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EXTRACELLULAR VESICLES AS VEHICLES OF AUTOANTIGENS IN AUTOIMMUNE BULLOUS DISEASES

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INTRODUCTION

Autoimmune Bullous Diseases

Autoimmune bullous diseases (AIBDs) are chronic relapsing rare diseases with significant morbidity and mortality. Pathogenesis of AIBDs is largely unknown and currently targeted therapies on pathogenic pathways are scant (1). AIBDs are characterised by pathogenic autoantibodies targeting junctional proteins of keratinocytes, which are responsible for cohesion between keratinocytes or between epidermis and dermis. The target of the autoantibodies varies depending on the type of AIBD. Namely, in pemphigus autoantibodies target the cadherins of the inter-keratinocyte junctions and in bullous pemphigoid the targets are components of the haemidesmosomes of the dermo-epidermal junction (1).

Bullous pemphigoid

Bullous pemphigoid (PB) is a rare organ-specific autoimmune bullous disease characterised by the presence of autoantibodies, mostly IgGs and to a lesser extent IgEs, directed against specific targets represented by the haemidesmosomal proteins of the dermal-epidermal junction, namely BP230 and BP180 (particularly the NC16A portion). BP is the most common autoimmune bullous disease with an incidence of 1-7/100,000 cases per year (2, 3). BP predominantly affects individuals over 65 years of age, with no gender or racial predilection (2). Clinically, BP often begins with diffuse itching and it is characterised by large, taut-roofed blisters with a serum-haematic content that arise on erythematous, urticarial or eczematous patches (4). Anti-BP180 IgGs and IgEs in bullous pemphigoid were shown to be pathogenic in in vitro (5-7) and in mouse models of the disease (7-9). However, many aspects of the pathogenesis remain unclear. These include, for example, the initial mechanism by which keratinocytes release proteins from the haemidesmosomal junctions and how these are presented as antigens to the immune system

with the consequent activation of adaptive immunity and the simultaneous loss of control of the capacity for immune regulation and suppression.

Pemphigus vulgaris

Pemphigus vulgaris (PV) is a rare AIBD characterised by loss of keratinocyte intercellular cohesion, resulting in intraepidermal bullous lesions. PV has an approximate incidence of 0.5 cases per 100,000 per year, which varies considerably depending on geographical area and ethnicity (10). PV is a serious, potentially fatal disease if left untreated. Clinically, it is characterised by extensive skin and mucosal erosions. In PV autoantibodies are directed against inter-keratinocyte junctions, namely desmoglein (Dsg) 3 and 1 and cause the formation of intraepidermal blisters in the skin or intraepithelial blisters in the mucosae (1). Murine models of pemphigus vulgaris have shown that direct binding of IgGs to Dsg3 results in loss of mechanical adhesion, causing the bullous lesions (11). As in bullous pemphigoid, the initial phases of stimulation of the immune system and loss of tolerance against skin antigens are unknown.

Extracellular vesicles

Extracellular vesicles (EVs) are small membranous structures delimited by a lipid bilayer. They encapsulate a variegate cargo, including proteins, microRNA, mRNA and lipids, representing pivotal mediators of intercellular communication both in physiological and pathological processes, including autoimmune diseases (12). Based on size, origin and function, EVs can be classified into three classes: exosomes, microvesicles and apoptotic bodies (13). Since EVs are often isolated based on a size criterion a more recent classification divides them into “small EVs” (EVs) < 100nm or < 200nm and “medium/large EVs” (m/lEVs) > 200nm. Thus, EVs comprise both exosomes and microvesicles. Exosomes are nano-sized EVs (40-160nm), with

an endocytic origin. They result from late-stage endosomes, which evolve in multi-vesicular bodies and merge with the plasma membrane, releasing exosomes into the extracellular environment (13). Microvesicles (100-1000nm) originate by an outward budding at the plasma membrane, while apoptotic bodies (50-5000nm) are released in the extracellular space by dying cells (14). Exosomes have been implied in numerous functions, such as paracrine cell-to-cell interactions and protein recycle and disposal processes (13). Microvesicles were originally thought to be, like exosomes, a cellular dumping or maintenance mechanism, by which the cell would get rid of unwanted material (15). However, it has since been understood that microvesicles are involved in cell–cell communication between local and distant cells (14).

Extracellular Vesicles, tissue autoantigens presentation and immune system modulation

Notably, it has been reported that EVs can carry tissue autoantigens implicated in organ specific autoimmune diseases pathogenesis, such as type 1 diabetes mellitus (16, 17), rheumatoid arthritis (18) and lupus erythematosus (19, 20). In particular, EVs transport citrullinated proteins in rheumatoid arthritis (18) and islet antigen 2 (IA2), anti-glutamate acid decarboxylase (GAD65), proinsulin and insulin in type 1 diabetes mellitus (16, 17). Corresponding circulating autoantibodies can be found in rheumatoid arthritis [anti-cyclic citrullinated peptide (CCP) and anti-mutated citrullinated vimentin (MCV) antibodies] and in type I diabetes mellitus (anti-IA2, anti-GAD65, anti-insulin antibodies) (16, 17). Moreover, in type I diabetes, EVs containing autoantigens of pancreatic origin can stimulate and activate antigen presenting cells in vitro (16).

A similar phenomenon occurs in organ transplants. In the event of rejection after organ transplantation, the number of circulating EVs, which containing self-antigens related to the failing organ attacked by the recipient's immune system, increases (21-24). The transport of tissue-specific antigens by EVs in organ transplant rejection represents a marker of disease

severity that correlates with the intensity of organ damage and immune aggression (13, 21). Moreover, in mouse models of allogeneic heart and skin grafting, exosomes derived from the grafted organ express MCH-II are able to activate the recipient's T cells (22, 25).

Thus, EVs containing tissue antigens may be a possible direct (26) or indirect way (*via* dendritic cells) (16) of antigen presentation without intracellular contact (27).

Extracellular vesicles and keratinocytes

Human keratinocytes were reported to act as non-professional antigen-presenting cells without intercellular contact (26). Indeed, Cai et al. found that exosomes derived from human keratinocytes support superantigen-induced proliferation of resting T cells. The Authors found that exosomes of keratinocyte origin, contained MHC I and II, could transfer antigens to recipient cells, and could elicit superantigen-associated immunity by inducing the proliferation of CD4+ and CD8+ T cells in vitro (26). Chavez-Muñoz et al. identified exosome-associated proteins from *in vitro* undifferentiated and differentiated human keratinocytes using proteomic analysis (28). Differentiated human keratinocytes in physiological conditions shed EVs containing inter-keratinocyte adhesion proteins (E-cadherine) and parts of the haemidesmosome (laminin 332). Dsg1, Dsg3, BP180 or BP230 were not detected (28).

Extracellular vesicles and autoimmune bullous diseases

To date, only two studies link EVs to AIBDs, namely bullous pemphigoid, investigating proteome of blister fluid-derived exosomes from BP patients, by using liquid chromatography with tandem mass spectrometry (LC–MS/MS) (29, 30). More specifically, Fang et al. directly studied proteome of blister fluid-isolated exosomes, while Solimani et al. carried out a comparison between whole and exosome-depleted blister fluid proteome.

Both authors found in the exosomal proteome from blister fluid signature proteins of BP-related inflammation such as proteins of neutrophilic, eosinophilic and keratinocyte origin. However, BP targeted autoantigens were not identified in blister fluid derived exosomes.

Fang and colleagues also demonstrated the potential pathogenic role of exosomes extracted from blister fluid derived from patients with bullous pemphigoid (29). The internalisation of BP blister fluid-derived exosomes by cultured human keratinocytes. Following this, keratinocytes underwent pro-inflammatory activation with the production of cytokines and chemokines, including IL-6, TNF-alpha and CXCL8.

STUDY RATIONALE

The presence of targeted autoantigens in EVs in PV has not been specifically investigated yet. BP targeted autoantigens were not identified in blister fluid derived EVs and were never investigated in serum derived EVs of BP patients. Moreover, although Fang and colleagues have partially elucidated the role of exosomes in the pathogenesis of BP, several aspects remain to be investigated. In particular, the relationship between extracellular vesicles and the production of pathogenic autoantibodies directed against BP antigens (BP230 and BP180) (25).

Finding targeted autoantigens in serum or blister fluid derived EVs in patients with AIBDs could help elucidate AIBDs pathogenesis and it might have a prognostic value:

1. EVs containing cutaneous autoantigens might have a pathogenetic role in AIBDs:
    1.1. Autoantigens carried by EVs could represent a direct or indirect route of antigen presentation without intercellular contact in autoimmune bullous diseases.
    1.2. The discharge of junctional proteins in EVs could contribute to the mechanical instability of the skin and the formation of bullous lesions in AIBDs
    1.3. EVs might spread among adjacent keratinocytes signals of internalization or depletion of adhesion molecules without direct cellular contact
2. EVs containing skin autoantigens in ABIDs could be a prognostic marker of disease severity, as already demonstrated for other autoimmune diseases and for organ transplant rejection.

STUDY OBJECTIVES

Primary Objective

To investigate the presence in serum or blister fluid derived EVs of cutaneous autoantigens, namely BP180 and BP230 in bullous pemphigoid and desmoglein 1 and 3 in pemphigus vulgaris.

Secondary objectives

1. to attribute pathogenetic significance to the finding of EVs containing skin antigens (antigen presentation or formation of bullous lesions)
2. to correlate circulating EVs containing skin antigens with the clinical severity of autoimmune bullous disease.

MATERIALS AND METHODS

Study population

We enrolled patients with BP or PV, confirmed by histological and direct immunofluorescence (DIF) findings [linear deposition at the dermal-epidermal junction (DEJ) of IgGs and C3 in BP and intercellular deposition of IgGs and C3 in pemphigus], and serological findings [positive ELISA testing for circulating anti-BP180 IgGs in BP and Dsg3 in PV (titre > 3 times cut-off, 20UI/l)].

Internationally validated clinical severity scales will be used, namely the Bullous Pemphigoid Disease Area Index (PBDAI) and the Autoimmune Bullous Skin Disorder Intensity Score (ABSIS).

For the control population, healthy subjects were enrolled, for blood or blister fluid collection. Blister fluid was obtained in three cases by suction blistering on the volar surface of forearms of healthy volunteers by using Hijama cups (31), in two cases from burn blisters and in one case from a pressure blister.

All patients and healthy controls gave informed consent. The study was approved by the local Ethical Review Board (Comitato Etico Regione Liguria: N. Registro CER Liguria: 12/2021 - DB id 11151).

Serum and blister fluid collection and processing

Serum and blister fluid were centrifugated twice at 4°C at 2500RPM for 10 minutes to remove major debris and then frozen and stocked at -20°C.

Extracellular Vesicle isolation

EVs were isolated from serum and from blister fluid by size exclusion chromatography (SEC), which represents an efficient method for separation of EVs from circulating proteins (32). EVs were isolated using 500 µl qEV70 columns (Izon Science, Christchurch, New Zealand), that contain a resin with an approximately 70 nm pore size. Proteins and other contaminating molecules smaller than 70 nm enter the pores of the resin and are delayed in their passage through the column, eluting in later fractions. Prior to EV isolation, SEC columns were conditioned by washing with 15 ml of freshly 0.2µm filtered phosphate-buffered saline (PBS). Thawed serum (500 µl) was added to the sample reservoir and EVs were eluted in PBS, which was added to the sample reservoir as the last of the serum entered the column. The initial six fractions of flow-through were discarded, EVs were collected in the pooled fractions 7 to 9 using Protein LoBind tubes, while contaminating proteins were eluted from fraction 10 to fraction 22. The elution profile of both EVs and contaminating proteins has been assessed measuring the absorbance at 280 nm.

EV characterization by flow cytometry

EV characterization by flow cytometry was performed as previously described (33). For each preparation, one tube has been stained with 1 µM CFDA-SE at 4°C (Vybrant™ CFDA SE Cell Tracer Kit, ThermoFisher Scientific), as control to verify CFDA-SE specificity, and one tube containing EVs was stained with the same amount of CFDA-SE at RT to visualize intact EVs and set the correct dimensional gate. A mixture of fluorescent beads of varying diameters (Megamix-Plus FSC and Megamix-Plus SSC, Biocytex) was used following the manufacturer's instructions to discriminate EV size. Expression of typical vesicle markers CD9 (APC Mouse Anti-Human CD9, Biolegend, 312108), CD63 (PE-CyTM7 Mouse Anti-Human CD63, BD Biosciences) and CD81 (BV421 Mouse Anti-Human CD81, BD Biosciences) and the

corresponding isotype controls APC Mouse IgG1, κ Isotype Ctrl (FC) Antibody (Clone MOPC-21, Biolegend), PE-Cy7 Mouse IgG1, κ Isotype Ctrl (FC) Antibody (555872, BD Horizon), and BV421 Mouse IgG1, κ Isotype Ctrl Antibody (562438, BD Biosciences), as well as disease-associated markers COL17A1 (Polyclonal Rabbit anti-Human COL17A1, LSBio), DSG-3 (anti-human Dsg3 AF647, Santa Cruz Biotechnology), and DSG-1 (Dsg1, APC, Novus) were evaluated within the CFDA-SE positive events using the BD FACS Aria II (BD Biosciences).

Western Blot analysis

For western blot analysis, blister fluid EVs were resuspended in RIPA buffer (1% NONIDET p-40, 0.1% SDS, 0.1% Sodium deoxycholate, protease inhibitor cocktail 1x, in PBS pH 7.5) and protein content was quantified by BCA assay. Afterwards, 2 μg of proteins for each sample, were loaded on 4%–12% NuPAGE Bis-Tris gel (Life Technologies, Carlsbad, California, USA). For epidermic extracts, 50 μg and 750 μg were loaded. Electrophoresis was performed at 150V and proteins were blotted on a polyvinylidene fluoride (PVDF) membrane (Millipore, Burlington, Massachusetts, USA). After blocking nonspecific sites with 5% non-fat dry milk (EuroClone, Italy) in Tris Buffered Saline with Tween 20 (TTBS, 20mM Tris pH 7.5, 500mM NaCl, 0.05% Tween 20), blot membrane was incubated overnight at 4°C with specific primary antibodies for: rabbit anti-human BP180 (1:1000), goat anti-human DSG1 (1:1000, R&D), goat anti-human DSG3 (1:1000, R&D), mouse anti-human CD63 (1:1000 dilution, 10628D, Invitrogen), prepared in 2.5% non-fat dry milk/TTBS. After three washes with TTBS, membranes were incubated with a specific HRP-conjugated goat anti-rabbit/mouse secondary antibody (1:2000 dilution, Cell Signaling Technology, Danvers, Massachusetts, USA). Positivity was highlighted by providing the substrates for the chemiluminescence reaction of HRP (Amersham ECL Prime Western Blotting Detection Reagent, GE Healthcare, Chicago, Illinois, USA) and impressing a photographic sheet by autoradiography (GE Healthcare).

Images were scanned using the Epson perfection 1260 scanner. Gel running was performed under not denatured conditions for the detection of CD-63 and under denatured conditions for other markers.

RESULTS

Study Population

Bullous Pemphigoid Patients

Seventeen BP patients (mean age 75.6 ± 8.9; F:M=1:1) were enrolled (Table 1). Thirteen patients had idiopathic BP and 4 patients had drug-induced BP, namely 3 were induced by gliptins (BP1, BP13, BP17) and 1 was induced by nivolumab (BP3).

Twelve cases were new diagnoses, and five cases were flares in known patients (BP3, BP4, BP14, BP16, BP17). Mean Bullous Pemphigoid Disease Area Index (BPDAI) score was 33.5 (range 2-68) and mean Autoimmune Bullous Skin Disorder Intensity Score (ABSIS) score was 77.5 (range 7.5-135). According to BPDAI score stratification, 6 patients (BP1, BP3, BP10, BP11, BP14, BP16) had mild forms of BP (BPDAI ≤19), 7 patients (BP2, BP4, BP5, BP7, BP8, BP9, BP17) had moderate forms of BP (BPDAI ≥20 and ≤56) and 4 patients (BP6, BP12, BP13, BP15) had severe forms of BP (BPDAI ≥57) (34). Seven patients were on immunosuppressive therapy at the time of enrolment: two patients (BP2 and BP) were applying once daily a topical super potent steroid (clobetasol 0,05% cream); Five patients (BP3, BP7, BP14, BP16) were on systemic immunosuppressants.

On average the disease onset was 2.4 months (range 1-12 months) prior to enrolment. ELISA testing showed that 8 patients had only circulating anti-BP180 antibodies, 6 had both anti-BP180 and anti-BP230 antibodies and 3 patients had neither (but had linear IgG / C3 deposits at the DEJ at DIF).

Only from 11 patients blister fluid was collected (BP1-BP11). The present thesis focuses on this subpopulation of BP patients and the analysis of EVs in blister fluid. In detail this study population had a mean age of 77.7±6.7 years and a male to female ration of 1:1. Nine patients

had idiopathic BP and two patients had drug-induced BP, namely one was induced by gliptins (BP1) and 1 was induced by nivolumab (BP3).

Nine cases were new diagnoses, and two cases were flares in known patients (BP3, BP4). Mean Bullous Pemphigoid Disease Area Index (BPDAI) score was 33.8 (range 3-67) and mean Autoimmune Bullous Skin Disorder Intensity Score (ABSIS) score was 78.8 (range 7.5-135). According to BPDAI score stratification, 4 patients (BP1, BP3, BP10, BP11) had mild forms of BP (BPDAI ≤19), 6 patients (BP2, BP4, BP5, BP7, BP8, BP9) had moderate forms of BP (BPDAI ≥20 and ≤56) and 1 patient (BP6) had severe forms of BP (BPDAI ≥57) (34). Seven patients were on immunosuppressive therapy at the time of enrolment: two patients (BP2 and BP8) were applying once daily a topical super potent steroid (clobetasol 0,05% cream); two patients (BP3, BP7) were on systemic immunosuppressants.

On average the disease onset was 1.9 months (range 1-7 months) prior to enrolment. ELISA testing showed that 4 patients had only positive circulating anti-BP180 antibodies, 4 had both anti-BP180 and anti-BP230 antibodies, and 3 patients had neither (but had linear IgG / C3 deposits at the DEJ at DIF).

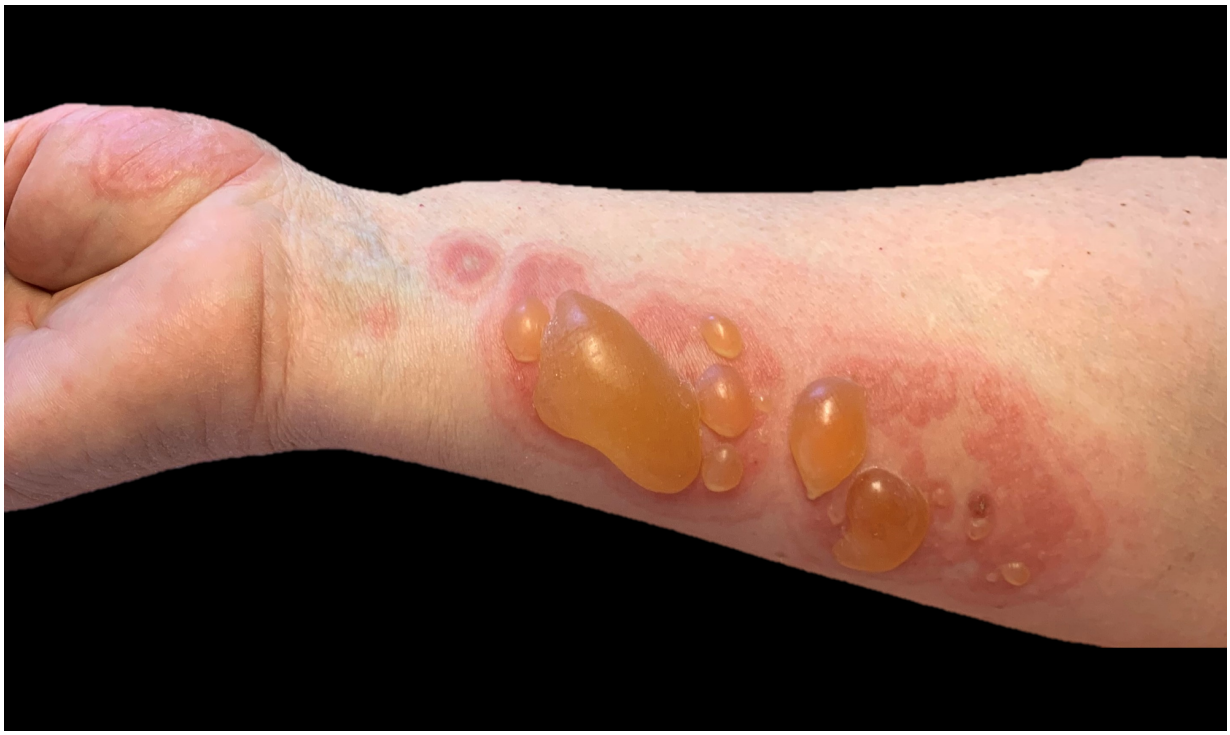


Figure1. clinical image of patient BP6 showing the bullous lesions from which EVs were isolated

Pemphigus Vulgaris Patients

Four patients were enrolled (mean age 74 years; both males). Both patients had muco-cutaneous manifestations of PV and the serum samples were collected during a flare of the disease while they were not on immunosuppressive therapy. ABSIS score for both was 135, showing a severe form of PV. Blister fluid was not collected, as blisters in PV are extremely fragile and break immediately after formation, leaving eroded areas.

Table 1. Demographic, clinical and immunological characteristics of patients.

ID	AGE	SEX	DIAGNOSIS	DRUG INDUCED	CLINICAL TYPE	BPDAI	ABSIS	CLINICAL COURSE	DISEASE ONSET (months)	IMMUNOSUPRESSIVE THERAPY	FLUID TYPE	Anti-BP180 Ab	Anti-BP230 Ab	Anti-Dsg1 Ab	Anti-Dsg3 Ab	DIF /IIF / SSS-IIF
BP1	77	F	Bullous pemphigoid	Yes (gliptin)	Bullous inflammatory	8	18	New diagnosis	1	No	Serum & blister	>200	30	-	-	Nd
BP2	70	F	Bullous pemphigoid	No	Bullous	30	135	New diagnosis	1	Clobetasol 0.05%	Serum & blister	>200	Neg	-	-	DIF: linear deposits IgG, C3
BP3	78	M	Bullous pemphigoid	Yes (nivolumab)	Bullous inflammatory	8	27	Flare	7	Prednisone 25mg QD	Serum & blister	104	180	-	-	Nd
BP4	80	F	Bullous pemphigoid	No	Bullous inflammatory	25	38	Flare	3	Prednisone 37.5mg azathioprine 50mg QD	Serum & blister	>200	Neg	-	-	Nd
BP5	84	M	Bullous pemphigoid	No	Bullous	50	135	New diagnosis	1	No	Serum & blister	164	Neg	-	-	DIF: linear deposits C3
BP6	66	F	Bullous pemphigoid	No	Bullous inflammatory	67	135	New diagnosis	1	No	Serum & blister	80	Neg	-	-	DIF: linear deposits C3
BP7	74	M	Bullous pemphigoid	No	Bullous inflammatory	54	135	New diagnosis	1	Prednisone 25mg QD	Blister	136	124	-	-	SSS-IIF positive roof
BP8	85	M	Bullous pemphigoid	No	Bullous inflammatory	42	135	New diagnosis	3	Clobetasol 0.05% QD	Blister	91	10	-	-	Nd
BP9	69	F	Bullous pemphigoid	No	Bullous inflammatory	25	38	New diagnosis	1	No	Blister	Neg	Neg	-	-	IIF: linear deposits C3, IgG
BP10	82	M	Bullous pemphigoid	No	Bullous pauci-inflammatory	3	7.5	New diagnosis	1	No	Blister	Neg	Neg	-	-	IIF: linear deposits C3, IgG
BP11	82	M	Bullous pemphigoid	No	Bullous pauci-inflammatory	5	7.5	New diagnosis	1	No	Blister	Neg	Neg	-	-	DIF: linear deposits C3, IgG IIF: linear deposits IgG,C3
BP12	68	F	Bullous pemphigoid	No	Bullous inflammatory	60	81	New diagnosis	1	No	Serum	>200	>200	-	-	DIF: linear deposits IgG,C3
BP13	80	M	Bullous pemphigoid	Yes (gliptin)	Bullous inflammatory	67	135	New diagnosis	1	No	Serum	>200	22	-	-	DIF: linear deposits IgG,C3
BP14	71	F	Bullous pemphigoid	No	Bullous inflammatory	2	7.5	Flare	3	Prednisone 25mg QD	Serum	75	Neg	-	-	Nd
BP15	51	F	Bullous pemphigoid	No	Bullous inflammatory	68	135	New diagnosis	1	No	Serum	>200	Neg	-	-	Nd
BP16	83	M	Bullous pemphigoid	No	Bullous inflammatory	4	13.5	Flare	12	Prednisone 25mg QD	Serum	>200	Neg	-	-	Nd
BP17	85	M	Bullous pemphigoid	Yes (gliptin)	Bullous inflammatory	53	135	Flare	2	No	Serum	200	Neg	-	-	DIF: linear deposits C3
PV1	73	M	Pemphigus vulgaris	No	Muco-cutaneous	-	135	Flare	6	No	Serum	-	-	105	182	DIF: ICS deposits IgG C3
PV2	75	M	Pemphigus vulgaris	No	Muco-cutaneous	-	135	Flare	5	No	Serum	-	-	Neg	221	Nd

DIF= direct immunofluorescence; IIF = indirect immunofluorescence; SSS-IIF = salt split skin indirect immunofluorescence; ICS = intracellular substance

Healthy controls

Healthy controls included 4 blood donors (mean age 33 years; M:F=3:1) and 6 subjects from whom blister fluid was obtained (3 suction blisters, 2 burn blisters and 1 pressure blister, mean age 51 ± 12.9 years; M:F=2:1).

All controls had no other relevant inflammatory or autoimmune skin disease.

Table 2. Demographic and clinical characteristics of healthy controls

	AGE	SEX	FLUID TYPE
CTRL1	60	M	Blister (Suction)
CTRL2	71	M	Blister (Suction)
CTRL3	36	M	Blister (Suction)
CTRL4	52	M	Blister (Burn)
CTRL5	40	F	Blister (Burn)
CTRL6	47	F	Blister (Pressure)
CTRL7	41	F	Serum
CTRL8	31	M	Serum
CTRL9	32	F	Serum
CTRL10	28	F	Serum

Extracellular vesicle isolation from serum

EVs were efficiently isolated from the serum of both patients and healthy donors. Figure 2 reports a representative elution profile obtained using the SEC columns.

Most of the contaminating proteins contained in the starting material were efficiently separated from EVs with a diameter greater than 70nm. In any case, some lipoproteins, and in particular Very Low-Density Lipoproteins (VLDL) and chylomicrons might be still present in our final vesicle preparation, having a similar dimension. For this reason, we prefer to define our vesicle preparation as a "mixed nanoparticle" suspension enriched in EVs.

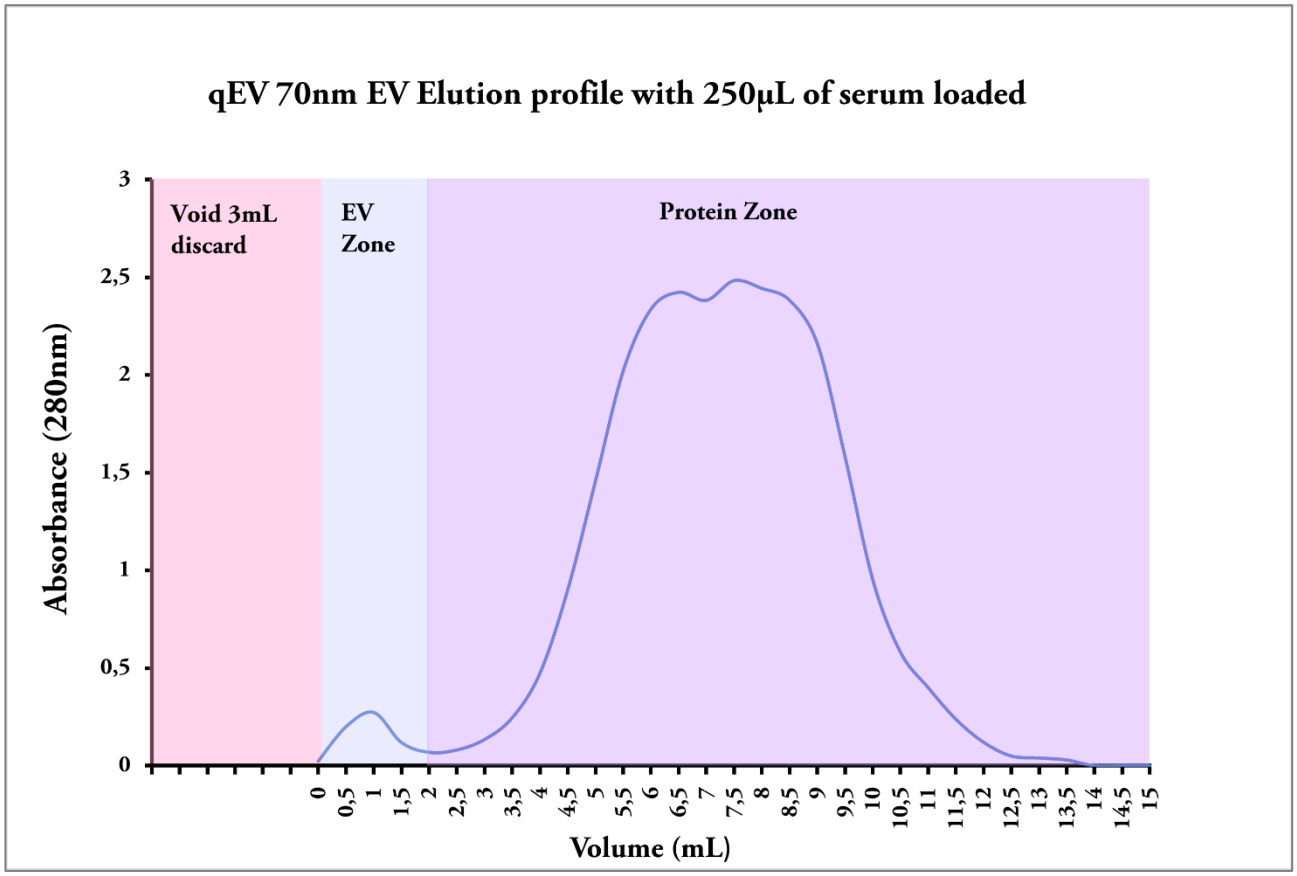
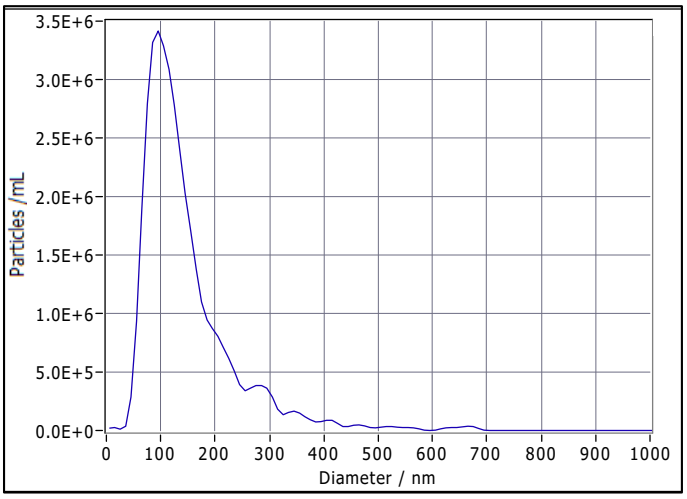


Figure 2. Representative elution profile of EVs isolated from 250 µl of serum.

The EV-enriched suspension has been analysed by nanoparticle tracking analysis (NTA) to define their size distribution and concentration and by flowcytometry to evaluate the expression of specific disease-associated markers.

NTA analysis indicated that the EV-enriched fractions were enriched in particles with a size distribution characterizing small-EVs (exosomes). Indeed, the main peak was present at 98.7 nm, and a median size distribution of 117.3 nm, while the concentration of vesicles retrieved from 250 µl was $3.9x10^{10}$ particles/ml (Figure 3).



Peak Analysis (Concentration)

Diameter / nm	Particles/mL	FWHM / nm
98.7	3.4E+6	91.9

X Values (all sizes are given in nm)

	Number	Concentration	Volume
X10	67.9	67.9	144.5
X50	117.3	117.3	335.0
X90	252.4	252.4	642.9
Span	1.6	1.6	1.5
Mean	147.1	147.1	369.1
StdDev	87.4	87.4	176.5

Figure 3. Representative NTA analysis of EVs isolated from 250 µl of serum.

Flow cytometry analysis confirmed that the retrieved nanoparticle suspension was enriched in EVs. Samples were stained with the cell-permeant, fluorescein-based CFDA-SE tracer that is useful to discriminate intact vesicles from debris and membrane fragments, together with a mixture of fluorescent beads of known varying diameters (Figure 4A). Since CFDA-SE is able to passively diffuse within vesicles and interact with intra-vesicular enzymes at room temperature (RT) (Figure 4C), we ensured that at 4°C the cloud of particles was under the level of the dimensional gate in the FL1 intensity channel (Figure 4B). After an accurate titration of antibodies and the use of related isotype controls, the expression of the typical vesicular markers

CD81 and CD63, belonging to the tetraspanin family was evaluated in EV-enriched suspension (Figure 4D and 4E). Serum-derived EVs expressed both CD81 and CD63, indicating that the suspension collected from the SEC column was mainly composed by EVs.

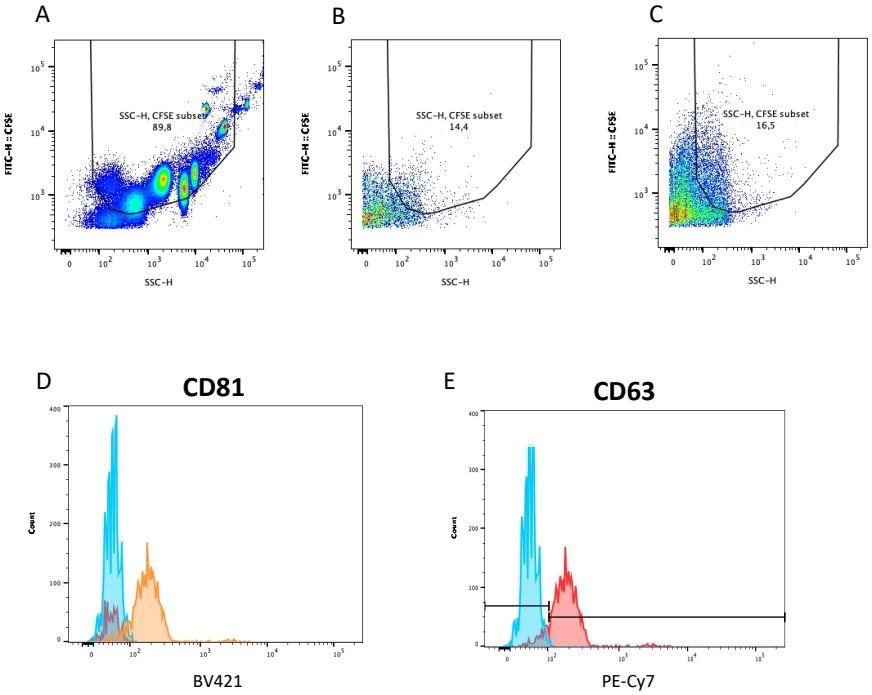


Figure 4. Representative flow cytometry analysis of serum derived EVs. (A) Dot plot representing the fluorescent (FL1 channel) beads with known diameters ranging from 100 nm to 900 nm. (B, C) Representative dot plots of EVs stained with 1 µM CFDA-SE at 4°C and RT, respectively. Representative histograms reporting the percentage of CD81 (D) and CD63 (E) positive EVs within the CFDA-SE-positive events.

Extracellular vesicle isolation form blister fluid

EVs were efficiently isolated from the blister fluid of both patients and healthy donors. Most of the contaminating proteins contained in the starting material were efficiently separated from EVs with a diameter greater than 70 nm. Once again, some lipoproteins, and in particular Very Low-Density Lipoproteins (VLDL) and chylomicrons could be still present in our final vesicle preparation, having a similar dimension. Thus, vesicle preparation can be defined as a "mixed nanoparticle" suspension enriched in EVs.

The EV-enriched suspension has been analysed by nanoparticle tracking analysis (NTA) to define their size distribution and concentration and by flowcytometry to evaluate the expression of specific vesicle markers. NTA analysis indicated that the EV-enriched fractions were enriched in particles with a size distribution characterizing small-EVs (exosomes). Indeed, the main peak was present at 94.5nm, and a median size distribution of 123.9 nm, while the concentration of vesicles retrieved from 250µl was 6.5x1010 particles/ml (Figure 5A).

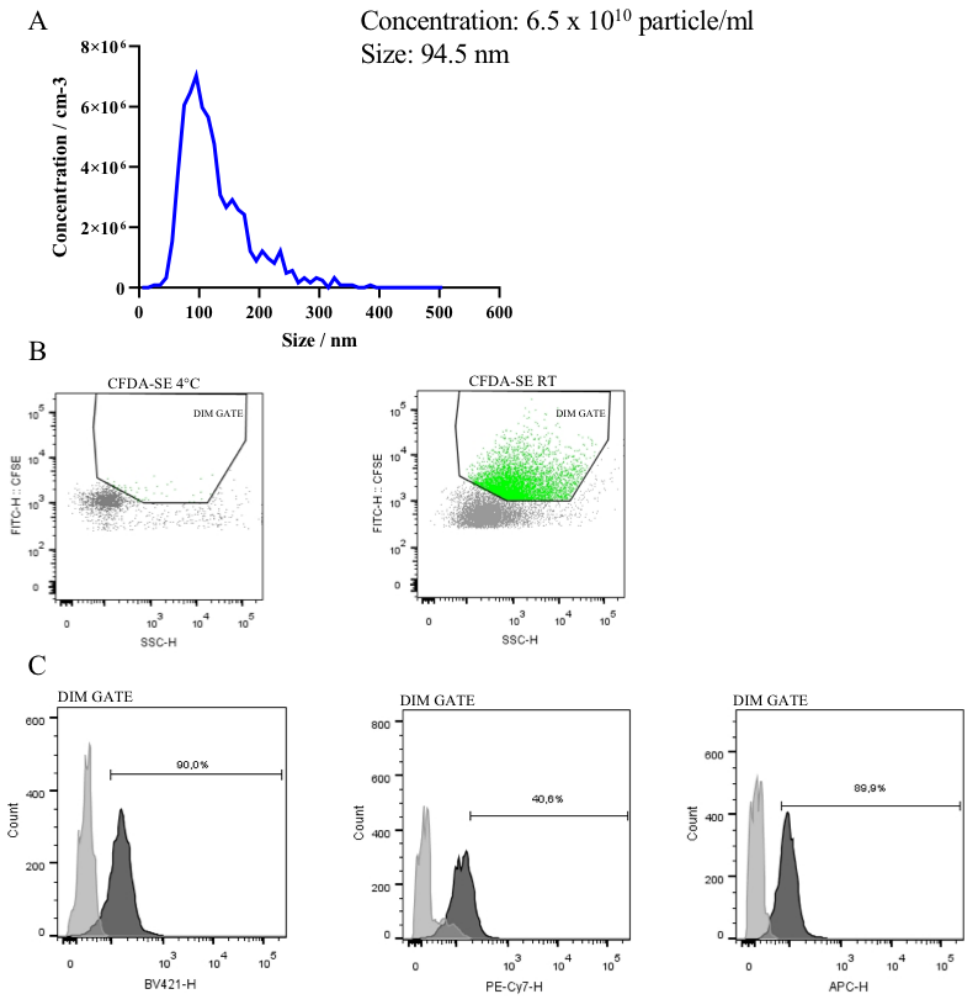


Figure 5. Blister-fluid-derived EV characterization. (A) Representative size distribution for blister fluid-EVs, analysed by Zetaview NTA. (B) Representative bidimensional dot plots (FL1-H vs. SSC-H, in logarithmic scale) for CFDA-SE specificity: EVs stained with CFDA-SE at 4°C (left panel) and EVs stained with CFDA-SE at room temperature (RT) (right panel). (C) Flow cytometry analysis of blister fluid-EVs. Areas under the black lines identify vesicles reacting with CD81 (left panel), CD63 (middle panel), and CD9 (right panel). Areas under the grey lines indicate the interactions of vesicles with corresponding non-reactive immunoglobulin of the same isotype.

Flow cytometry analysis confirmed that the retrieved nanoparticle suspension was enriched in EVs. Samples were stained with the cell-permeant, fluorescein-based CFDA-SE tracer that is useful to discriminate intact vesicles from debris and membrane fragments, together with a mixture of fluorescent beads of known varying diameters (data not shown). Since CFDA-SE is able to passively diffuse within vesicles and interact with intra-vesicular enzymes at room temperature (RT) (Figure 5B), we ensured that at 4°C the cloud of particles was under the level of the dimensional gate in the FL1 intensity channel. After an accurate titration of antibodies and the use of related isotype controls, the expression of the typical vesicular markers CD81, CD9 and CD63, belonging to the tetraspanin family was evaluated in EV-enriched suspension. BF-EVs expressed high levels of both CD81 and CD9 (84.6% and 84.2%, respectively), while the expression of CD63 was lower (5.6%), indicating that the suspension collected from the size exclusion chromatography (SEC) column was mainly composed by EVs.

Surface autoantigen expression in serum derived extracellular vesicles by Flow cytometry analysis

Data indicated that the serum of patients were enriched in circulating EVs. Therefore, we evaluated whether circulating EVs could express on their surface disease-associated markers, that is BP180, namely COL17A1, in 4 BP patients (BP2, BP3, BP12, BP13), Dsg1 and Dsg3 in 2 PV (PV1, PV2) patients and COL17A1, Dsg1, and Dsg3 in 4 healthy controls (CTRL7, CTRL8, CTRL9, CTRL10). After having defined the dimensional gate thanks to the use of CFDA-SE staining (Figure 6A, 6B, 6E, 6F), we evaluated the percentage of CD81-positive (Figure 6C and 6G) and COL17A1-positive (Figure 6D and 6H) events falling within the dimensional gate. The percentages of CFDE-SE positive Col17A1-positive EVs were very high

among (approximately 80%) both patients and donors, with no significant differences in intensity of expression.

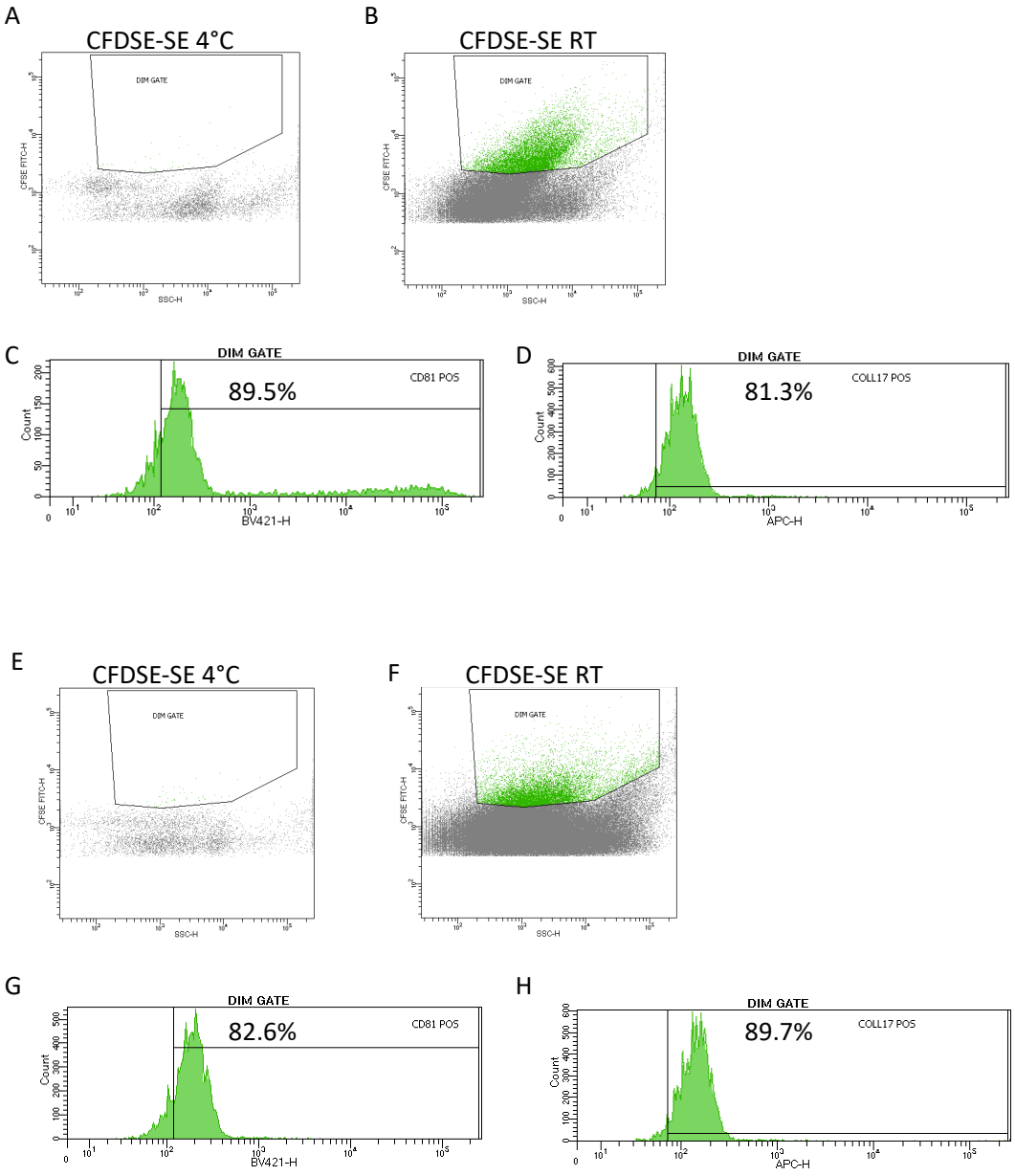


Figure 6. Representative flow cytometry analysis of EVs isolated from the serum of a patient and from the serum of a healthy donor. (A, E) Representative dot plots of EVs derived from a patient (A) and from a donor (E) stained with 1 µM CFDA-SE at 4°C. (B, F) Representative dot plots of EVs derived from a patient (B) and from a donor (F) stained with 1 µM CFDA-SE at room temperature (RT). (C, D) Representative histograms reporting the percentage of CD81- (C) and Col17A1- (D) positive EVs isolated from the serum of a patient. (G, H) Representative histograms reporting the percentage of CD81- (G) and Col17A1- (H) positive EVs isolated from the serum of a donor.

Similar results were obtained for PV patients. Data obtained from both PV patients and healthy controls, indicated that there were no significant differences in Dsg1 and Dsg3 expression, which was very high, approximately expressed by 80% of EVs, in both patients and healthy controls, on the surface of circulating EVs (Figure 7).

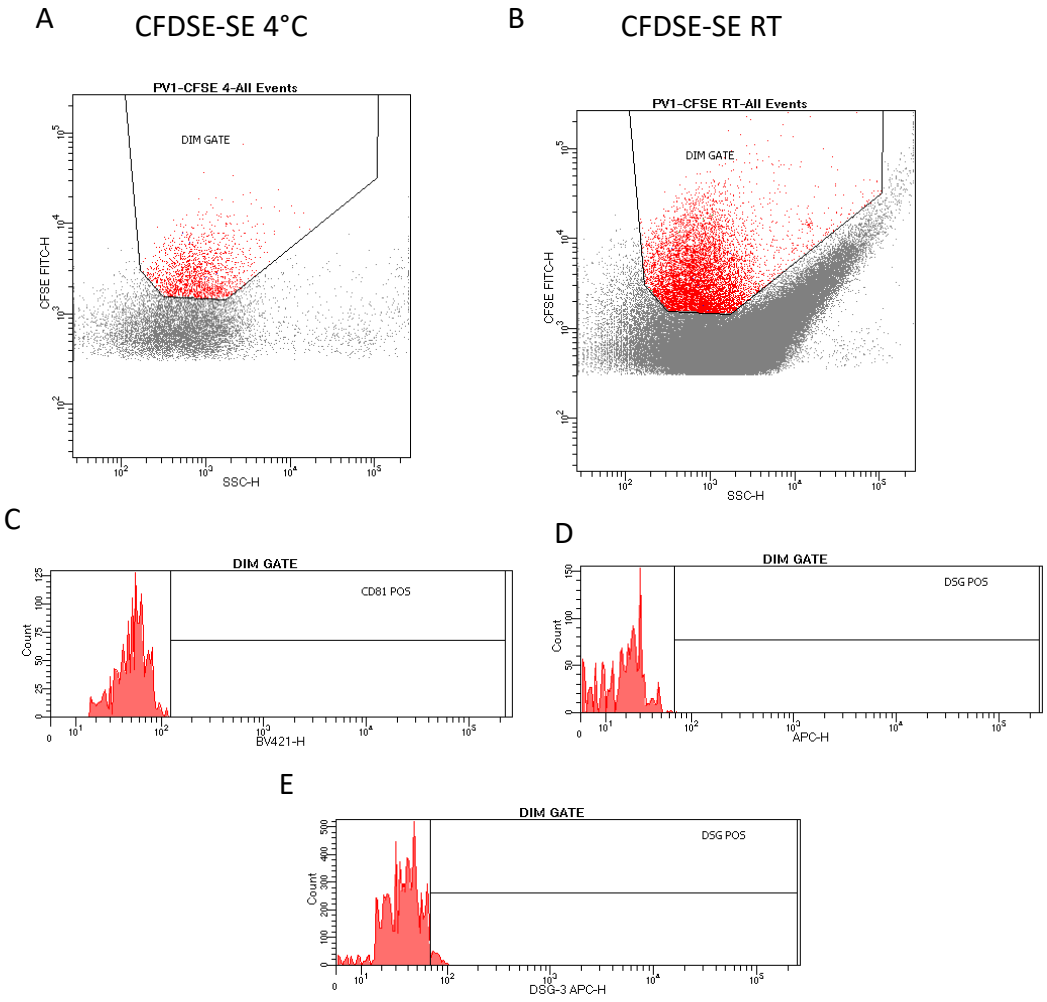


Figure 7. Representative flow cytometry analysis of EVs isolated from the serum of a patient and from the serum of a healthy donor. (A) Representative dot plots of EVs derived from a patient (A) stained with 1 µM CFDA-SE at 4°C. (B) Representative dot plots of EVs derived from a patient (B) stained with 1 µM CFDA-SE at room temperature (RT). (C, D) Representative histograms reporting the percentage of CD81- (C) and Dsg1- (D) and Dsg3 (E) positive EVs isolated from the serum of a patient.

Autoantigens in blisters fluid derived extracellular vesicles by Western Blot Analysis

A band at 180kDa was detectable at variable intensity in 6 out of 11 patients (BP3, BP4, BP6, BP7, BP8, BP9). A band at 120KDa was clearly visible in 3 cases (BP6, BP8, BP9) and a faint band was detectable in 3 cases (BP4, BP5, BP7). Bands at a molecular weight of approximately 150KDa were visible in four cases (BP1, BP4, BP7, BP8, BP11) (Figure 8, Table 3). Overall, in 9 out of 11 cases full length BP180 and/or of its fragments were found in BF-EVs.

Neither BP180 nor its cleaved forms were expressed by EVs from the blisters of healthy controls.

BP230 and Dsg1 were not detectable in any blister fluid nor from BP patients nor from suction blisters.

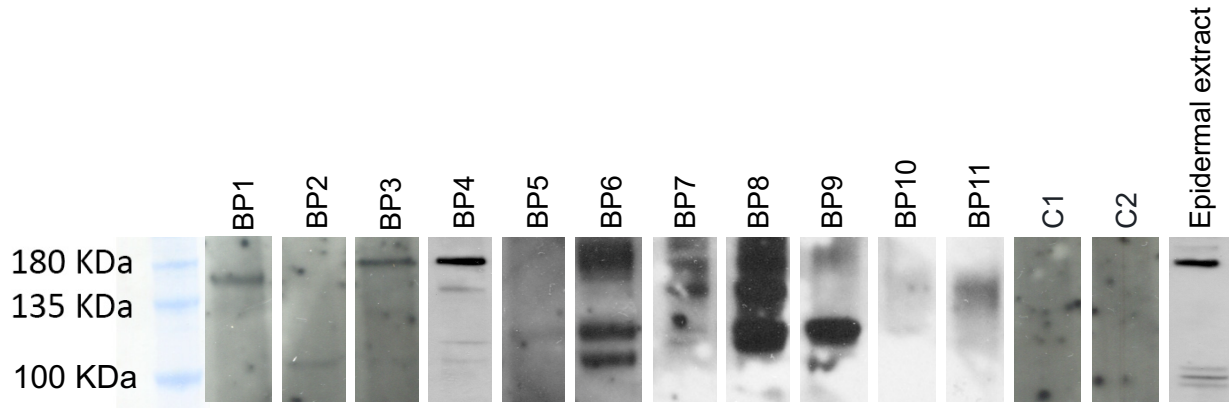


Figure 8. Western blot results, on BF-EVs derived from BP patients and healthy controls and on epidermic extract.

Table 3. WB results and patients' clinical and immunological characteristics

ID	Age	Sex	Drug induced	Clinical type	BPDAI	ABSIS	Clinical course	Disease onset (months)	Immunosupressive therapy	Anti-BP180 Ab	Anti-BP230 Ab	DIF /IIF / SSS-IIF	180kDa	150kDa	120kDa
BP1	77	F	Yes (gliptin)	Bullous inflammatory	8	18	New diagnosis	1	No	>200	30	Nd	NEG	POS	NEG
BP2	70	F	No	Bullous	30	135	New diagnosis	1	Clobetasol 0.05%	>200	Neg	DIF: linear deposits IgG, C3	NEG	NEG	NEG
BP3	78	M	Yes (nivolumab)	Bullous inflammatory	8	27	Flare	7	Prednisone 25mg QD	104	180	Nd	POS	NEG	NEG
BP4	80	F	No	Bullous inflammatory	25	38	Flare	3	Prednisone 37.5mg azathioprine 50mg QD	>200	Neg	Nd	POS	POS	POS faint
BP5	84	M	No	Bullous	50	135	New diagnosis	1	No	164	Neg	DIF: linear deposits C3	NEG	NEG	POS faint
BP6	66	F	No	Bullous inflammatory	67	135	New diagnosis	1	No	80	Neg	DIF: linear deposits C3	POS	NEG	POS
BP7	74	M	No	Bullous inflammatory	54	135	New diagnosis	1	Prednisone 25mg QD	136	124	SSS-IIF positive roof	POS	POS	POS faint
BP8	85	M	No	Bullous inflammatory	42	135	New diagnosis	3	Clobetasol 0.05% QD	91	10	Nd	POS	POS	POS
BP9	69	F	No	Bullous inflammatory	25	38	New diagnosis	1	No	Neg	Neg	IIF: linear deposits C3, IgG	POS	NEG	POS
BP10	82	M	No	Bullous pauci-inflammatory	3	7.5	New diagnosis	1	No	Neg	Neg	IIF: linear deposits C3, IgG	NEG	NEG	NEG
BP11	82	M	No	Bullous pauci-inflammatory	5	7.5	New diagnosis	1	No	Neg	Neg	DIF: linear deposits C3, IgG IIF: linear deposits IgG,C3	NEG	POS	NEG

DISCUSSION

The experiments we performed by Flow Cytometry Analysis for the identification of autoantigens on the surface of serum derived EVs did not yield the expected results. Indeed, our data showed that about 80% of the serum derived EVs of both AIBD patients and healthy controls expressed skin autoantigens. It seems unlikely that all three adhesion proteins of keratinocyte origin are actually so highly expressed in serum EVs of both patients and controls. Possibly, this was due to low specificity of the antibodies we used for flow cytometry analysis. In fact, only few anti-BP180 and anti-Dsg1 and Dsg3 antibodies for flow cytometry analysis were commercially available. Probably, because such protein targets are not often investigated in circulating EVs or cells, as BP-180, Dsg1 and Dsg3 are adhesion molecules principally expressed in the skin. Otherwise, this result could be explained by the physiological presence of BP180 and Dsg1 and 3 in other types of circulating EVs (not strictly of keratinocyte origin), also found in the blood of health subjects. To this regard Dsg1 and 3 have been reported in few other studies on circulating EVs investigating other conditions that used mass spectrometry for the analysis on EV content and incidentally found Dsg1 (35-37) or Dsg3 (38, 39). However, the latter hypothesis appears to be less likely and we are inclined to believe that the antibodies we used for flow cytometry analysis were not specific enough.

Since the results on skin antigen expression in serum derived EVs were unclear (methodological limitation vs expression in physiological conditions) we decided to focus on blister derived EVs, which could more strictly represent the pathological process involving the skin in AIBDs. Thus, the rest of the experiments focused on AIBD blister fluid and suction/burn/pressure blister derived EVs.

We were able to identify BP180 in blister fluid derived EVs derived from BP patients. To the best of our knowledge, this is the first time that BP180 was found in BF-EVs of BP patients. BP180 in BF-EVs was detectable in 6 out of 11 BP patients (approximately 50% of cases) and in none of suction blister fluid EVs from healthy controls. Moreover, in some cases, bands at 120kDa. A 120-kDa protein, namely LAD-1, is a well-known soluble fragment derived from the cleavage of the ectodomain of BP180 (40). In BP and other autoimmune bullous diseases involving the dermal-epidermal junction, mainly linear IgA dermatosis, anti-LAD-1 autoantibodies are found in the patient's sera (41). A band at 150kDa was also visible in some patients, this could be another degradation product of BP180 or due to slightly abnormal migration of BP180 in the WB lane. Due to their soluble nature, fragments of BP180 can also be found in the extracellular medium and it could be argued that are not specifically found in EVs. However, full-length BP180 has a hydrophobic transmembrane portion which can only be found bound to a lipid bilayer. Thus, is seem plausible that full-length BP180 in blister fluid is bound to lipid vehicles such as small size EVs.

Notably, previous studied that extensively analysed the BP patient blister fluid EVs proteasome by mass spectrometry were not able to identify BP-180 or its degradation products in BF-EVs (29, 30). In these studies, blister fluid was collected from 16 patients (29) and 8 patients (30) were investigated respectively. These studies investigated the whole proteasome of EVs and did not specifically investigate the presence of BP autoantigens.

Many variables likely contribute to the presence of BP180 in BF-EVs. No specific clinical characteristics seemed to correlate to the presence of BP180 in BF-EVs. In fact, no difference was found between newly diagnosed patients and patients experiencing flares, or between patients on immunosuppressive therapy or not. Also, EV-BP180 did not seem to correlate with the titres of circulating autoantibodies. Possibly, finding BP180 in BF-EVs could be related to the stage of blister formation. The blister fluid of BP6 was collected from a newly formed blister

and the patient was experiencing a very violent blister eruption. Nonetheless, these considerations must be thoroughly investigated in a larger number of cases.

Other parts of the hemidesmosome, which are also targeted by autoreactive antibodies in BP, such as BP230, was not detected in EVs from BP nor in EVs from suction blisters. Indeed, despite a few cases of BP characterized only by anti-BP230 antibodies have been documented (42) in most cases anti-BP230 antibodies play marginal role in the pathogenesis (43). Four of our patients had circulating anti-BP230 antibodies (BP1 and BP3) and neither had BP230 in BF-EVs

Dsg1 was considered as a negative control, as it is not a disease specific targeted autoantigen but at the same time it is of keratinocyte derivation, therefore it may help distinguish an unspecific keratinocyte antigen release from a disease specific antigen release. It was not found in neither BP patients blister fluid nor in suction blister fluid. The absence of Dsg1 in BF-EVs from BP patients suggests that BP180 release via BF-EVs in the extracellular fluid is specific phenomenon correlated to BP.

The presence of BP180 in BP in BF-EVs might have three possible pathogenic meanings in BP (Figure 9).

The first potential mechanism could be antigen presentation without cell-to-cell contact. Indeed, it has been demonstrated that EVs containing autoantigens may be a direct (26) or indirect pathway (via dendritic cells) of antigen presentation to the immune system (16, 27). As it has already been demonstrated in type I diabetes in vitro models, human pancreatic islets can release the intracellular b-cell autoantigens in human T1D, GAD65, IA-2, and proinsulin in exosomes, which can be taken up by and activate dendritic cells (16). Moreover, human keratinocytes may act as non-professional antigen-presenting cells and could, under pathological conditions, present antigens (e.g. staphylococcal superantigens) directly to the immune system via exosomes (26). Also, in physiological conditions mature human keratinocytes shed plasma membrane adhesion proteins in exosomes such as cadherins and laminin 332 (remarkably, BP180 nor BP230 were ever described in exosome of keratinocyte origin) (28). Therefore, hypothetically keratinocytes could stimulate cutaneous resident dendritic cells via EVs containing BP180.

The second hypothetic mechanism might be that EVs contribute to BP180 recycling and depletion from the plasma membrane in basal keratinocytes during blister formation. In fact, one of the first demonstrated roles of EVs was protein disposal and recycling during reticulocyte maturation process (44). Blister formation in BP happens via two pathogenic mechanisms: a complement mediated and complement independent pathway. The complement independent pathway consists of a mechanical detachment at the dermal-epidermal junction, due to BP180 depletion. It was demonstrated that BP180 is depleted in cultured keratinocytes incubated with anti-BP180 IgG derived from patients' sera, determining cellular instability and reduction in the adhesive capacity of the cells (5, 6, 45, 46). The depletion from the plasma membrane of basal keratinocytes was explained by internalization of BP180 bound to

pathogenetic antibodies via micropinocytosis (5, 45). Once internalized, BP180 was demonstrated to be depleted via ubiquitination and proteasomal degradation (45, 46). While lysosomal pathway of degradation of endocytosed BP180 by macropinocytosis seems unlikely as, on electron microscopy examination macropinosomes did not merge with lysosomes (45). MG132 (inhibitor for proteasome) suppressed BP180 depletion in both in vitro mouse and human keratinocytes stimulated with BP IgG (46). However, in vivo in mice models, the intraperitoneal injection of MG132 prior to the injection of recombinant monoclonal human IgG4 did not prevent the induction of blister formation in the treated mice. Also, the entity of BP180 depletion, when MG132 was used, was not quantified in these studies. Possibly, there is another way for BP180 disposal; EVs might contribute to such depletion, by dumping BP180 in extracellular environment.

The third hypothesis might be that EVs spread among adjacent keratinocytes signals of internalization or depletion of BP180 without direct cellular contact. In neurodegenerative diseases, such Parkinson's or Alzheimer's disease mediated by abhorrent protein accumulation, exosomes are implicated in the transport damaged cellular material, which eventually contributes to the spread of pathological misfolded proteins within the brain, thus promoting the neurodegeneration process (47, 48). BP180 in keratinocytes derived exosomes in blister fluid might propagate a signal to adjacent cells internalize or eliminate BP180. Notably, a way of internalization of exosomes is through macropinocytosis (49).

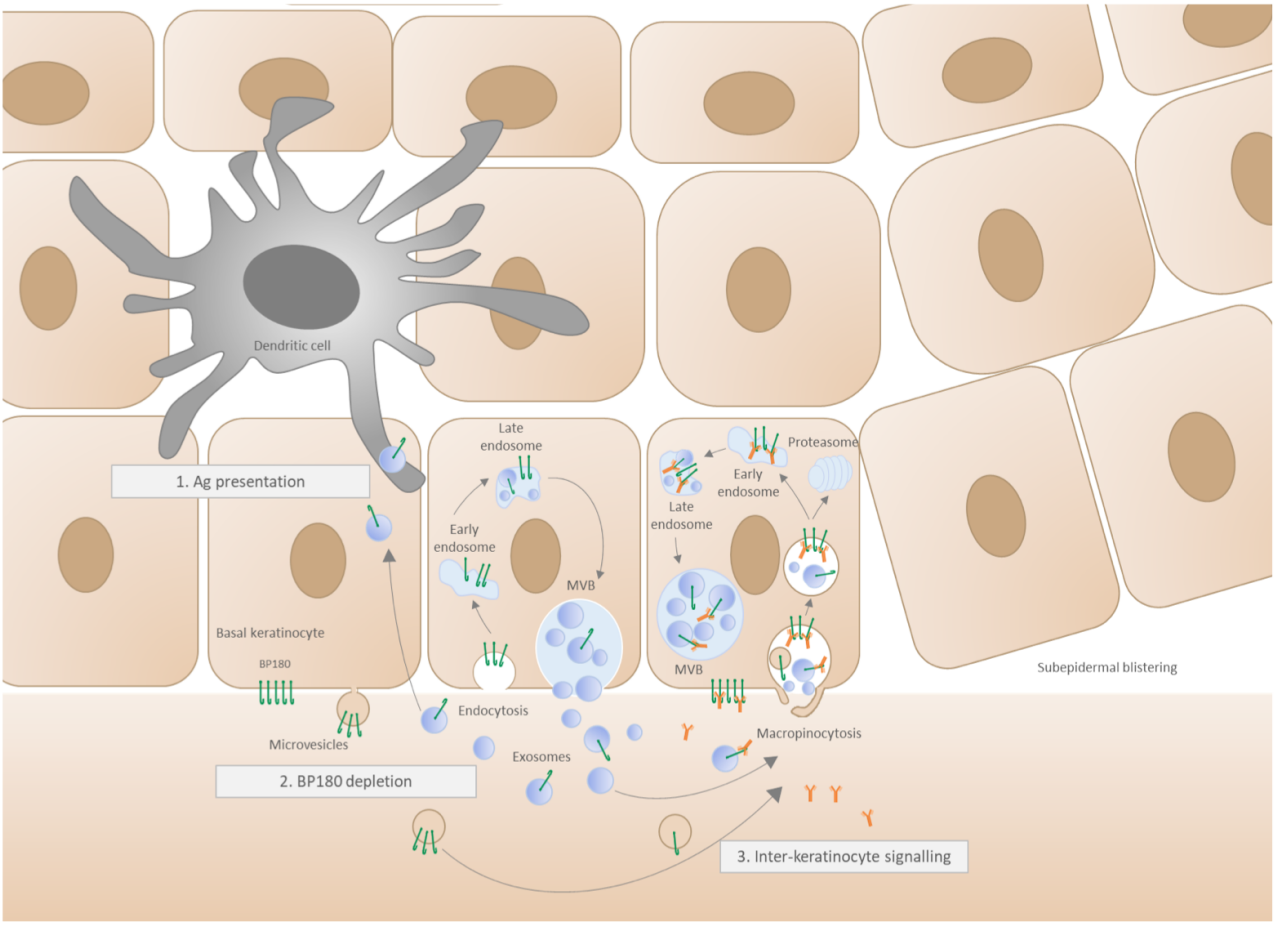


Figure 9. Hypothetic pathogenic mechanisms involving BP180 transport in EVs in BP:

1. Antigen presentation without cell-to-cell contact: resident DCs could uptake of EVs of keratinocyte origin containing BP180.

2. IgG-bound BP180 internalized by micropinocytosis could be sorted into early endosomes, late endosomes and finally multivescicular bodies, parallelly to proteasome destruction.

3. EVs containing BP180 or IgG-bound BP180 could mediate inter-keratinocyte signalling, inducing further internalization of BP180.

Moreover, the transport of tissue-specific antigens by EVs in autoimmune diseases and organ transplant rejection could represent a marker of disease severity that correlates with the intensity of organ damage caused by the immune system aggression (13, 21). Further studies correlating clinical severity to EV-associated BP180 are needed.

The discovery of BP180, the most important autoantigen target in BP, in EVs derived from blister fluid represents a milestone in the understanding of BP pathogenesis. Nonetheless, this is a preliminary study and many aspects of the pathological process regarding EVs release of BP180 are still to be unravelled.

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