5 domains of Dsg1 grafted on the extracellular domain of Dsc1. The C-terminal end of these chimeric molecules has a His-tag **(Fig. 1).** We observed that IgG4 and IgG1 fractions from the same patient immunoprecipitated EC1 and EC5 domain-swapped molecules **(Fig.**

2). Thus, these findings suggest that while IgG4 and IgG1 bind Dsg1 at epitopes within EC1 and EC5 domains, only the IgG4 autoantibodies are pathogenic and therefore responsible for blister formation.

The prevalence of IgG4 anti-EC1 autoantibodies in FS sera was tested by ELISA **(Fig. 3).** We tested the sera of 55 FS patients and 55 normal donors, which included 23 sera from endemic areas of FS in Brazil and 22 from UNC Blood Bank. IgG4 anti-EC1 autoantibodies were detected in 37 FS sera (67%) and only in one of the normal human sera from endemic areas **(Fig. 3).** These findings strongly support the hypothesis that the presence of anti-EC1 IgG4 autoantibodies is a key hallmark of FS.

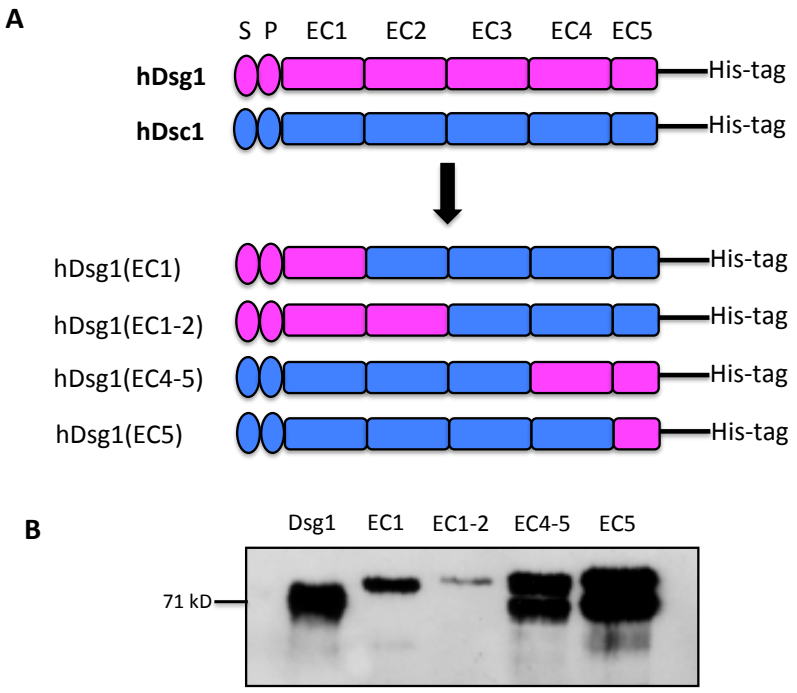

Figure 1. ***Domain-swapped hDsg1/Dsc1 recombinant proteins produced in baculovirus expression system.*** **A)** Molecular structures of 4 domain-swapped molecules bearing different regions of hDsg1 ectodomain using Dsc1 as a backbone. **B)** Immunoblot of the insect cells supernatants containing the secreted recombinant proteins. Monoclonal anti-His antibody was used as a probe. The double band is due to the expression of mature and immature forms of recombinant proteins in baculovirus system. S=signal sequence, P=propeptide sequence.

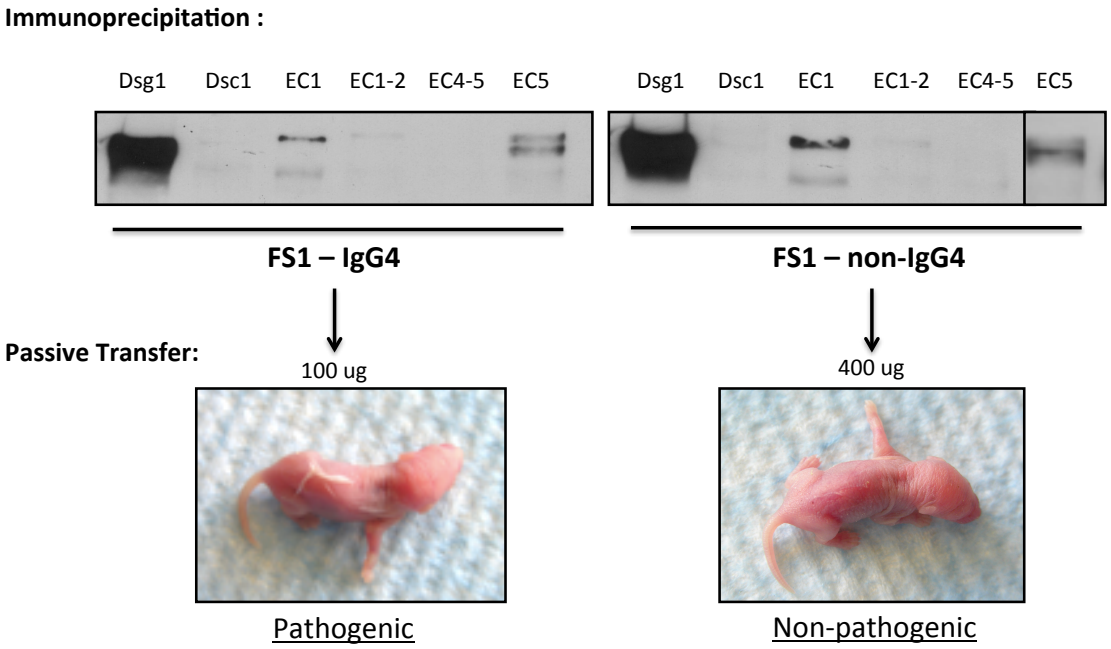

Figure 2. ***Analysis of IgG4 and non-IgG4 autoantibodies from FS patient serum by IP and Passive Transfer.*** *Top panel*: IP/IB using purified swapped-domain proteins. Monoclonal anti-His antibody was used as a probe. IgG4 (97% pure fraction) and non-IgG4 (92% IgG1) recognized the EC1 and EC5 domains of Dsg1. *Bottom panel*: Different doses of both fractions were passively transferred to neonatal Balb/c mice. *Bottom left:* mouse injected with 100 ug of IgG4 showed extensive blistering at the injection site. *Bottom right*: Mouse injected with 400 ug of non-IgG4 did not develop blisters. Two mice per group were used.

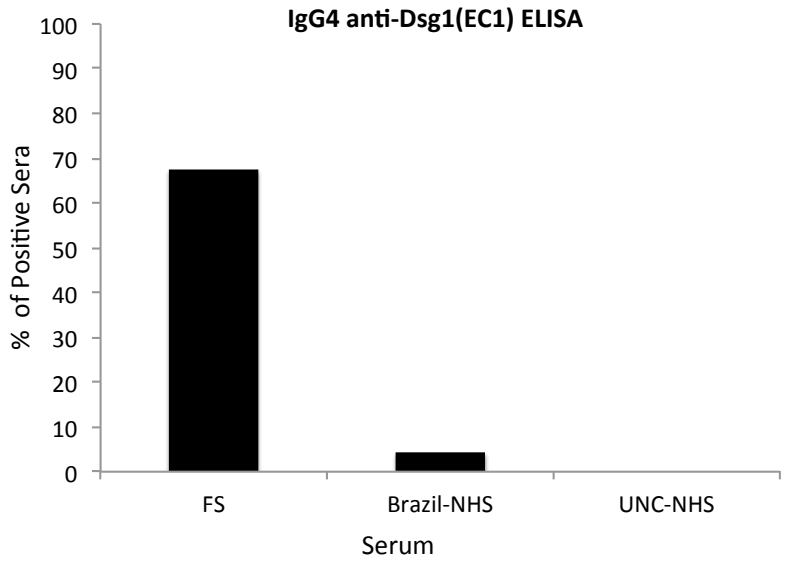

Figure 3. ***Prevalence of IgG4 autoantibodies against Dsg1(EC1) by ELISA***. Purified swapped-domain protein bearing EC1 domain of Dsg1 was used to coat ELISA plates in order to test the immunoreactivity of IgG4 anti-EC1 across human sera: FS (n=55), Brazil-NHS (n=23), UNC-NHS (n=22). Monoclonal anti-human IgG4-HRP antibody was used as a secondary antibody. A cutoff of 7.54 defined positive sera. NHS= normal human sera.

2.3.2. Dominant conformational epitopes for pathogenic IgG4 FS autoantibodies are located within EC1 and EC2 regions of Dsg1 extracellular domain.

To further refine the mapping of the epitopes recognized by IgG4 anti-Dsg1 autoantibodies, we purified IgG4 fractions from 20 FS sera using the llama anti-human IgG4-Sepharose

matrix. The IgG4 fraction from each serum was immobilized on CNBr-activated Sepharose followed by incubation with purified recombinant human Dsg1 extracellular domain. As described in Methods and shown in **Fig. 4**, the immobilized immune complex, i.e. hDsg1-IgG4, was digested with trypsin/chymotrypsin and the IgG4-protected Dsg1 peptides (epitopes) were later eluted and analyzed by MALDI-TOF/TOF-MS/MS.

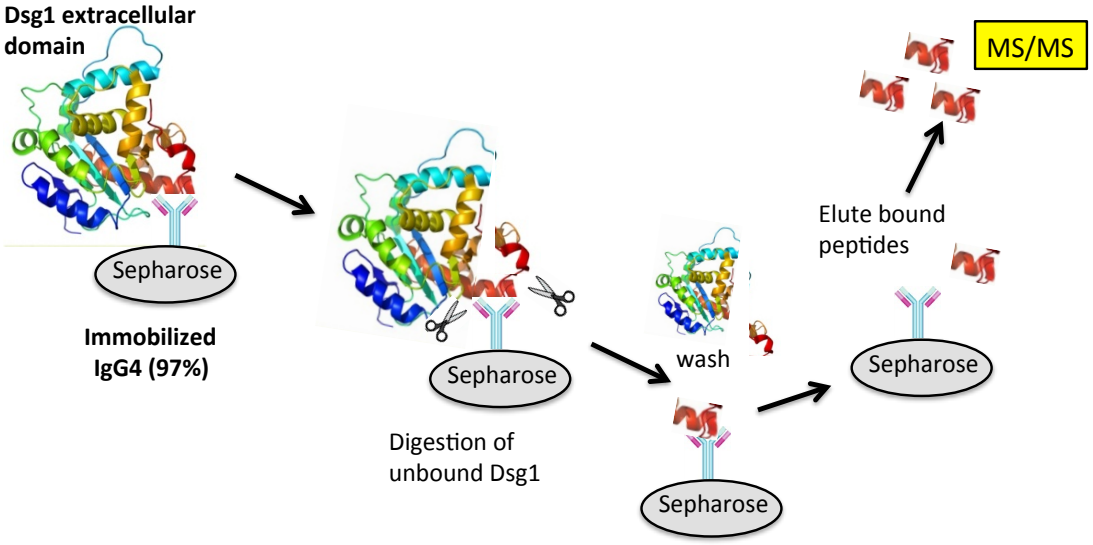

Figure 4. ***Epitope mapping by epitope excision and mass spectrometry.*** A schematic representation of the epitope mapping strategy used in this study. Immobilized pure IgG4 binds to recombinant Dsg1 extracellular domain, followed by digestion of the unbound portions with chymotrypsin/trypsin. After several washes, bound peptides are dissociated and analyzed by MALDI-TOF/TOF-MS/MS.

Two dominant epitopes were identified. One is located within EC1 (residues A129 – R144) and the second is within EC2 (residues Q201-R213). The epitope in EC1 was recognized by 19/20 FS sera (95%) and the epitope in EC2 was recognized by 17/20 FS sera (85%). Additional epitopes located within the propeptide sequence, EC4, and EC5 were also identified but in lower frequencies (5-45%). The summary of these results are shown in **Tables II and III.**

To validate our epitope mapping results we utilized the IgG4 fraction purified from healthy human serum, which did not recognize any epitopes in Dsg1 by our epitope excision method. Additionally, when recombinant hDsg1 extracellular domain was digested with trypsin/chymotrypsin prior to incubation with FS IgG4-Sepharose, no relevant epitopes were detected by MALDI-MS/MS. These results suggest that the majority of identified epitopes bound by FS IgG4 are conformational. The two major epitopes (peptides) identified within EC1 and EC2 were synthesized alone or linked to biotin. The synthetics peptides were used to perform ELISAs and inhibition assays. None of the FS sera or purified IgG4 fractions recognized the synthetic peptides, as demonstrated by direct ELISA, or ELISAs on Streptavidin-coated microtiter plates (data not shown). These results strongly suggest that both epitopes are conformational.

Table II. Conformational Epitopes in the extracellular domain of human Dsg1 targeted by IgG4 autoantibodies from 20 FS patients.

Patient ID	propeptide	EC1				EC2			EC4			EC5
	W41-R45	F54-R59	E60-R67	I73-R86	A129-R144	Q201-R213	N214-R219	T220-R227	V404-R422	Y423-R438	Y460-R473	D538-F546
FS1	+				+	+					+	
FS2	+	+				+	+				+	
FS3	+				+	+		+			+	
FS4	+				+	+					+	+
FS5	+				+	+		+			+	+
FS6	+			+	+	+		+	+	+	+	
FS7	+				+	+		+				
FS8					+	+					+	+
FS9					+	+					+	+
FS10	+				+	+		+				+
FS11					+	+						
FS12					+	+		+		+		
FS13	+				+							
FS14					+	+		+		+		
FS15					+	+						
FS16					+							
FS17			+		+							
FS18					+	+						
FS19					+	+						
FS20					+	+						
TOTAL	**9**	**1**	**1**	**1**	**19**	**17**	**1**	**7**	**1**	**3**	**8**	**5**

Table III. Frequency of IgG4 anti-Dsg1 autoantibodies targeting conformational epitopes identified by MALDI-MS/MS analysis.

Region in Dsg1 ectodomain	Epitope Amino Acid sequence	Frecuency of FS IgG4 (n=20)
Propeptide	WHSIR	45 %
EC1	FAAACR	5 %
	EGEDNSKR	5 %
	IHSDCAANQQVTYR	5 %
	ALNSMGQDLERPLELR	**95%**
EC2	**QEPSDSPMFIINR**	**85 %**
	NTGEIR	10 %
	TMNNFLDR	35 %
EC4	VGDFVATDLDTGRPSTTVR	5 %
	YVMGNNPADLLAVDSR	15 %
	YQGTILSIDDNLQR	40 %
EC5	DLLSDNVHF	25 %

Molecular modeling of the Dsg1 extracellular domain was performed to predict the atomic structure of the molecule and the location of the EC1 and EC2 epitopes recognized by FS IgG4. As shown in **Fig. 5**, the structure of hDsg1 was predicted in its homodimeric adhesive conformation. One monomer is dark gray and the second monomer is in light grey. The dimeric adhesive conformation can be recognized by the extension of the light and dark strands in EC1 and their interactions with the other monomer. A pocket within EC1 of the first monomer will contain its own W51 in the monomeric state but contains the W51 from the second monomer in the swapped state, which is the adhesive conformation. The first 2 residues (AL) of the epitope in EC1: A129-R144, are part of the cell-adhesion recognition site RAL of Dsg1, a motif important for maintaining desmosomal adhesion.

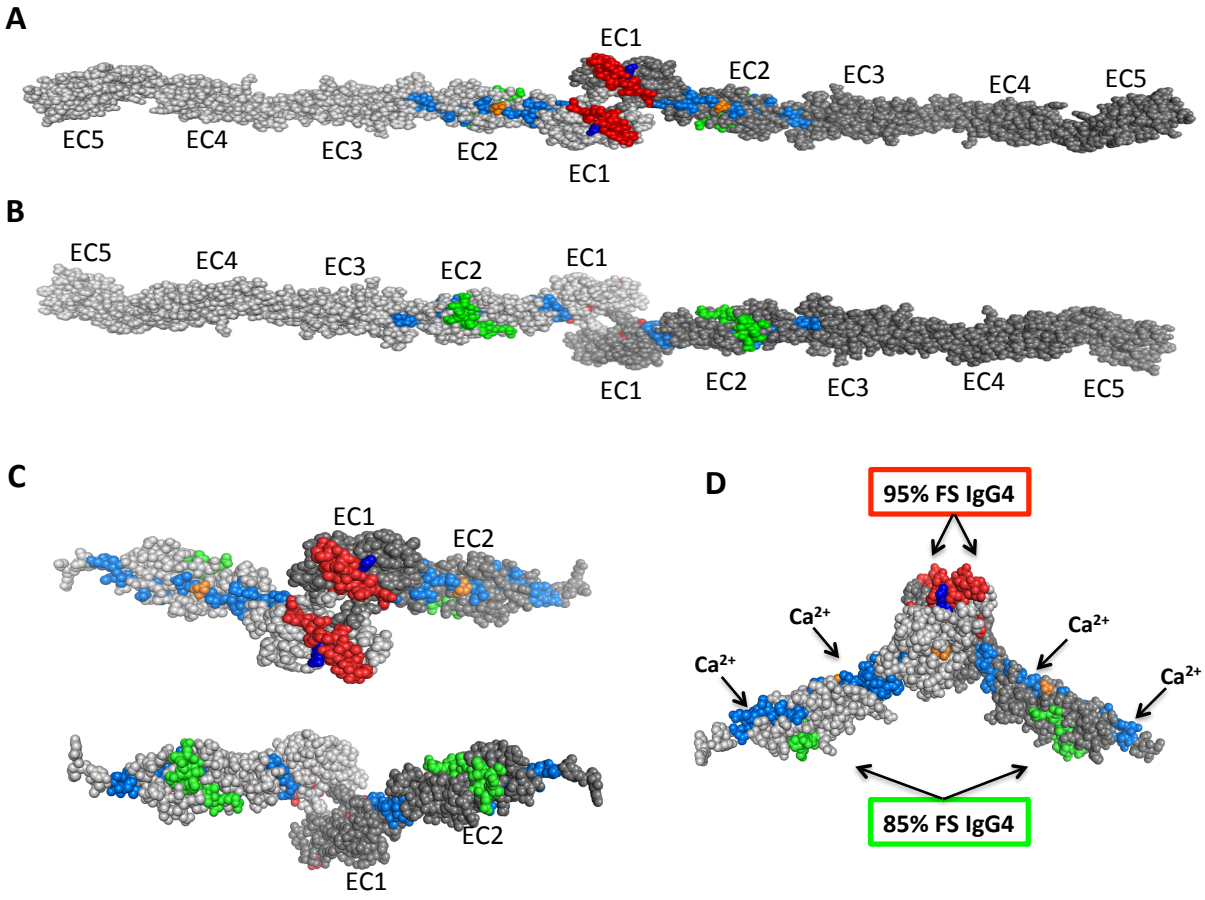

Figure 5. ***Location of 2 conformational epitopes recognized by FS IgG4 autoantibodies in a predicted model of hDsg1 extracellular domain.*** Predicted atomic structure of hDsg1 was generated by molecular modeling using classical cadherins as a template. Homodimeric adhesive conformation model is shown with one monomer in light gray and the second monomer in dark gray. **A)** Anterior view of hDsg1 homodimer showing epitope in EC1: ALNSMGQDLERPLELR in red. **B)** Posterior view of hDsg1 homodimer showing the epitope in EC2: QEPSDSPMFIINR in green. **C)** Blow up of hDsg1 homodimer in the context of EC1 and EC2 domains. Note that the extension of the light and dark strands in EC1 defines the dimeric adhesive conformation. A pocket within EC1 of the first monomer will contain its own W51 in the monomeric state but contains the W51 from the second monomer in the swapped state, which is the adhesive conformation. **D)** Bottom view of hDsg1 dimer showing the nearness of the EC1 epitopes in the dimer conformation. R of RAL adhesive motif is in dark blue and AL residues are shared with the EC1 epitope (red). Calcium binding sites are in light blue. Two N-glycosilation sites in orange.

2.3.3 Residues M133 and Q135 in EC1 region of hDsg1 extracellular domain are key components of the conformational epitope for pathogenic IgG4 autoantibodies in FS.

In order to define the key components of the conformational epitope for pathogenic IgG4 autoantibodies in FS, we first compared the amino acid sequence of the two major epitopes identified in this study across homologous cadherins. The alignment of both epitopes sequences in hDsg1 showed that there are more conserved amino acids in hDsg4 than in other desmosomal cadherins. This observation is in agreement with previous reports that anti-Dsg4 antibodies from pemphigus patients cross-react with Dsg1 but are not pathogenic in the mouse model [20]. Moreover, Dsg4 extracellular domain is not recognized by the 20 FS sera used in this study by ELISA.

The sequence of the conformational epitope in EC1 of hDsg1 differs in two residues from hDsg4 (residues M133 and Q135), whereas in the EC2 epitope of Dsg1, three residues are different from Dsg4 (residues D205, S206 and I211) **(Fig. 6B).** Two point-mutated recombinant Dsg4 extracellular domains were generated by site-directed mutagenesis: Dsg4-MEC1 (containing M133 and Q135 from Dsg1) and Dsg4-MEC2 (containing D205, S206 and I211 of Dsg1). In addition, we generated two point-mutated recombinant Dsg1 extracellular domains: Dsg1-MEC1 (containing R133 and E135 from Dsg4) and Dsg1-MEC2 (containing G205, A206 and L211 from Dsg4) **(Fig. 6C)**.

The four mutated recombinant proteins were tested by ELISA in order to determine the effect of the mutations in the reactivity of IgG4 autoantibodies from 20 FS against Dsg1 or Dsg4. Mutation of residues M133 and Q135 in Dsg1 (Dsg1-MEC1) dramatically reduced (77%) the reactivity of IgG4 autoantibodies against Dsg1, demonstrating the crucial role that

these residues play in forming the recognized epitope **(Fig. 7A)**. Mutations in EC2 (Dsg1-MEC2) did not show significant difference (13% reduction) in reactivity of IgG4 against Dsg1. On the other hand, mutations in Dsg4 did not restore recognition of this molecule by IgG4 autoantibodies from FS patients **(Fig. 8)**, suggesting that there may be additional residue(s) in other regions of Dsg1 that are absent in Dsg4 and are necessary to achieve the proper protein conformation that makes the conformational epitope accessible to IgG4 autoantibodies.

To further confirm these results, we performed immunoprecipitation (IP) using recombinant hDsg1 extracellular domain and the two point-mutated recombinant hDsg1 proteins. By IP, IgG4 from FS patients did not immunoprecipitate Dsg1-MEC1 but did recognized Dsg1-MEC2 **(Fig. 7B)**, mirroring our ELISA results. Taken together, our results indicate that residues M133 and Q135 located in EC1 region of Dsg1 extracellular domain are required to achieve the proper conformation of the epitope for pathogenic IgG4 autoantibodies in FS.

Figure 6. ***Site directed mutagenesis in the extracellular domains of hDsg1 and hDsg4.*** **A)** Amino acid sequence of the N-terminal end of Dsg1. Showing the sequence of EC1 domain (purple box), EC2 domain (blue box), signal sequence (black box) and propeptide sequence (orange box). Peptides recognized by FS IgG4 antibodies in EC1 and EC2 are in red and green respectively. RAL sequence is highlighted. **B)** Alignment of amino acid sequences of the two dominant conformational epitopes identified in hDsg1 with its homolog cadherin hDsg4 that is not recognized by FS IgG4 autoantibodies. Non-conserved residues are labeled with a star (*). **C)** Diagram of constructs of wild type (WT) and point-mutated (MEC1 or 2) molecules expressed in baculovirus system. Residues 133,135,205,206 and 211 were replaced in the extracellular domain of hDsg1 or hDsg4 by site-directed mutagenesis.

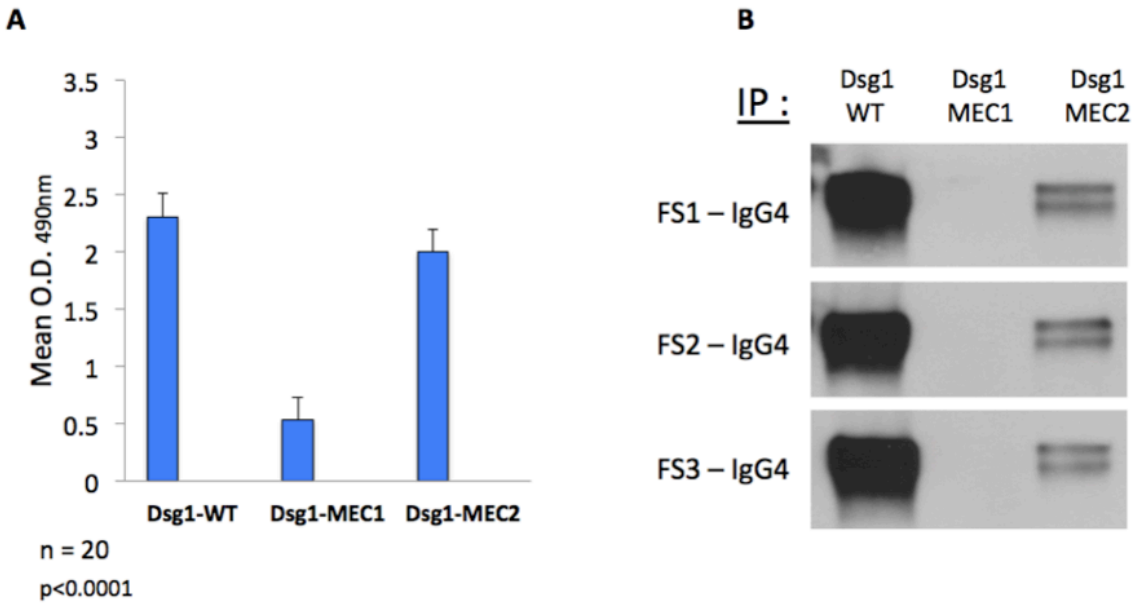

Figure 7. ***Mutation of residues M133 and Q135 in EC1 domain of Dsg1 dramatically reduces the binding of IgG4 autoantibodies to Dsg1 extracellular domain.*** **A)** Purified IgG4 fractions from 20 FS sera were tested by ELISA for reactivity against Dsg1-WT and Dsg1-mutants. Dsg1-MEC1 (with mutations in M133 and Q135) showed diminished recognition (77%) by IgG4 FS autoantibodies. Mutations in EC2 showed no significative reduction (13%) in binding by IgG4. Commercially available human IgG4 (Abcam) and normal human serum (NHS) or IgG purified from NHS were used as negative controls in each ELISA. Monoclonal anti-hIgG4-HRP conjugated antibody was used as a secondary antibody. Mean O.D. values are shown. **B)** Immunoprecipitation (IP) results from IgG4 fractions purified from 3 FS patients are shown. Fractions were pathogenic in mouse passive transfer model. hDsg1 with mutations in EC1 (M133 and Q135) is not recognized by any of the 3 fractions. Monoclonal anti-His-HRP conjugated antibody was used for detection.

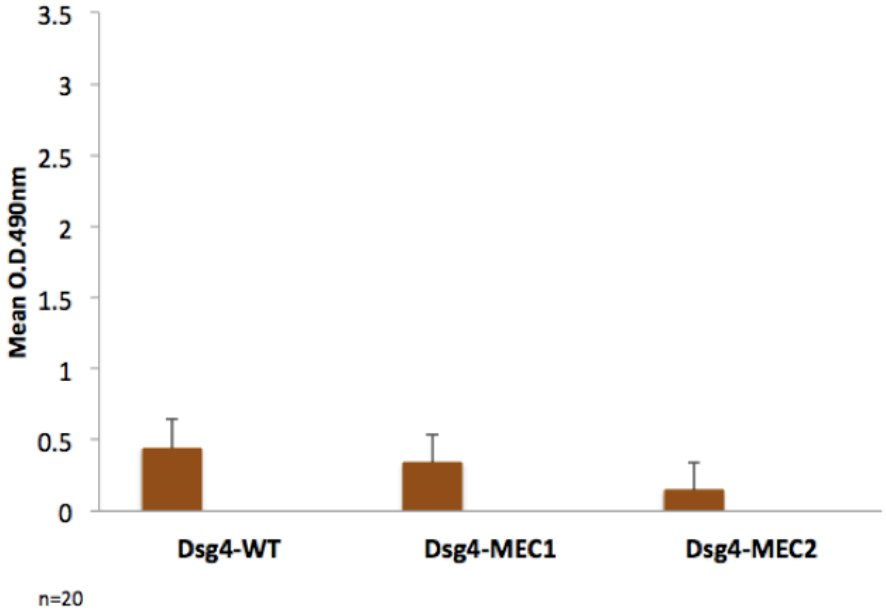

Figure 8. ***Detection of IgG4 autoantibodies against Dsg4-WT and Dsg4-mutants by ELISA.*** Purified IgG4 fractions from 20 FS sera were tested for reactivity against Dsg4 WT and Dsg4 mutants. Commercially available human IgG4 (Abcam) and normal human serum (NHS) or IgG purified from NHS were used as negative controls in each ELISA. Monoclonal anti-hIgG4-HRP conjugated antibody was used as a secondary antibody. Mean O.D. values are shown.

2.4. DISCUSSION

In this study, we have successfully located two dominant conformational epitopes within the EC1 and EC2 domains of Dsg1 by using improved IgG4 purification techniques and a powerful epitope mapping strategy. Most importantly, we identified 2 residues within the calcium- and conformational-dependent EC1 epitope that appear to be required for the binding of pathogenic IgG4 anti-Dsg1 autoantibodies.

The low levels of IgG4 subclass present in normal human serum (4% of IgG) [23] has made purification of IgG4 antibodies challenging. Our group and others have used different separation methods such as Ion Exchange Chromatography on DEAE or a combination of positive and negative affinity chromatography using monoclonal antibodies [4, 24, 25]. However, these "multistep" methods have limitations such as time, cost, efficiency, and most importantly, the so-called IgG4 enriched fractions exhibit significant IgG1 contamination (12-30%). In this study we overcame such limitations to obtain highly purified IgG4 fractions by using an affinity matrix containing a 12kD llama antibody fragment that recognizes human IgG4 without cross reactivity to other human IgG subclasses 1,2 or 3. The use of llama antibodies as affinity ligands offers several advantages over conventional antibodies [26-28]. These antibodies lack the entire light chain and the CH1 domain found in conventional antibodies. Thus the antigen binding domain is a 12-15kD single protein, called VHH, which is much more stable compared to standard monoclonal antibodies [28-30]. Using this one step affinity purification method, we obtained 97% pure IgG4 fractions from FS patients' sera. Purified IgG4 was immunoreactive when tested by IP or ELISA and in the passive transfer mouse model. Furthermore, the observation that both bound (97% IgG4) and unbound fractions (92% IgG1) targeted the same domains in Dsg1 extracellular domain (EC1

and EC5) **(Fig. 2)** indicated the importance of using a highly purified reagent (IgG4) in order to dissect the specific epitope(s) within those domains.

Epitope spreading is a pathogenic process that leads to relapse and chronic progression of an autoimmune disease [31-33]. We have previously suggested that intramolecular epitope spreading occurs in FS [8], where the anti-Dsg1 autoimmune response evolves from a non-pathogenic response to the EC5 portion of the Dsg1 extracellular domain in healthy individuals to a pathogenic response against the EC1 and EC2 in FS patients.

The identification of the specific epitope(s) recognized by pathogenic FS antibodies is a key step in understanding the pathogenesis of FS blister formation. Previous attempts to elucidate the specificity of pemphigus autoantibodies within Dsg1 have shown that blister formation is driven by IgG autoantibodies specific for the N-terminal end of Dsg1 extracellular domain [14, 34, 35], which contains the adhesive interface of the molecule responsible for maintaining desmosomal adhesion[36] However, the fine epitope specificity of IgG4 anti-Dsg1 autoantibodies remains elusive. In this study, we refine previous epitope mapping findings and identify critical components of the conformational epitope for IgG4 autoantibodies from FS.

We prepared a recombinant Dsg1-Sepharose matrix to purify IgG4 anti-Dsg1 antibodies from the IgG4 fractions obtained from FS serum. We successfully obtained IgG4 anti-Dsg1 antibodies and they were also pathogenic when passively transferred into neonatal mice. Only 10 μg of anti-Dsg1 IgG4 per mouse were needed to produce extensive blister in the mouse, equivalent to the blistering induced by injection of 100 μg of IgG4 fraction **(Fig. 2)**. This result, coupled with the observation that injection of a higher dose (4-fold) of the unbound fraction from the previous step (containing 92% IgG1) doesn't induce blistering in

the mouse model, strongly suggests that the epitope(s) recognized by IgG4 autoantibodies is responsible for the blister formation in FS.

Consistently with previous findings [8] the majority of IgG autoantibodies from FS patients sera targeted the EC1 region of the Dsg1 extracellular domain, when using domain-swapped recombinant proteins in IP assays (data not shown). In addition, 67.3% (33/55) of FS patients serum possessed circulating IgG4 specific for the EC1 region of Dsg1 when tested by ELISA using domain-swapped Dsg1 chimeric protein. This result suggests that the epitope for pathogenic IgG4 autoantibodies may be located within EC1 region of Dsg1 extracellular domain.

Previous studies have shown that the binding of pemphigus antibodies is calcium- and conformation-dependent [11, 12], which indicates that most of these autoantibodies recognize three-dimensional structures that depend on protein folding. Unfortunately and to the best of our knowledge, there is no 3D crystal structure of Dsg1 available, making it difficult to pinpoint the specific sequence that constitutes the epitope for pemphigus autoantibodies.

In this study, we introduce the use of proteomics to the pemphigus field. Epitope mapping for pathogenic IgG4 autoantibodies from FS patients is assessed for the first time by epitope excision and mass spectrometry. Epitope excision is a form of protective assay, where the Dsg1-bound IgG4 protects the epitopes sequences from digestion because of the known resistance of the immobilized antibody to proteolysis [37] and the use of non-denaturing conditions, which allows the antigen to retain its conformation. Using polyclonal IgG4 fractions, we were able to identify two dominant conformational epitopes that are consistently recognized by almost all of the 20 FS patients (95% and 85%). We identified

additional epitopes but in much lower frequencies (5-45%). These additional epitopes may not be relevant to disease and/or may have been part of the epitope spreading cascade in earlier, perhaps non-pathogenic, stages of FS.

Using molecular modeling we predicted the atomic homodimeric structure of the Dsg1 extracellular domain using classical cadherins as a template. We localized the sequences of the two dominant conformational epitopes in EC1 and EC2 respectively. Both epitopes were flanked by calcium-binding sites in the model and were in opposite faces of the molecule. The most common epitope with residues 129A-144R located within EC1 shares 2 residues (129A and 130L) with the RAL sequence of Dsg1, that is part of the adhesive interface of this molecule. In addition, the nearness of the two EC1 epitopes from the dimer conformation suggests that the binding of IgG4 to this epitope may interfere with the dimer formation, depending on which residues it binds. The epitope with residues Q201–R213 located within EC2 seems to be far from the adhesive interface; however, a calcium-binding site separates this epitope from the one in EC1. It is possible that upon binding of calcium ions to Dsg1, the protein folds into a conformation that brings both epitopes into proximity, allowing the displayed epitope to incorporate residues from EC1 and EC2. Availability of Dsg1 crystal structure will be useful to prove this hypothesis.

Although we identified two conformational epitopes that are recognized by IgG4 autoantibodies from FS patients, further testing is needed to determine whether one or both of the epitopes are relevant to disease. Alignment of both epitopes sequences of Dsg1 with Dsg4 extracellular domain reveals that residues M133, Q135, D205, S206 and I211 are not conserved in Dsg4. Replacement of these residues in Dsg1 and Dsg4 by site-directed mutagenesis demonstrated that residues M133 and Q135 in EC1 of Dsg1 extracellular

domain are required for the binding of IgG4 autoantibodies from FS patients, suggesting that these residues may define the conformational epitope for pathogenic IgG4 autoantibodies in FS. Interestingly, the replacement of residues in EC1 or EC2 of hDsg4 in order to mimic both epitopes from hDsg1 did not cause any effect in the binding by IgG4 when tested by ELISA or IP. Therefore, there may be additional residues in other regions of Dsg1 that are absent in Dsg4, which help to create the conformation that displays the epitope in EC1. This may be the case for the epitope in EC2, where mutation of the three residues was not sufficient to block the binding of IgG4 to Dsg1. It is also possible that the IgG4 autoantibodies specific for the epitope in EC2 constitutes a small population of autoantibodies that may or may not be pathogenic.

This study identifies two dominant conformational epitopes recognized by pathogenic IgG4 autoantibodies in FS. In addition, it narrows down the residue requirement in EC1 for the binding of these autoantibodies. These findings provide a basis for targeted epitope-specific preventative and improved therapeutic strategies, as well as for identification of molecular mechanisms and/or environmental triggers(s) for FS.

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CHAPTER III

INDUCTION OF IgG4 ANTI-DESMOGLEIN 1 ANTIBODIES BY HEMATOPHAGUS INSECTS BITES[1]

3.1. INTRODUCTION

It is an accepted assumption that the interaction of unknown environmental factors with susceptibility genes of the host ensues the immune system to react to self-antigens causing an spectrum of autoimmune diseases [1]. The common thread amongst autoimmune diseases is the obscure etiology. Human organ specific autoimmune diseases targeting the skin comprise the pemphigus group, where pathogenic IgG4-restricted, anti-epidermal autoantibodies cause epidermal cell detachment that leads to blister formation [2]. The antigen recognized by these autoantibodies in PF is Dsg1. The idiopathic, non-endemic form of PF is known worldwide, whereas an endemic variety, FS is seen in certain regions of subtropical Brazil [3]. FS shows similar clinical, histological and immunological features to non-endemic PF, except for the unique epidemiology of FS. A case-control epidemiological study of FS in Brazil suggested that certain living conditions and exposure to hematophagous insect bites were risk factors of FS [4]. Exposure to bites of three insects is suspected to be linked to FS: *Lutzomyia longipalpis* (sand flies), reduviids (kissing bugs) and simuliids

[1] Partially reproduced from: Qian,Y., Jeong, J.S., Maldonado, M., Valenzuela, J.G., Gomes, R., **Evangelista, F.,** Qaqish, B., Aoki, V., Hans, G.Jr., Rivitti, E.A., Eaton, D., Diaz, L.A. *Cutting Edge: Brazilian pemphigus foliaceus anti-Desmoglein 1 autoantibodies cross-react with sand fly salivary LJM11 antigen.* J Immunol. 2012. 189(4): 1535-1539.
F. Evangelista contributed with the generation and purification of rDsg1, immunoprecipitation assays and additional experiments that are not part of the text, but supportive of the overall conclusion.

(black flies). They are vectors of leishmaniasis, Chagas disease and onchocerciasis respectively. Moreover, the sera of a large number of these patients possess anti-Dsg1 autoantibodies [5].

A recent study has demonstrated that not only IgM and IgG4 anti-Dsg1 autoantibodies are detected in the sera of FS but also IgE [6]. It is remarkable that IgG4 anti-Dsg1 autoantibodies are restricted and pathogenic in FS; however, the mechanisms involved in the emergence of these autoantibodies are completely unknown. The endemic nature of FS and the circumstantial evidence presented above allow us to test the hypothesis that salivary gland antigens from hematophagus insects are the source of sensitizing antigen that triggers the autoimmune disease in FS. We selected a well-defined system provided by *Lutzomyia longipalpis,* where the salivary gland proteins are well characterized [7, 8]. In this investigation we show that IgG4 autoantibodies from FS sera recognized salivary gland antigens from *Lutzomyia longipalpis* (SGLL). The major SGLL antigenic component recognized by FS sera is LJM11. In addition, sera from mice immunized with LJM11 also recognize human recombinant Dsg1. These results strongly support the notion that LJM11 induces cross-reactive antibodies in FS patients and experimental animals. This is the first evidence that a non-infectious agent may trigger a human autoimmune disease via molecular mimicry.

3.2. MATERIAL AND METHODS

Serum samples and anti-Dsg1 monoclonal antibodies from FS patients

FS sera (N = 45) and two IgG4 anti-Dsg1 monoclonal antibodies (4E4 and 2D11) derived from FS patients [9], were used. Sera from healthy donors (n = 43) from the University of

North Carolina blood bank were included as controls (HC-UNC). Ten sera from normal donors living in Brazilian endemic areas of FS were also included in some of the studies (HC-endemic). This study was approved by the Institutional review boards from universities of North Carolina, Chapel Hill and Sao Paulo, Brazil. The H and L chains of 4E4 and 2D11 [9] were cloned into pComb3XSS vector and expressed in Top10 F' cells [10]. A GST-tag was introduced to the 4E4 construct to increase the solubility of the recombinant 4E4 scFv, and 4E4-GST scFv was produced and purified by Genscript (Piscataway, NJ). The 4E4-GST scFv was not pathogenic when tested by passive transfer into neonatal mice [2] and the dispase assay [11] using concentrations up to 30ug/dose and 5ug/ml of the antibody respectively.

Recombinant Human Dsg1, Sand fly salivary gland extract, and sand fly salivary proteins

Recombinant Dsg1 was generated and purified as described [12]. Salivary gland extracts from *Lutzomyia longipalpis* (SGLL) and SGLL proteins LJM11, LJM17, and LJL143 were generated at the Laboratory of Malaria and Vector Research, NIAID by Valenzuela [8, 13].

ELISA

IgG4 anti-Dsg1 and anti-SGLL ELISAs were conducted as described in previous communications [6, 9, 14]. The ELISA assay to detect IgG4 antibody activity against LJM11, LJM17 and LJL143 SGLL proteins was also conducted as above with some modifications. Stripwell Microplates (Corning, Lowell, MA) were coated with 50ng/well with either LJM11, or LJM17, or LJL143 proteins. A 1:100 dilution of each of FS sera or scFv 4E4 and 2D11 anti-Dsg1 monoclonal antibodies (50ng/ml) was added and incubated.

The bound IgG4 antibodies from serum samples or scFv antibodies were detected with anti-human IgG4 HRP (Zymed, San Francisco, CA) or anti-HA HRP conjugate (Roche, Indianapolis, IN), respectively. Anti-Dsg1 ELISA using SGLL recombinant proteins immunized mouse sera was conducted according to regular anti-Dsg1 ELISA described above with following modification. The mouse serum was diluted 1:500 and goat anti-mouse IgG (Fc fraction) HRP conjugate (Jackson ImmunoResearch, Carlsbad, CA) was used to detect anti-Dsg1 antibodies from mouse sera.

Immunoprecipitation (IP)

Pan mouse IgG magnetic Dynabead (Invitrogen, Carlsbad, CA) was used for the IP according to manufacturer's instructions with modifications. Mouse anti-human IgG4 and Mouse anti-GST tag monoclonal antibodies (Zymed, San Francisco, CA) were used for the IP of sand fly salivary gland proteins LJM11, LJM17, and LJL143. All IPs were proceeded in the TBS buffer containing 0.5% Tween-20 and 5mM $CaCl_2$ (TBS-T-Ca), plus 0.1% BSA. First the Dynabeads (20μl of slurry) were blocked with 0.1% BSA in TBS-T-Ca for 1 hour at room temperature (RT) and incubated with mouse anti-GST (for 4E4-GST) or mouse anti-human IgG4 (for serum samples) for 1 hour at RT, and then 4E4-GST or human serum samples for 2 hours at RT. Finally Dynabeads were incubated with LJM11, LJM17, and LJL143, respectively for 2 hours at RT. For IP using SGLL recombinant protein immunized mouse sera, 2μl of each mouse serum samples were incubated with 450μl recombinant Dsg1 cell culture supernatant for 2 hours at RT and then with 20μl of Dynabead slurry for 1 hour. Each IP sample was subjected to SDS-PAGE and subsequent Western Blot. The membranes

were probed with anti-His HRP (Qiagen, Valencia, CA) and the bands revealed with chemiluminescent substrate (Pierce, Rockford, IL).

Statistical Analysis

Groups were compared by t-test. Correlation analysis was by the Pearson correlation.

3.3. RESULTS

3.3.1. FS patients possess IgG4 antibodies against *Lutzomyia longipalpis* salivary gland antigens.

We have recently reported that FS patients possess significantly higher levels of IgG4 anti-Dsg1 antibodies than control groups from non-endemic areas of Brazil and US [6]. It is possible that generation of these anti-Dsg1 autoantibodies maybe secondary to exposure and sensitization to an environmental antigen(s). The sera from FS patients, and healthy controls from US (HC-UNC) were tested for IgG4 antibody activity against SGLL by ELISA. As shown in **Fig. 1A**, the index values of IgG4 anti-SGLL antibodies are significantly higher in FS patients (n=45) than HC-UNC controls (n=43) ($p<0.001$). The IgG4 anti-SGLL antibody response in the FS group was significantly correlated with both the IgG4 anti-Dsg1 (Pearson correlation: $r=0.56$, $p<0.0001$) **(Fig. 1B).** These findings strongly suggest that the generation of potentially pathogenic IgG4 anti-Dsg1 in FS may be associated with the antigenic stimulation produced by salivary antigens from hematophagous insects.

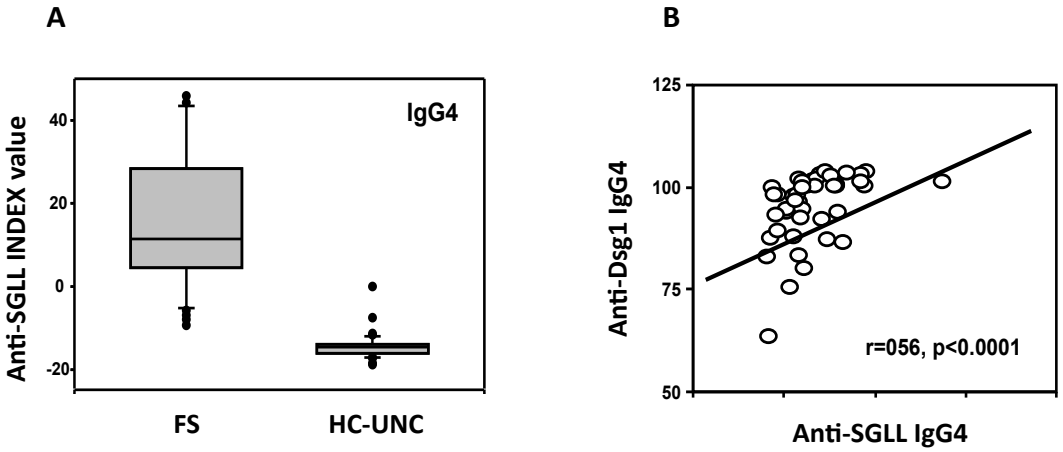

Figure 1. ***FS patients have high levels of anti-SGLL IgG4 antibodies. A)*** Boxplot analysis of the IgG4 response in FS patients (n=45) and UNC healthy controls (n=43) against SGLL antigens by ELISA. The index values are higher in the FS group than the HC-UNC group. ***B)*** Pearson correlation analysis between the IgG4 anti-SGLL and the IgG4 anti-Dsg1 responses in the same donors of the FS group.

3.3.2. The cross-reactivity of anti-Dsg1 antibodies to SGLL

To determine whether the anti-SGLL activity from FS sera is due to the cross-reactivity of anti-Dsg1 autoantibodies, ELISA was performed with two scFv IgG4 monoclonal anti-Dsg1 antibodies derived from two FS patients [9] against SGLL antigens. As shown in **Fig. 2A**, both scFv recognize SGLL. To confirm the cross-reactivity of the two monoclonal anti-Dsg1 autoantibodies with anti-SGLL antibodies, a competition ELISA assay was employed using human recombinant Dsg1 as an inhibitor of the binding of anti-SGLL antibodies to immobilized SGLL antigen. As shown in **Fig. 2B**, binding of both monoclonal antibodies to SGLL are inhibited in a dose dependent manner by soluble Dsg1. These

findings suggest that the anti-SGLL antibody activity in FS patients is, at least in part, due to cross-reactivity of the anti-Dsg1 autoantibodies.

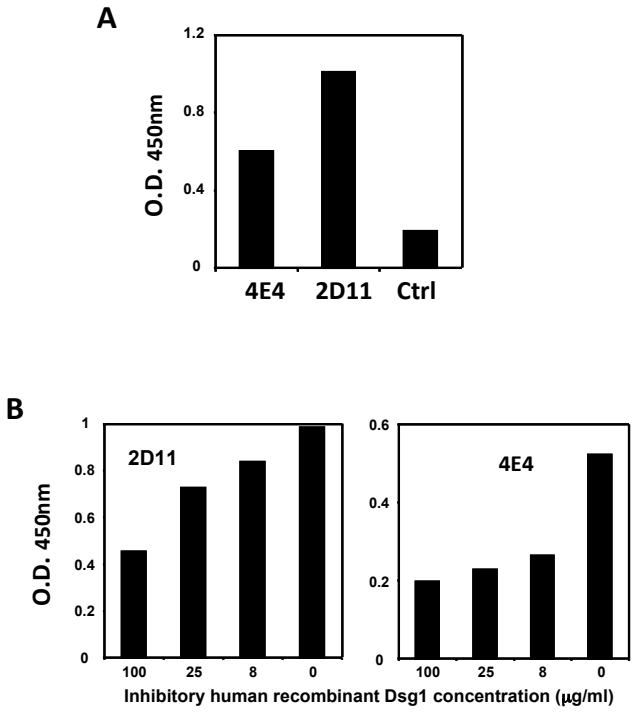

Figure 2. ***The cross-reactivity of two IgG4 anti-Dsg1 monoclonal antibodies from FS to SGLL by ELISA.*** **A**). Both IgG4 anti-Dsg1 monoclonal antibodies (scFv 4E4 and scFv 2D11) react to SGLL. (**B**). The binding of both monoclonal antibodies: 2D11 (left panel) and 4E4 (right panel) to SGLL is inhibited by Dsg1 in a concentration dependent manner. These results were repeated three times with similar results.

3.3.3. The LJM11 major immunogenic component of SGLL is recognized by FS sera and IgG4 monoclonal anti-Dsg1 antibodies

A large number of secreted proteins are present in *Lutzomyia longipalpis* saliva [7, 8, 13] and, LJM11 and LJM17 are the two main antigens that incite antibody responses in humans. Other salivary antigens such as the LJL143 are weakly recognized by humans [13].

These three recombinant proteins were chosen to determine whether they are recognized by IgG4 antibodies from FS patients and normal controls from either FS endemic regions (HC-endemic) or from non-FS endemic region (HC-UNC). Ten serum samples from each group were randomly selected and tested. As shown in **Fig. 3A**, we found that the reactivity of FS sera was consistently much stronger to LJM11 than to LJM17 and LJL143. In addition, the level of anti-LJM11 antibodies from FS is significantly higher than healthy donors from HC-endemic (t-test, p=0.00029) and HC-UNC (t-test, p=0.00013). These results show that IgG4 antibodies from FS sera chiefly recognize the LJM11 component of SGLL.

We next tested the reactivity of 4E4 and 2D11 IgG4 anti-Dsg1 monoclonal antibodies against LJM11, LJM17 and LJL143. As shown in **Fig. 3B**, both monoclonal antibodies bind strongly to LJM11, but weakly to LJM17 and LJL143. These findings are similar to those observed when testing FS sera. To further confirm that these two monoclonal antibodies cross-react to LJM11, an ELISA inhibition assay was conducted. A dose of 100μg/ml of recombinant Dsg1 incubated with these antibodies was able to inhibit 90% and 70% of the bindings of 4E4 and 2D11 to LJM11 respectively **(Fig. 3C)**. In addition, IP using monoclonal antibody 4E4, and FS patient serum show that both react strongly to LJM11 (~43kD), but weakly to LJM17 (~45kD) and LJL143 (~34kD) **(Fig. 4A)**. These findings suggest that LJM11 is the main component of SGLL that triggers the IgG4 immune response in humans that cross-react with Dsg1 autoantigen.

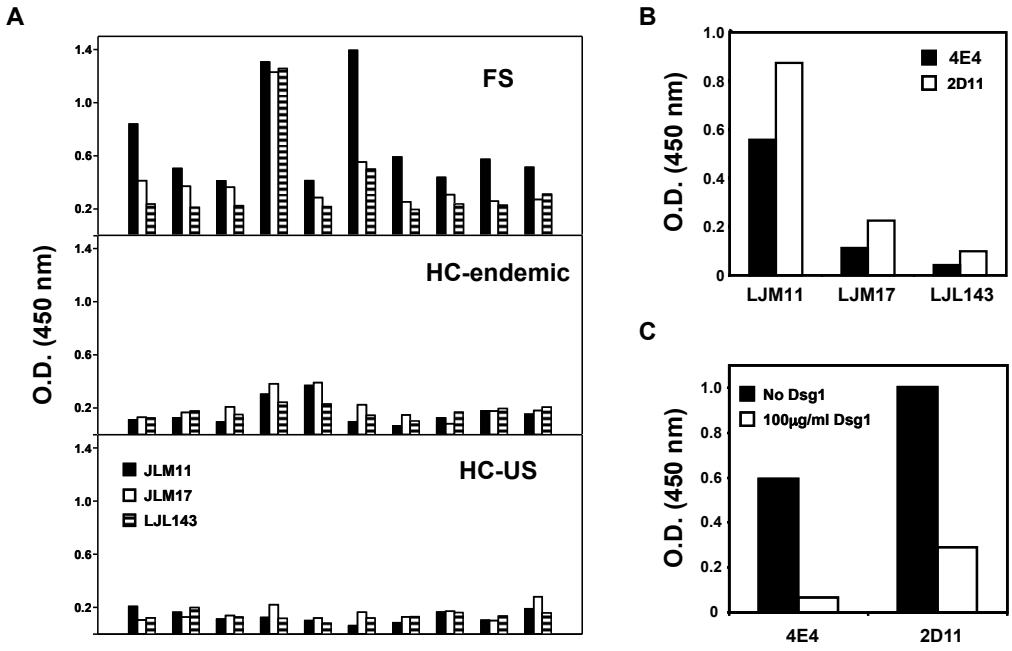

Figure 3. ***IgG4 antibodies from FS patients and two IgG4 monoclonal anti-Dsg1 antibodies derived from FS patients recognize LJM11, a protein component from SGLL.*** **A)** The reactivity of ten random-selected FS sera (top panel), is higher than donors from the HC-endemic (middle panel) and HC-UNC donors (bottom panel) when tested by ELISA with three SGLL main proteins, LJM11, LJM17, and LJL143. The reactivity is mainly with the LJM11 protein. The difference between HC-endemic and HC-UNC by the student-t test is not significant (p=0.3800). **B)** The 4E4 and 2D11 IgG4 monoclonal anti-Dsg1 antibodies also recognize the LJM11 component from SGLL. **C)** The binding of both anti-Dsg1 monoclonal antibodies to LJM11 is inhibited by Dsg1 protein.

3.3.4. The binding of anti-Dsg1 monoclonal antibodies to LJM11 is conformational and Ca^{2+} dependent.

Both 4E4 and 2D11 anti-Dsg1 monoclonal antibody failed to react with denatured LJM11, LJM17, and LJL143 by Western blot analysis (data not shown), suggesting that 4E4

antibody binding to the SGLL is conformational. Since binding of pathogenic autoantibodies to Dsg1 is conformational and Ca^{2+} dependent [15], we tested the reactivity of these two antibodies to LJM11 in the presence of either Ca^{2+} or EDTA. As shown in **Fig. 4B** the binding of these monoclonal antibodies from FS patients to LJM11 is also Ca^{2+}-dependent. These results suggest that the epitopes recognized by FS sera on SGLL, like those on Dsg1, are Ca^{2+} dependent and conformational.

3.3.5 Mice immunized with LJM11 produce anti-Dsg1 antibodies

If SGLL proteins introduced by sand fly bites induce antibodies that cross-react to both human Dsg1 and LJM11 in humans, it is expected that mice immunize with LJM11 also generate cross-reactive antibodies to human Dsg1. Anti-Dsg1 reactivity of the sera from these mice and control mouse were test by ELISA. As shown in **Fig. 4C (top panel)**, sera from all three mice immunized with LJM11 strongly react to human Dsg1. The Dsg1 reactivity of these mouse sera was also tested by IP. As shown in **Fig. 4C (bottom panel)**, the serum from a mouse immunized with LJM11 antigen also immunoprecipitates human Dsg1. Control serum and sera from mice immunized with LJM17 and LJL143 antigens do not show reactivity. These findings further confirm that LJM11 is the major component in SGLL that induces the cross-reactive antibodies in both human FS patients and experimental mice.

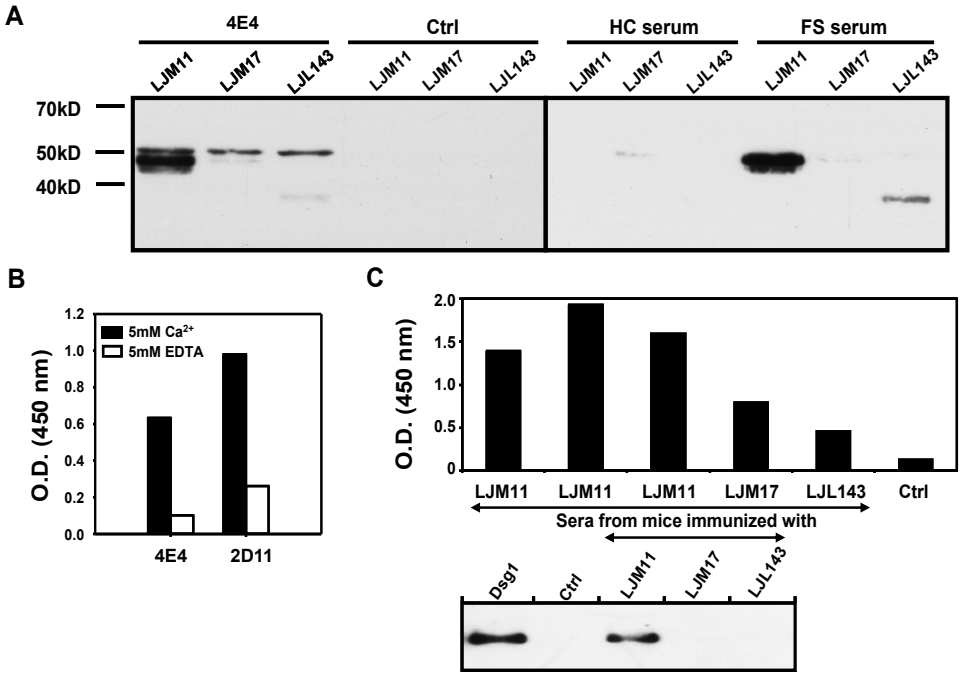

Figure 4. ***FS autoantibodies recognize LJM11 and sera from mice immunized with LJM11 bind human Dsg1.*** **A**)The 4E4 anti-Dsg1 monoclonal antibody (lanes 1,2,3), and control samples without 4E4 (lanes 4,5,6) **(left panel)**, HC-UNC (lanes 7,8,9) and FS serum (lanes 10, 11, 12) **(right panel**) were tested by IP against LJM11, LJM17 and LJL143 antigens. The LJM11 heavy band is precipitated by 4E4 anti-Dsg1 monoclonal antibody and FS serum. **B**) The reactions of 4E4 and 2D11 anti-Dsg1 monoclonal antibodies with LJM11 by ELISA in the presence of 5mM Ca2+ **(black columns)** are significantly inhibited in the presence of 5mM EDTA **(white columns). C**) Three sera from mice immunized with LJM11, show strong reactivity to human Dsg1 by ELISA as compared with sera from mice immunized with LJM17 and LJL143 and control mice. The bottom panel shows human Dsg1 (lane 1), a control murine serum (lane 2), mouse anti-LJM1 (lane 3), mouse anti-LJM17 (lane 4) and mouse anti-LJL143 (lane 5) tested against human Dsg1 by IP. The only serum that immunoprecipitates hDsg1 is from mouse anti-LJM11.

3.4. DISCUSSION

It is unusual that a population of antibodies would develop cross-reactivity to two evolutionarily distant molecules, the human Dsg1 and a sand fly salivary gland component. There is no amino acid sequence similarity between Dsg1 and LJM11 (data not shown). The Ca2+-dependent interaction suggests that autoantibodies react with both molecules via conformational, but not linear, epitopes. This could explain how two evolutionary distant molecules can both be recognized by cross-reactive antibodies since conformational epitopes of both molecules may be the same even though their linear structures do not show significant homology.

Our findings are also in line with molecular mimicry as the mechanism for how environmental factors induce autoimmune diseases [1, 16]. Unlike infectious agents that induce robust IgG immune response, the low dose of the antigens and the route of the exposure (skin) introduced by insect bites predictably induces an IgE response [17]. It is known that endemic regions of FS in Brazil are heavily infested of sand flies, and individuals living in these areas are constantly exposed to these pests. It is likely that these individuals mount IgE and IgG4 responses to salivary antigens such as the LJM11 protein. Similar antibody responses have been described in people undergoing constant bee stings or during immunotherapy of allergic patients [18-21]. It would be expected that those genetically predisposed individuals [22] living in endemic areas of FS are constantly exposed to sand fly bites (and be sensitized to salivary antigens, including the LJM11 protein), thus generating IgG4 anti-SGLL antibodies which, as shown in this investigation, may cross-react with unique epitopes on the ectodomain of human Dsg1. Epitope spreading, as previously reported [23], may be the underlying mechanism that leads to the generation of a more

diverse autoantibody response in FS. The cross-reactive antibodies may comprise a complex population of non-pathogenic and perhaps pathogenic antibodies. In this context, it would be feasible that certain FS IgG4 autoantibodies may exhibit distinct epitope specificity with those IgG4 anti-SGLL antibodies that cross-react with human Dsg1. It is clear however, that testing the pathogenicity of these cross-reactive antibodies is open for further investigations.

In summary, this investigation provides the first direct evidence that a non-infectious environmental agent may play a significant role in the initiation of an autoimmune disease via molecular mimicry.

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CHAPTER IV

SUMMARY AND FUTURE DIRECTIONS

4.1. SUMMARY

Traditionally, IgG4 has been considered to have a protective and anti-inflammatory function; however, recent studies demonstrate that this immunoglobulin can also induce disease [1, 2]. A restricted IgG4 immune response in humans has been reported in patients with parasitosis [3], autoimmune pancreatitis [4], as well as bee handlers [5], allergic individuals during desensitization therapy [6], and the recently reported IgG4-related disease [7, 8]. Pemphigus is not the exception for such IgG4 restriction, but information about its pathogenic role in skin disease is limited [9].

A predominantly IgG4 autoantibody response has been reported in patients with active endemic pemphigus foliaceus (FS) living in LV, Brazil. Moreover, FS IgG4 anti-epidermal antibodies are known to be pathogenic [10] and monovalent Fab fragments are sufficient to induce blistering in the mouse model of FS [11]. Thus, the pathogenicity of these autoantibodies is independent from their Fc portion [12]. Association with certain HLA-DR alleles in IgG4-mediated diseases such as autoimmune pancreatitis and myasthenia gravis has been reported [13, 14] and the alleles for FS susceptibility have been identified in the HLA-DRB1 gene [15]. This indicates a genetic predisposition to develop autoimmune diseases that are IgG4-restricted.

FS is a good model to study the progression of autoimmune disease. The epidemiology of FS and the fact that healthy individuals in the preclinical stage also possess antibodies against the autoantigen makes it possible to explore every step of disease development. The titers of anti-Dsg1 autoantibodies are directly related to the severity and activity of disease, and the B cell repertoire in FS is antigen driven during preclinical stage [16]. Subclass switching has been suggested to occur in FS, where an initial mixture of IgG1 and IgG4 response is observed in healthy individuals and overtime, a dramatic rise in IgG4 anti-Dsg1 autoantibodies coincides with the onset of active disease [17]. In addition, IgG4 anti-Dsg1 autoantibodies are very sensitive predictors of disease in FS [18], which points to the IgG4 response as a key step in the development of FS.

The prevalence of FS is determined by proximity to an endemic area, suggesting that environmental risks factors are potential triggers of the anti-Dsg1 autoimmune response observed in these patients. As described in Chapter 1, hDsg1 has five calcium- binding cadherin repeats (EC1 to EC5) in its extracellular domain, with EC5 being proximal to the cell membrane. A previous study showed that the total IgG response against Dsg1 evolves from a non-pathogenic response targeting the EC5 domain in healthy individuals (no skin lesions) to a pathogenic response against the EC1-EC2 ectodomains of Dsg1 in FS patients. Additionally, the response shifted back to EC5 upon remission [19]. These findings demonstrate that the autoimmune response against Dsg1 is dynamic and that EC1 and EC2 domains of Dsg1 are relevant to FS acantholysis.

It is possible that the dominant epitope in Dsg1 recognized by FS autoantibodies exhibits molecular mimicry with an environmental trigger. Also, it appears that the epitopes for IgG1 and IgG4 are different because of the different outcomes observed when each

subclass is predominant. Therefore, identification of specific epitopes in Dsg1 for pathogenic and non-pathogenic FS autoantibodies and their association with an environmental trigger will be a key step in our efforts to understand the pathogenesis of FS **(Fig. 1).**

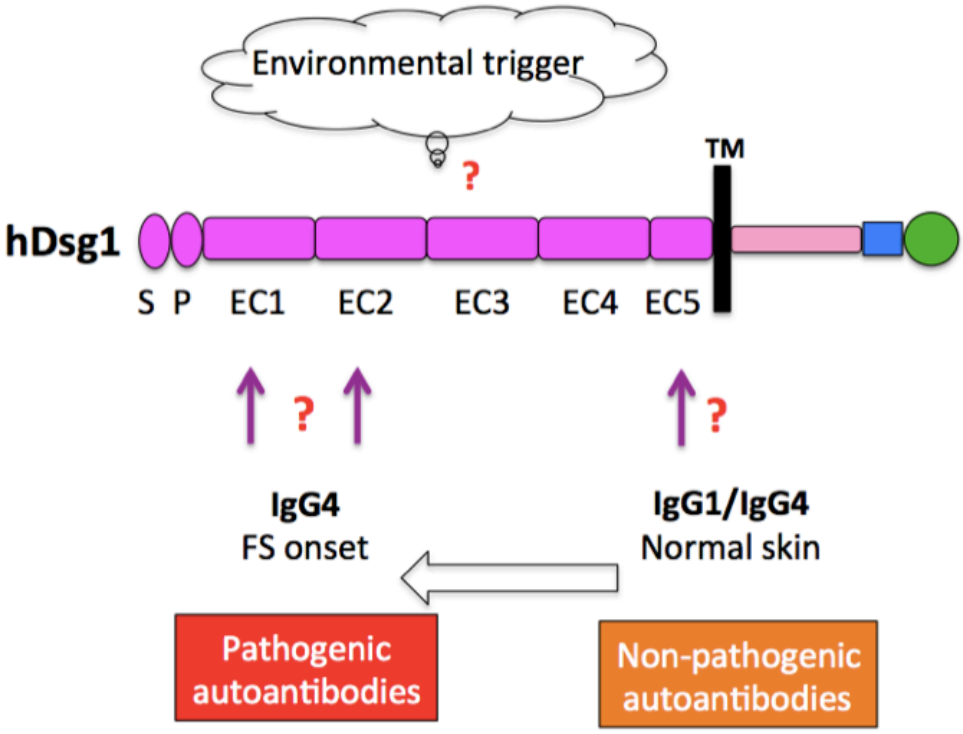

Figure 1. A working model for FS immunopathogenesis. An "unknown" environmental trigger induces the production of autoantibodies against EC5 domain, these antibodies are of the IgG1 and IgG4 subclasses. Overtime, a pathogenic autoimmune response against EC1 and/or EC2 is generated, and this response is restricted to IgG4.

Previous epitope mapping studies using domain-swapped molecules suggested that the N-terminal end of Dsg1 is the target for potentially pathogenic FS autoantibodies. These studies were only able to identify regions at least 100 residues long using a mix of IgG autoantibodies, leaving unknown the exact sequence recognized by FS autoantibodies relevant to disease. In this dissertation, I have identified the amino acid sequences for two dominant epitopes (16 and 13 residues long) in Dsg1 that are recognized by pathogenic IgG4 autoantibodies purified from FS patients sera. Moreover, these 2 epitopes are conformational and are located within EC1 and EC2 domains respectively. Additionally, 2 residues (M133 and Q135) in the EC1 epitope are shown to be required for the conformation of this epitope and therefore for the binding of the pathogenic FS IgG4 autoantibodies. The EC1 epitope overlaps both the homophilic adhesive interface of the molecule defined by amino acid residues in positions 51(W51) and 130 (A130) and the cell-adhesion recognition site motif RAL, strongly supporting the steric hindrance model of FS pathogenesis and suggesting that the specific binding of IgG4 autoantibodies to this epitope disrupts the homophilic adhesion of Dsg1 leading to acantholysis.

A case-control epidemiological study suggests that certain living conditions and exposure to hematophagus insect bites are risk factors for FS [20]. Three insects are suspected to be involved: *Lutzomyia longipalpis* (sand flies), reduviids (kissing bugs) and simuliids (black flies). These insects are vectors of leishmaniasis, Chagas disease and onchocerciasis, respectively. Chapter 3 of this dissertation showed a novel cross-reactive antigen present in the saliva of a hematophagus insect (*Lutzomya longipalpis)* that is recognized by IgG4 antibodies from FS patients. The binding to this cross-reactive antigen (LJM11) is also calcium-dependent, and the absence of significant homology between

LJM11 and hDsg1 structures suggests that the epitopes recognized in both molecules are conformational.

Much work remains to fully elucidate the etiology and immune pathogenesis of FS. The findings of this dissertation advance our understanding FS acantholysis, by providing supportive evidence for the concepts of steric hindrance and molecular mimicry as mechanisms inducers of FS autoimmunity.

4.2. FUTURE DIRECTIONS

The data presented in this dissertation refines the understanding of key factors that contribute to the development of FS. However, it also opens many questions for future research.

The pathogenicity of IgG4 FS antibodies has been confirmed and the presence of IgG1 anti-Dsg1 autoantibodies seems to be irrelevant to active disease. However, it has been suggested that the initial immune response in FS is IgG1-mediated but doesn't induce blistering. Identification of the hDsg1 epitopes recognized by IgG1 and assessment of the structural similarities of hDsg1 epitopes and potential environmental triggers in future studies may help elucidate the mechanisms for the initial non-pathogenic FS autoimmune response.

More than one environmental factor appears to be involved in FS pathogenesis. It has been suggested that proteins in the saliva of black flies cross-react with the EC5 domain of hDsg1, which may be the trigger for the initial response and is perhaps driven by IgG1 anti-Dsg1 antibodies. The cross-reactivity of IgG4 antibodies with a salivary protein reported in this dissertation also suggests that there may be a second environmental trigger that induces

epitope spreading and therefore a pathogenic response. These observations need to be further investigated in order to dissect the functions of both subclasses in FS pathogenesis.

The epitope identified in EC2 seems to not be recognized by majority of IgG4 antibodies. However it is common across FS patients and its pathogenicity has not been ruled out. The relevance of this and the other minor epitopes needs to be explored and perhaps will gives us more insight in the epitope spreading cascade especially in the early stages of the disease.

A crystal 3D structure of hDsg1 is urgently needed to help identify the different conformations that display the epitopes in Dsg1. This information will enable researchers to match the Dsg1 epitopes with the potential epitopes found in the sequence of salivary proteins from insects (such as LJM11). These data will provide a clearer picture of how an environmental trigger induces disease, and perhaps will provide insight into the sequencial epitope spreading events in FS.

While there is evidence that anti-Dsg1 autoantibodies might simply disrupt adhesive Dsg1 dimers, others have suggested that antibody binding could initiate signaling events that drive the disassembly of desmosomes to reduce intercellular adhesion [21, 22]. My epitope mapping studies support the notion of direct disruption of adhesion. However, it would be interesting to investigate if whether IgG1 or IgG4 is the responsible for activation of signaling transduction or apoptosis upon binding to Dsg1.

Finally, research into the involvement of non-desmosomal components of intercellular junctions may provide more answers to the questions that surround FS etiology and pathogenesis. For example, early EM-based studies have shown that upon binding of pemphigus autoantibodies to the epidermal cell surface, the first structural change observed

was widening of the intercellular spaces between desmosomes where E-cadherin mediates cell adhesion. Only later did desmosomes begin to split in two halves with subsequent complete cell separation [23]. I have previously reported that anti-E-cadherin autoantibodies are detected in the serum of pemphigus patients [24], which suggests that pemphigus autoantibodies target the ectodomain of adhesion molecules in both desmosomes and adherens junctions, thus, dysfunction of adhesion mediated by both types of molecules may contribute to the pemphigus epidermal injury.

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