4.1 Final publishable summary report

Executive summary

The overall goal of the translational network “Pemphigus – from autoimmunity to disease” is to provide a link from preclinical disease models to pathogenetic in vitro-studies, the identification of novel diagnostic tools and potential therapeutic targets to prospective clinical trials in pemphigus, a potentially devastating autoimmune disease of the skin. Although rare, pemphigus is the prototype of an organ-specific autoimmune disease due to it well-understood pathogenesis. Still, the standard immunosuppressive treatment of pemphigus is complicated by potentially severe side effects.

The intimate collaboration of basic researchers and clinicians has resulted in a tremendous gain of knowledge on the critical role of pathogenic autoantibodies in pemphigus, the nature of the targeted antigens, the molecular events associated with the characteristic skin pathology, and a general understanding of how immune tolerance to self antigens is regulated. Moreover, the consortium has joined forces to define ad validate clinical and immunologic outcome measures in this hard-to-treat autoimmune disease. Specifically, three different mouse models were utilized to analyze fundaments aspects of autoimmunity, i.e. the role of the interaction of autoaggressive CD4+ T cells and B cells, the HLA class II restriction of this autoimmune response and the regulation of central and peripheral tolerance to the pemphigus autoantigen, desmoglein 3. In addition, the repertoire of autoaggressive memory B cells from pemphigus patients and the spectrum of pathogenic human autoantibodies was extensively studied including epitope specificity, isotype profile, and recognition of novel, not yet pathogenetically understood target molecules. Finally, for the first time, two novel, internationally accepted clinical parameters of pemphigus, the autoimmune bullous disorder intensity score, ABSIS, and the pemphigus disease area score, PDAI were utilized in a multicenter trial as outcome measures of disease activity.

The major achievements of this network are made available to the scientific community and the public community by publications in scientific journals and a public website, “pemphigus.eu”. Overall, the establishment of an international pemphigus network has a major impact on the progress of basic and clinical research in pemphigus and autoimmune diseases in general in Europe and beyond.

Descritption of the project context and the main objectives.

Pemphigus represents a potentially life-threatening organ-specific autoimmune disorder affecting skin and mucous membranes. Clinically, pemphigus patients present with severe painful erosions of the mucous membranes and the skin. Pemphigus is considered as a paradigm of an autoantibody (autoAb) mediated autoimmune disease, primarily associated with autoAb against desmosomal adhesion proteins, which are responsible for the cohesion of epidermal keratinocytes providing mechanical integrity of skin and mucosa. The exact mechanisms finally leading to autoAb-mediated loss of keratinocyte adhesion are presently not completely understood. In general, a more comprehensive understanding of disease-underlying autoimmune processes (e.g. signalling events upon binding of autoAb, autoreactive T cell-B cell-interactions, identification of non-desmosomal autoantigens, characterization of epitope specificity of autoAb etc.) is a major goal of current investigations in the pemphigus field. The overall goal of this collaborative research project was to obtain novel insights in fundamental immune dysregulation leading to autoimmunity in pemphigus. Finally, the more defined knowledge of the autoimmune cascade in this disease will enable both researcher and clinicians to develop improved diagnostic assays and to apply new and presumably more effective therapeutic regiments, respectively.

In this regard, major emphasis was put on defining outside-in-signalling events following binding of desmoglein (Dsg) 3-specific autoAb to the target molecules. Dsg3 is the major autoantigen of pemphigus vulgaris (PV), the most common form of pemphigus diseases in Europe. It could be shown, that transadhering non-junctional Dsg3 acts as a genuine signalling receptor at the transition between proliferation and terminal differentiation in keratinocytes and that non-junctional Dsg3 is associated with EGF-R at cell cycle exit under normal homeostatic conditions. It seems that EGF-R is rapidly activated after autoAb binding and contributes functionally to cellular alterations. Furthermore, PV antibodies alter epidermal stem cell dynamics and functions. The involvement of several intracellular effector molecules, such as PI3K, EGF-R, c-Myc, p38 MAPK, caspases, Src kinase, JNK kinase, TGFβ-R and γ-secretase/Notch, in the pathogenesis of PV was confirmed by application of respective inhibitors. The obtained results provide the basis for testing of therapeutic drugs in PV mouse models and for identification of novel first line treatments for patients of this orphan disease.
In addition to the major autoantigens Dsg1 and Dsg3, a subset of pemphigus sera contains autoAb against other desmosomal and hemidesmosomal proteins. Therefore, an important step in the elucidation of the disease pathogenesis is the obtained generation of the expression vectors for various desmosomal (desmoplakin, periplakin and plakoglobin) and hemidesmosomal (plectin, BP230) antigens and the production of these proteins in mammalian cells. Sera from patients suffering from paraneoplastic pemphigus (PNP), which is a devastating pemphigus variant associated with neoplasia, recognized a protein with apparent molecular mass of 170kDa. After several years of fruitless search, the identity of this protein is uncovered quite now. In this regard, it could be shown, that several PNP sera, but none of sera from PV, PF, BP patients and healthy controls, respectively, were reactive against the 170kDa protein. Establishment of stable cell lines expressing this 170kDa protein is currently in progress. Use of all of these proteins will be employed to characterize the antigenic determinants recognized by pemphigus sera and to

evaluate intermolecular epitope spreading during the course of disease. This will allow to develop improved diagnostic tools for pemphigus diseases, to better adapt the therapeutic regimens resulting in improved quality of life and life expectancy and finally in reduction of the associated medical costs.

It was recently published, that both autoreactive Th1 and Th2 cells are involved in the regulation of the autoAb production by B cells in PV. In this regard, distinct HLA class II alleles shape the autoimmune response to Dsg3. Immunization of mice, which are transgenic for the PV-associated HLA class II haplotype HLA-DRB1*0402/DQ8 and the human CD4 co-receptor, with recombinant human Dsg3 allowed the identification of immunodominant Dsg3 epitopes recognized by in vivo-induced Dsg3-reactive T cells. In this regard, the HLA-anchor motifs and the TCR-binding residues of particular T cell epitopes were successfully identified. This knowledge makes an essential condition for development of antigen-specific treatment approaches. Furthermore, it was found that after immunization of mice with recombinant Dsg3 the specificity of Dsg3-reactive autoAb is initially directed against COOH-terminal epitopes of Dsg3. Repeated application of Dsg3 protein resulted in intramolecular epitope spreading leading to additional IgG reactivity to NH2-terminal Dsg3 domains.

A second mouse model introducing Dsg3-deficient mice allows to study the spontaneous development of autoimmunity against Dsg3. Phenotypically, these mice show pronounced erosions of skin and mucosa. Transplantation of bone marrow stem cells from Dsg3-expressing donors resulted in recovery of bone marrow with more than 50% of Dsg3+ donor derived cells. Moreover, the skin of transplanted Dsg3-/- mice revealed a reconstituted epidermal structure with basal Dsg3+ keratinocytes and no signs of an upcoming blistering disease or an immune response against the Dsg3 neoantigen.

Transformation and immortalisation of human Dsg3-specific memory B cells, which may be exploited as producers of pathogenic autoAb, using EBV in the presence of TLR agonists represent crucial steps for analysis of fine specificity and genetical and functional characterization of autoAb. The frequency of Dsg3-reactive memory B cell precursors in the peripheral blood of PV patients ranged from 0.01 – 2.12%. Interestingly, the frequency of Dsg3-specific memory B cells appeared to vary from patient to patient and was not related to treatment and disease activity, respectively. However, it was to our knowledge the first successful isolation of human Dsg3-reactive monoclonal antibodies (Mabs) from immortalised memory IgG+ B cells of PV patients. Most occurring isotypes of the sustained Mabs were IgG1 and IgG4, which is similar to the autoAb isotypes found in sera of pemphigus patients. Pathogenic potential of generated human Mabs has been tested in a recently described in vitro assay using normal human keratinocytes. The proposed advanced cloning of Dsg- or other epithelial antigen-reactive B cells from pemphigus patients will result in the availability of human Mabs, which could be exploited for more specific research as well as for diagnostic and therapeutic purposes.

Indeed, the aforementioned results promote the comprehension of the etiopathogenesis of pemphigus and provide crucial additional insights into basic mechanisms leading from autoimmunity to autoimmune disease. However due to the low prevalence of pemphigus, control trials are difficult to conduct and the efficacy of treatments is difficult to compare. At present, there is no generally accepted clinical scoring system for the follow-up of pemphigus patients, which may serve as a valid parameter for the extent and activity of disease. A common clinical score is critical for future multi-centre trials. Therefore, a clinical study has been initiated which will evaluate disease activity and response to treatment using two clinical scoring systems, i.e. ABSIS and PDAI, in order to facilitate and advance clinical trials. The

study was considered as a prospective, multi-centre, observational (non-interventional) study by the local Ethics committees and Ethics approval has been obtained for the participation of 36 European centres. At present, 23 newly diagnosed pemphigus patients have been included in the study and enrolment of the intended 100 pemphigus patients should be finished in about 18 months. Scoring systems will be compared over time to serological markers, corticosteroid doses and life quality assessment. A consistent objective scoring system for pemphigus, which is effective and easy to use, would be indispensable to compare both the extent and activity of disease and the efficacy of different treatment regimens, including novel therapeutic approaches.

Description of the main S & T results/foregrounds

The main scientific results of the project are reported referring to the main objectives, including

1. Definition of the molecular mechanisms in the immune pathogenesis of pemphigus *(work package 1).*
2. Analysis of the effector phase of the autoimmune response against epidermal adhesion proteins in pemphigus *(work packages 2,3).*
3. Characterization of the molecular events following antibody binding to the target autoantigens *(work package 4).*
4. Spectrum of the autoimmune B cell response and the impact of therapeutic strategies on the cellular and humoral autoimmune response in pemphigus *(work package 5).*
5. Clinical read-out parameters for prospective studies in pemphigus *(work package 6).*

Objective 1 (work package 1)

Activity 1.1: *HLA class II transgenic mouse model of PV.*

The HLA class II transgenic mouse model provide the opportunity to study the interaction of CD4+ T cells and B cells upon immunization with recombinant Dsg3-protein under in vivo conditions. Immunization of HLA class II-transgenic mice carrying the PV-associated HLA-DRB1*0402/-DQ8 haplotype as well as the human CD4 receptor and are deficient for murine MHC class II molecules (I-Aβ-/-) with recombinant human desmoglein 3 (Dsg3) leads to Ab production against this protein. Ab production was induced by injecting complete Freund´s adjuvant (CFA) containing a total of 50µg recombinant human Dsg3 protein (entire ectodomain) subcutaneously into the plantar surface of both hind paws (partner 1). Two weeks later this procedure was repeated utilizing incomplete Freund´s adjuvant (IFA) followed by intraperitoneal injection of Dsg3-containing Alum on day 21. This immunization protocol results in a pronounced generation of IgG Ab directed against human Dsg3, as demonstrated by ELISA and indirect immunofluorescence using human skin samples and monkey oesophagus, respectively. The pathogenicity of these in vivo induced Dsg3-reactive IgG Ab was confirmed by Ab-mediated loss of adhesion between cultured normal human keratinocytes. Additionally, incubation of human skin biopsies ex vivo with serum samples of Dsg3-immunized mice revealed an acantholytic split formation at the suprabasilar level of the epidermis, accompanied by positive direct immunofluorescence showing a PV-like intercellular staining pattern. To investigate the requirement of CD4+ T cell help for the production of Dsg3-reactive IgG antibodies in Dsg3-immunized HLA class II transgenic animals, two separate series

of experiments were performed. First, HLA transgenic mice were depleted of CD4+ T cells using the mouse CD4-specific monoclonal antibody GK1.5. Treatment of the animals with the GK1.5 antibody around the time of Dsg3-immunization completely abrogated the secretion of Dsg3-reactive IgG. Interestingly, depletion of CD4+ T cells after the first Dsg3-immunization did not seem to influence the course of Dsg3-specific IgG development compared to isotype control treated animals. Referring to the results of this experiment, CD4+ T cell help does not appear to be crucial in an ongoing immune response whereas it is crucial for the initiation of a Dsg3-specific IgG response. Concordantly, interrupting the CD40-CD40 ligand interaction by using the anti-CD40 ligand monoclonal antibody MR-1, completely abrogated the IgG response to Dsg3 in HLA transgenic mice. Development of a distinctive PV phenotype after Dsg3 immunization, i.e. appearance of oral erosions and patchy hair loss, was not observed, presumably owing to maintenance of adhesion between epidermal keratinocytes in the epidermis of HLA-transgenic mice by continuously expressed murine Dsg3.

Activity 1.2*: Defining Dsg3 epitopes that induce experimental pemphigus.*
Autoreactive CD4+ T cells are considered to be crucial in initiating and perpetuating the development of autoAb secretion by autoreactive B cells. To identify immunodominant T cell epitopes, HLA-DRB1*0402-DQ8-transgenic mice were immunized with recombinant human Dsg3 protein. Proliferation of CD4+ T cells in response to defined Dsg3 epitopes, that had previously been described to be recognized by autoreactive CD4+ T cell clones in PV patients, was observed in vitro in CD4+ T cells isolated from different lymphoid organs, i.e. spleen and lymph nodes. In this regard identification of HLA- and TCR-binding residues of T cell epitopes was performed using T cell hybridomas specific for the Dsg3-peptide aa505-521, which were provided by fusion of T cells from draining lymph nodes of immunized HLA-transgenic mice with the murine thymoma cell line BW5147.
Based on the in vitro results, HLA-DRB1*0402-DQ8-transgenic mice were immunized with a set of five immunodominant Dsg3-epitopes (partner 1). CFA containing 50µg peptid-pool was administered subcutaneously into each hind paw, followed by repetitive intraperitoneal peptid-pool/Alum injections. Sera of the mice contained IgG Ab recognizing human Dsg3 as detected by indirect immunofluorescence and Dsg3-ELISA. Pathogenic properties of the respective IgG Ab were assessed by incubation of human skin biopsies with serum samples of Dsg3 epitope-immunized mice resulting in acantholytic split formation at the suprabasilar level of the epidermis. Interestingly, only immunization with a pool of immunodominant peptides, but not with single immunodominant peptides, leads to production of Dsg3-reactive Ab in HLA-transgenic mice. Animals immunized with control Dsg3-peptides, i.e. non-immunodominant peptides (random peptides), did not develop a pathogenic Ab response. These results provide direct in vivo evidence for the hypothesis, that PV is an HLA-class II-restricted, CD4+ T cell-driven autoimmune disease.

Activity 1.3: *Ex vivo detection of autoreactive T cells with HLA class II-Dsg3-peptide tetramers.*
CD4+ T cell epitopes of the Dsg3 autoantigen have been identified previously by our group and others using human CD4+ T cell clones reactive to human Dsg3. Immunodominant CD4+ T cell epitopes were used to generate HLA-DR0402-tetramers to detect autoreactive Dsg3-specific T cells. The single alpha and beta-chain of the HLA-DR0402 molecule were produced using the baculovirus expression system. Figure 2B shows the results of an immunoblot detecting HLA-DR0402

proteins harbouring the Dsg3 peptide aa505-521 which was previously identified in HLA class II-transgenic mice upon Dsg3-immunization. The HLA protein was successfully purified from culture supernatants of baculovirus infected insect cells (high five cells) using either the monoclonal anti-HLA-DR antibody (lane 2 and 4) and an anti-His tag antibody (lane 7, 9 and 10). The next step is the tetramerization of four HLA-DR0402-Dsg3-peptide molecules in order to obtain the tetramer tool for ex vivo detection of autoreactive CD4+ T cells with defined Dsg3-epitope specificity. Development of HLA class II-tetramers composed of the PV-associated HLA-DRB1*0402 molecule harbouring immunodominant Dsg3 peptides is currently in progress (partner 1). Ex vivo detection of autoreactive CD4+ T cells with defined epitope specificity by these tetramers will be started as soon as the respective tetramers will be available.

Activity 1.4: *Epitope spreading of the autoantibody response against human Dsg3.*
Applying protein constructs spanning single extracellular domains (EC) of the Dsg3 protein in an ELISA system enables monitoring of development of the IgG response to human Dsg3 protein after immunization of HLA-transgenic mice. Interestingly, specificity of Dsg3-reactive Ab was initially directed against EC5 located at the COOH-terminus of Dsg3. Repetitive application of Dsg3 results in an intramolecular epitope-spreading characterized by additional IgG reactivity to epitopes of the NH2-terminal EC1 and EC2 domains. In line with recent publications describing that Ab directed against epitopes of the NH2-terminal adhesive region of Dsg3 are pathogenic, whereas antibodies recognizing epitopes of the COOH-terminus are non-pathogenic, mouse sera showing exclusively EC1-reactivity induce acantholysis in human skin biopsies, while COOH-reactive serum samples seem to have no pathogenic capability (partner 1).

Activity 1.5: *T-cell based tolerance induction in experimental PV mice.*
Since immunodominant CD4+ T cell epitopes of Dsg3 have been shown to induce a Dsg3-reactive IgG response in HLA transgenic mice, current investigations apply high doses of immunodominant Dsg3 peptides to induce anergy of Dsg3-reactive T cells in these mice. Furthermore, Dsg3-specific human CD4+ T cell clones are incubated with high doses of their respective Dsg3-peptide in order to induce anergy in vitro. (partner 1).

Activity 1.6: *Priming conditions required for the development of anti-Dsg3-antibodies.*
In addition to HLA-transgenic mice, Dsg3-/- mice were used to investigate priming conditions required for the development of anti-Dsg3 Ab. To determine the relative contribution of immunological tolerance to disease pathogenesis in Dsg3-/- mice mutant conditions of bone marrow chimeras were generated. Dsg3-/- mice were transplanted with bone marrow-derived stem cells from Dsg3+/+ mice. Following transplantation these mice recovered bone-marrow with more than 50% of Dsg3+ donor derived cells. Original data suggested that the skin of the transplanted Dsg3-/- mice was reconstituted by basal donor-derived cells. These donor-derived cells were originally thought to be keratinocytes; yet subsequent characterization of the donor-derived cells, labelled either with EGFP or with DsRed revealed that at least the vast majority of the donor-derived cells expressed markers characterizing hematopoietic cells. Ten weeks after BMT it was still not clear, whether bone-marrow-derived cells are capable of giving off springs to keratinocytes. To further study the fate of the donor cells and their future differentiation we performed FISH and electron

microscopy. These data show that donor-derived cells were able to migrate into the epidermis.Moreover – electron microscopy still suggest that single donor-derived may migrate through the basal membrane. Single cells even showed a hybrid structure, with features of stem cells and features of keratinocytes. Yet, until now we could not definitely prve, whether donor-derived bone marrow cells can turn into keratinocytes. Also other recent reports face similar problems (e.g. Tamai K et al. PNAS USA 2011; 108:6609), and it is still not clear whether donor-derived hematopoietic cells can turn into keratinocytes. Importantly, these chimeric mice showed no signs of an immune response against donor-derived cells and no signs of rejection of the neoantigen-expressing (EGFP; DsRed) cells. We also did not ind the induction of specific Dsg3 autoantibodies in the transplanted Dsg3-/- mice.As some data suggest that the reconstitution by fusion of donor-derived cells may take up to one year and more, we are currently desining studies addressing whether reconstitution with donor-derived Dsg3-expressing cells can be achieved in long term grafted mice. Data from humans tranlsplanted with cardiac stem cells or lung stem cells support such an approach (Bolli R et al. Lancet 2011;3778:1847; Kajastura J et al. N Engl J Med 2011;364:1795).

Activity 1.7: *Analysing the IgG isotypes, epitope spreading and antibody affinity.*
The original aim of characterizing Dsg3-specific Ab according to isotypes, epitope spreading and Ab affinity in all four groups of mice (A: wild type mice substituted with memory B cells from immunized Dsg3-/- mice, B: Dsg3-/-xDsg3+/+ bone marrow chimeras (BMC) on a Dsg3-/- background (Dsg3-/-xDsg3+/+bmcDsg3-/-); C: Dsg3-/-xDsg3+/+ bone marrow chimeras (BMC) on a Dsg3+/+ background (Dsg3-/-xDsg3+/+bmcDsg3+/+); D: HLA class II-transgenic mice) has not been performed, yet due to technical issues regarding the bone marrow transplantation (see activity 1.6). Instead we analyzed a a cohort of well characterized sera of pemphigus patients were investigated regarding IgG isotype and IgE reactivity to Dsg3, respectively. Serum samples were analyzed from a total 93 PV patients classified according to their clinical disease activity, i.e. acute onset PV (n=37), chronic active PV (n=42) and remittent PV (n=14). Sera from 71 healthy individuals served as controls. Both Dsg3-ELISA and indirect immunofluorescence using monkey esophagus were performed to characterize IgG and IgE autoantibody responses. AutoAb titers were correlated with clinical disease activity. As previously described, total IgG as well as IgG1 and IgG4 autoAb titers correlated well with disease activity (figure 11 A-C), i.e. PV patients with active disease demonstrated significantly higher Dsg3-reactive autoAb titers compared with patients in remission. Moreover, PV patients with acute disease showed highest levels of Dsg3-reactive IgE as dermined both by ELISA and indirect immunofluorescence. In order to characterize the IgG reactivity to Dsg3 in more details, single cadherin domains of the extracellular portion of the Dsg3 protein were produced using the baculovirus expression system. Applying these single Dsg3 extracellular domains in an ELISA system allows for a more defined characterization of the epitope specificity. The objective of the following investigation was to study whether the B cell depleting anti-CD20 monoclonal antibody, rituximab, has any impact on the profile of IgG autoAb against defined regions of the Dsg3 ectodomain in a cohort of 22 PV patients. The study patients were followed up clinically and serologically by Dsg3 ELISA over 12-24 months. Prior to rituximab treatment, all the 22 PV patients showed IgG against Dsg3 (Dsc3EC1-5). Specifically, 14/22 showed IgG reactivity against the Dsg3EC1 subdomain, 5/22 patients against Dsg3EC2, 7/22 against Dsg3EC3, 11/22 against Dsg3EC4, and 2/22 against Dsg3EC5. Figure 5 A demonstrates that all rituximab treated patients experienced a clinical remission of

both mucosal and cutaneous erosions within 6 months after treatment. Rituximab induced B cell depletion resulted in a decline in Dsg3-reactive IgG as shown in figure 5B. The novel Dsg3-extracellular domain ELISA system enables us to follow-up on changes in the autoAb profile, i.e. epitope specificity, in PV patients.

Activity 1.8: *Compare all four groups of mice for the development of biologically active antibodies in order to address the relative contribution of central and peripheral tolerance in antibody maturation.*
Using transplanted Dsg3-/- mice peripheral tolerance mechanisms, that are not based on deletion or Treg induction, but based on the deviation of the cytokine profile of pathogenic T cells might represent the first approach allowing to induce a targeted (peptide-specific) peripheral tolerance under therapeutic conditions. This is basically different from all 'immuno-suppressive' strategies developed for human autoimmune diseases until now (all other therapies were based on pharmacological silencing – or the intuction of regulatory T cells; the latter did not yet work in humans under therapeutic conditions). Partner 6 has analyzed in detail the mode of action underlying non deletional T cell tolerance that is required to establish stable graft acceptance. Long ongoing ROS stress has been identified to deeply silence the capacity of antigen presenting cells to produce IL-6, IL-12, or IL-23. On the contrary it enhances their capacity to produce IL-10. These data provide important mechanisms explaining on the one side the status of post transplant immune suppression; on the other side they allow to establish non-deletional tolerance towards grafted cell expressing neo-antigens and that thus may stabilize neo-antigen expressing cells, a status that is needed for long term reconstitution (Ghoreschi et al. J Exp Med 2011208:2291; Hoetzenecker et al. Nat Med, 2011)

Activity 1.9: *Characterization and quantification of T cell responses.*
The characterization and quantification of T cell responses using the ELISPOT technique is ongoing. The basic protocol using pemphigus patients' PBMC stimulated with Dsg3 protein and Dsg3-peptides has been established successfully. At the moment more patient samples are being analyzed.

Activity 1.10: *Local factors influencing disease manifestation of PV.*
Partner 6 did not find local proteases involved in the disease process of blister formation using the Dsg3-/- BMT mouse model. In contrast partner 6 found that ROS stress delivering small molecules like fumarates are capable of establishing a state of immunity protecting against harmful T cell actions (see activity 1.8).

Objective 2 (work packages 2,3)

Activity 2.1: *Determination of pathogenic domain-specific Dsg1 and Dsg3 autoAb in PV and PF.*
Activity 2.2: *Antigenic role of other desmosomal and hemidesmosomal proteins in PV and PF.*
AutoAb specificities and subtypes will be analysed in PV and PF patients using recombinant proteins of Dsg1, Dsg3 and extracellular domains EC1-EC5 of Dsg3. The recombinant forms of desmogleins (Dsg) 1 and 3 and of the Dsg 3 extracellular domains expressed in baculovirus-infected insect cells as well as several hemidesmosomal proteins expressed in mammalian cells needed for this study were expressed and prepared for their use in immunoassays. Recombinant forms of collagen VII and XVII were designed and expressed. The cDNA has been PCR

amplified from a pool of cDNA obtained by reverse transcription of keratinocyte mRNA and, subsequently, cloned into vectors for prokaryotic or eukaryotic expression systems. Two of the the immunossays using non-desmosomal proteins have been developed and thoroughly characterized by Partner 4 in cooperation with Partners 1 and 2 (Csorba et al, Orphanet J Rare Dis. 2011 May 28;6:31; Licarete et al, manuscript submitted). To evaluate the IgA reactivity against collagen XVII, we have expressed a soluble recombinant form of the collagen XVII ectodomain in mammalian cells. Reactivity of IgA autoantibodies from patients with IgA pemphigoid was assessed by immunofluorescence microscopy and immunoblot analysis. ELISA test conditions were determined by chessboard titration experiments. The sensitivity, specificity and the cut-off were determined by receiver-operating characteristics analysis. The optimized assay was carried out using sera from patients with IgA pemphigoid (n = 30) and healthy donors (n=105). By receiver operating characteristics (ROC) analysis, an area under the curve of 0.993 was calculated, indicating an excellent discriminatory capacity. Thus, a sensitivity and specificity of 83.3% and 100%, respectively, was determined for a cut-off point of 0.48. As additional control groups, sera from patients with bullous pemphigoid (n=31) and dermatitis herpetiformis (n = 50), a disease associated with IgA autoantibodies against epidermal transglutaminase, were tested. In 26% of bullous pemphigoid patients, IgA autoantibodies recognized the ectodomain of collagen XVII. One of 50 (2%) of dermatitis herpetiformis patients sera slightly topped the cut-off value. We developed the first ELISA for the specific and sensitive detection of serum IgA autoantibodies specific to collagen XVII in patients with pemphigoids. This immunoassay should prove a useful tool for clinical and translational research and should essentially improve the diagnosis and disease monitoring of patients with IgA pemphigoid. Moreover, our findings strongly suggest that IgA pemphigoid and IgG bullous pemphigoid represent two ends of the clinical spectrum of an immunological loss of tolerance against components of hemidesmosomes, which is mediated by both IgG and IgA autoantibodies (Csorba et al, Orphanet J Rare Dis. 2011 May 28;6:31; URL: http://www.ojrd.com/content/6/1/31). Using this novel immunoassay, we subsequently analyzed sera from patients with pemphigus (n=20) for reactivity against the ectodomain of collagen XVII. In a separate set of experiments, based on in silico antigenic analysis and previous wetlab epitope mapping data, we designed a chimeric collagen VII construct containing all collagen VII epitopes with higher antigenicity. ELISA was performed with sera from patients with EBA (n=50), Crohn disease (CD, n=50), ulcerative colitis (UC, n=50), bullous pemphigoid (BP, n=76), and pemphigus vulgaris (PV, n=42) and healthy donors (n=245). By ELISA, the receiver operating characteristics analysis yielded an area under the curve of 0.98 (95% CI: 0.9638-1.005), allowing to set the cut-off at 0.32 OD at a calculated specificity of 98% and a sensitivity of 94%. Running the optimized test showed that serum IgG autoantibodies from 47 EBA (94%; 95% CI: 87.41%-100%), 2 CD (4%; 95% CI: 0%-9.43%), 8 UC (16%; 95% CI: 5.8%-26%), 2 BP (2.63%; 95% CI: 0%-6.23%), and 4 PV (9.52%; 95% CI: 0%-18.4%) patients as well as from 4 (1.63%; 95% CI: 0%-3.21%) healthy donors reacted with the chimeric protein (Figure 1). Further analysis revealed that in 34%, 37%, 16% and 100% of sera autoantibodies of IgG1, IgG2, IgG3, and IgG4 isotype, respectively, recognized the recombinant autoantigen. Our results show a low prevalence of collagen VII-specific autoantibodies in pemphigus and bullous pemphigoid. Furthermore, we show that the autoimmune response against collagen VII is dominated by IgG4 autoantibodies (Licarete et al, submitted).

Activity 2.3: *Epitope spreading during the chronic course of pemphigus.*
Partners 1-4 and 7 recruited and evaluated pemphigus patients, including obtaining serum samples during the observation period. In parallel different immunoassays to evaluate the epitope spreading were developed and characterized by Partners 1, 3, 4 and 8. On this basis, at the end of the observation period for the recruited patients, Partner 4 together with the other Partners involved will asses the patients for epitope spreading using the developed serological assays.

Activity 2.4: *Relating IgG reactivity against distinct regions of Dsg3 ectodomain to the clinical phenotype in pemphigus.*
Partner 1 studied the autoantibody profile in 22 PV patients, which showed IgG against Dsg3 (Dsc3EC1-5). Specifically, 14/22 showed IgG reactivity against the Dsg3EC1 subdomain, 5/22 patients against Dsg3EC2, 7/22 against Dsg3EC3, 11/22 against Dsg3EC4, and 2/22 against Dsg3EC5. During the entire observation period, 6/22 PV patients experienced a clinical relapse which was associated with the reappearance of IgG against previously recognized Dsg3 subdomains, particularly against the Dsg3EC1 (Müller et al; Dermatol Res Pract. 2010;2010:321950). Interestingly, in pemphigus herpetiformis anti-Dsg3 antibodies associate with only cutaneous involvement for a long period. This finding indicate that the proposed Dsg compensation theory cannot always explain the clinical phenotype, changes in which may occur without apparent modification of the autoantibody profile and antibody specificity. Hence, additional factors, such as Fcgamma-dependent neutrophil activation, may critically affect the clinical presentation of pemphigus (Lebeau et al, Clin Exp Dermatol. 2010 ;35:366-72). In further experiments, Partner 1 investigated the pathogenicity of Dsc3-reactive IgG autoantibodies that were identified previously in a subset of patients with atypical pemphigus. We here demonstrate that IgG against Dsc3 causes loss of adhesion of epidermal keratinocytes. Specifically, IgG against Dsc3 was purified from Dsc3-reactive pemphigus sera by affinity column chromatography using recombinant human Dsc3. Affinity purified IgG was functionally active and did not only react with recombinant Dsc3 but also with epidermis and cultured human keratinocytes. Moreover, Dsc3-reactive IgG induced loss of adhesion of epidermal keratinocytes in a dispase-based keratinocyte dissociation assay that was reversed on pre-adsorption with human Dsc3 but not Dsg3. These findings demonstrate that IgG autoantibodies against an additional component of the desmosomes, Dsc3, induce loss of keratinocyte adhesion and thus may contribute to blister formation in pemphigus (Müller et al, Clin Exp Dermatol. 2009; 34:898-903.; Rafei et al, Am J Pathol. 2011; 178:718-23).

Activity 2.5: *Parallel detection of multiple autoAb reactivities.*
To optimize the detection of autoantibodies to different antigen fragments, in cooperation with Partner 3, we are developing an autoantigen chip containing major epitopes/autoantigens of pemphigus and other autoimmune blistering diseases. For this we provide recombinant forms of collagen XVII ectodomain and chimeric collagen VII (see above). In addition, for this purpose, we have generated a His-tagged recombinant form of collagen XVII containing 5 copies of the immunodominant 16th non-collagenous domain (NC16A). For the initial testing of the autoantigen chip, Partner 4 selected PV (n=20) and control (n=20) sera, including sera from the pemphigus study patients, which were provided to Partner 3, who coordinates these experiments. The new immunoassays should prove useful tools for clinical and translational research and should improve the routine diagnosis and disease monitoring in pemphigus and other autoimmune blistering diseases.

Activity 2.6: *AutoAb specificities as a response to therapy of PV and PF.*
Partner 1 investigated the effects of different treatment options on two B-cell mediators, B-cell-activating factor (BAFF) and a proliferation-inducing ligand (APRIL), in 19 PV patients on immunosuppressive drugs alone or in combination with immunoadsorption and anti-CD20 antibody, respectively. Serum BAFF and APRIL levels, circulating Dsg-reactive autoantibodies, and serum IgG specific for varicella-zoster virus (VZV) and Epstein-Barr virus (EBV) were determined by ELISA before and at different time points after initiation of the respective therapy. In contrast to immunosuppressive therapy alone and in combination with adjuvant immunoadsorption, respectively, rituximab treatment led to a strong and significant elevation of BAFF, but not of APRIL levels, which normalized upon recovery of peripheral CD19(+) B cells. Moreover, rituximab treatment led to a statistically significant increase of anti-VZV-IgG and anti-EBV-IgG titers, whereas anti-dsg1 and -3 specific autoantibody titers decreased significantly. (Nagel et al, J Invest Dermatol. 2009;129:2202-10). Partner 1 and 7 further studied the effect of the B cell depleting anti-CD20 monoclonal antibody, rituximab, on the profile of pathogenic IgG against distinct regions of the Dsg3 ectodomain in 22 PV patients who were followed up clinically and serologically by Dsg3 ELISA over 12-24 months. Prior to rituximab, all the 22 PV patients showed IgG against Dsg3 (Dsc3EC1-5). Specifically, 14/22 showed IgG reactivity against the Dsg3EC1 subdomain, 5/22 patients against Dsg3EC2, 7/22 against Dsg3EC3, 11/22 against Dsg3EC4, and 2/22 against Dsg3EC5. Within 6 months after rituximab, all the patients showed significant clinical improvement and reduced IgG against Dsg3 (5/22) and the various subdomains, that is, Dsg3EC1 (7/22), Dsg3EC2 (3/22), Dsg3EC3 (2/22), sg3EC4 (2/22), and Dsg3EC5 (0/22). During the entire observation period, 6/22 PV patients experienced a clinical relapse which was associated with the reappearance of IgG against previously recognized Dsg3 subdomains, particularly against the Dsg3EC1 (Müller et al; Dermatol Res Pract. 2010;2010:321950). These results suggest that elevated BAFF levels might exert a differential effect on the induction of autoreactive versus pathogen-specific IgG antibody production in PV patients, possibly due to promotion of antibody release of pathogen-specific long-lived plasma cells. However, rituximab only temporarily depletes pathogenic B cell responses against distinct subdomains of Dsg3 which reappear upon clinical relapse.

Activity 3.1: *PNP patients sampling & HLA-typing.*
Due to the rarity of the disease, the number of PNP patients is by definition very restricted. Therefore, a particular electronic database for record of PNP patients is not established, the database used in WP6 keeps track on these patients.
During the reporting period biological samples (skin biopsy, peripheral blood mononuclear cells and serum) from two PNP patients, who died a few days after diagnosis, have been collected and stored (partner 1, partner 2). The diagnosis has been established by direct and indirect immunofluorescence and immunoprecipitation analysis. Sera of several PNP patients were provided by partner 1, 2, 4, 5B, 6 and 7.

Activity 3.2: *170kDa PNP autoantigen identification.*
Among the autoantigens commonly recognized by autoAb in the serum of patients suffering from PNP one of them, with an apparent molecular mass of 170 kDa (p170), had not been identified. Using an immunoprecipitation and mass spectrometry based approach we have identified p170 as alpha-2-macroglobuline-like-1 (A2ML1), a

broad range protease inhibitor expressed in stratified epithelia and other tissues damaged in the PNP disease course. This conclusion is based on several lines of evidence: 1) the tryptic mass profile of the immunoprecipitated p170 had a significant match with that expected for A2ML1; 2) p170 was recognized by anti-A2ML1 antibodies and could be immunoprecipitated from culture media of human keratinocytes, in the same manner as A2ML1; 3) PNP sera immunoprecipitated recombinant A2ML1 from cell extracts, whereas binding to A2ML1 was never observed with sera obtained from normal volunteers (n = 52) as well as patients with autoimmune bullous diseases of the skin (n = 56); 4) p170-reactive PNP sera selectively labeled transfected cells expressing recombinant A2ML1; 5) pre-incubation of p170-reactive PNP sera with recombinant A2ML1 selectively abrogated reactivity of the PNP sera with p170 by immunoblot or immunoprecipitation, and further reduced the labeling of the epidermal granular cell layers, where A2ML1 is predominantly expressed. Epitope mapping revealed that the amino-terminal half of A2ML1 was more antigenic than the carboxyl terminal half. Together, our data unravel a novel class of proteins targeted by autoantibodies in patients suffering from this devastating multiorgan disease (Schepens I, et al., PLoS One. 2010 Aug 18;5(8):e12250). In connection with the detection of autoantibodies in pemphigus patients (see 3.6), we have cloned the coding sequence of A2ML1 with an H6 tag and two streptavidin tags, to facilitate its purification. Recognition of A2ML1 by autoantibodies is strongly dependent on the conformation of the protein. So far, detection of autoantibodies against A2ML1 is based on an immunoprecipitation assay from extracts of keratinocytes radiolabeled in culture, as A2ML1 is not detected by patient autoantibodies on standard Western blots. This is a cumbersome procedure. Immunoprecipitation of A2ML1 from culture medium of transfected cells and its detection with an anti-tag is already much simpler than the standard method. Nevertheless, our final goal is to spot the purified native protein on microarrays (see 3.6).

Activity 3.3: *Plakin and DSG epitope mapping.*

Recombinant forms of the major autoantigens in pemphigus and bullous pemphigoid diseases: the carboxyl terminus region of desmoplakin, the carboxyl terminus region of plectin, the whole plakin domain and globular carboxyl terminus region of periplakin, the whole plakin domain and globular carboxyl terminus domain of envoplakin, the whole plakin domain and globular carboxyl terminus domain of envoplakin, the whole extracellular domain of DSG1 (partner 1), and the whole extracellular domain of DSG3 (partner 1) were expressed and purified by affinity chromatography for the development a protein microarray (see 3.6). Partner 4 (see WP2) completed the panel of autoantigens playing an important role in bullous diseases, which could be later spotted on microarrays (see 3.6).

We analyzed 12 PNP sera by standard immunoprecipitation of in vivo radiolabelled keratinocyte extracts and identified several autoantigens by WB (Schepens I, et al. PLoS One. 2010 Aug 18;5(8):e12250). Moreover, in a collaborative study with Hendri Pas (Department of Dermatology, Centre for Blistering Diseases, University Medical Centre Groningen, 9713 GZ Groningen, the Netherlands) we are testing the best method to diagnose PNP: indirect immunofluorescence on monkey esophagus substrate, indirect immunofluorescence on salt-split skin substrate, indirect immunofluorescence on rat bladder substrate, immunoblotting on keratinocyte cell extract substrate, immunoprecipitation of keratinocyte cell extract substrate, envoplakin ELISA, Dsg1 ELISA, Dsg3 ELISA, BP230 ELISA, BP180 (NC16A) ELISA. Then we will test pemphigus-bullous pemphigoid-PNP microarray with the same sera

(see 3.6). Partner 1 compared the recognition of DSG3 extracellular subdomains (ECDs) by PNP and pemphigus vulgaris autoantibodies (epitope mapping) and typed auto-IgGs. Interestingly, PNP autoantibodies predominantly recognized the ECD 4 and 5, considered as not or much less pathogenic epitopes than ECD1 and 2, usually decorated by pemphigus vulgaris IgGs. PNP anti-DSG3 IgGs were predominantly of subclass 1 and 4.

Activity 3.4: *Characterization of memory B cell repertoire.*
Selection of PNP memory B cells from one PNP patient against permeabilized keratinocytes by immunofluorescence was performed by partners 2 and 8. Five clones were isolated, secreting antibodies recognizing proteins with an apparent molecular weight of 250,000 (four clones) and 210,000 (one clone), probably corresponding to desmoplakin and envoplakin, respectively. Isolation of B cell clones secreting antibodies against A2ML1 was not successful. PNP anti-DSG3 IgGs were predominantly of subclass 1 and 4, as analyzed by partner 1.

Activity 3.5: *Characterization and quantification of T cell responses.*
This activity was not performed on the two samples collected during the first period because of lack of time and low priority rank among the milestones to be reached by partner 1.

Activity 3.6: *Luminex technology for PNP autoAb testing.*
The Luminex technology for the simultaneous, sensitive and specific detection of different antibody reactivities is an expensive technology, requiring the covalent conjugation in solution of the antigens to the fluorescent microbeads. Unfortunately, recombinant proteins expressed in bacteria are often insoluble, while retaining their immunoreactive properties, even in a denatured conformation. The company Qiagen was exclusively selling fluorescent beads with a functional group reactive to sulfydryl groups, which could also be used in the presence of high concentration of the denaturing agent urea, in which most proteins can be solubilized. Unfortunately, the company stopped their whole program based on this technology by the end of 2009. To be independent on the solubility of recombinant proteins, a surrogate technology to Luminex had to be found. Protein microarray seemed to fulfil the requested criteria. In collaboration with partner 4 and Lionel Fontao, Dermatology, University Hospital of Geneva, Switzerland, and the Proteomics Group of Geneva, we tested this technology, as a proof of concept with the soluble GST protein fused to the carboxyl terminus of BP230, an epitope often recognized by PNP sera and by bullous pemphigoid sera. The proteins were sprayed onto nitrocellulose membrane (Gentel Biosciences). Initial results were promising. Two antibodies recognizing the COOH-terminus of BP230 (positive controls) and 2 out of 5 human sera were positive, in agreement with BP230 ELISA results. The next step was the spotting of denatured proteins in urea. It turned out that despite all their efforts the piezo electric sprayer in Geneva could not handle samples containing urea. Therefore, we contacted the Functional Genomics Centre of Zürich (FGCZ), which also has a protein microarray platform. We tested the spraying of proteins dissolved in 8 M urea on three different slides, epoxysilane, hydrogel, and the nitrocellulose membrane FAST. The piezo electric sprayer in Zürich could handle samples in up to 8 M urea. Results obtained with antibodies and sera were again very promising on two out of the three types of slides tested, epoxysilane (E) and nitrocellulose (FAST), whereas the hydrogel matrix failed (data not shown), suggesting that this technology could deliver the results we were looking for. Consequently, we officially collaborate with the FGCZ on this topic

(see annex). Then we spotted the extracellular domain of DSG1 and DSG3, the detection of which is conformational dependent. Again, results, obtained with the microarrays and ELISA, were in agreement with each other, confirming the potential of this technology. Based on these good results, we have a performed a larger scale experiment, testing 20 pemphigus, 20 bullous pemphigoid, and 20 normal sera on a pemphigus-bullous pemphigoid sera. Except for BP180-NC16a, the immobilization of which on this series of slides was deficient for an unclear reason, the correspondence between ELISA and microarray results was good to excellent.
Presently, we are analysing the stability of the immunoreactivity of the spotted proteins versus time with E and FAST slides, stored in different conditions. Once this optimization test is finished, we are ready to test from 20 to 50 bullous pemphigoid, pemphigus and normal sera for each category, which have been analysed with commercial ELISA kits, to be able to compare the results with the method considered as standard, and publish the results.

Objective 3 (work package 4)

Activity 4.1: *In vitro model*
During this funding period, several additional long-term cultures to study PV antibody induced signaling have been established: Akt1-/- keratinocytes (gift B. Hemmings, FMI, Basel) used for in vitro analysis as described below, cultures of skin keratinocytes from EGFR-/- and Notch1-/- (gift Pr. M. Sibilia, University of Vienna and Pr. F. Radke, EPFL Lausanne, respectively) which are of particular importance to underscore an important role of EGFR and Notch signaling in PV. Long-term cultures of several transgenic mouse keratinocytes were expanded and used for in vitro analyses as described below. In addition, keratinocytes from Dsg3-/- mice (gift partner 6 and Prof J. Stanley, University of Philadelphia) were isolated, cultured and characterized within the current funding period. These cells are invaluable to underscore Dsg3-dependent signal pathway activation (partner 5A).

Activity 4.2: *Animal model*
Paraffin and OCT-embedded biopsies from PV mouse model were collected during the funding period. This model consists of neonatal C57bl/6J mice and Rag2-/- (to avoid interference of results with effects of the immune system) injected with pathogenic mouse monoclonal anti-Dsg3 Ab (AK23). This material serves the in vivo validation of results obtained in cultured keratinocytes. In addition, during this period, the PV mouse model was characterized and extensively used to demonstrate that AK23 Ab deplete the stem cell compartment (partner 5A). The gold standard to test PV sera consists of a passive transfer of human autoantibody into neonatal mice and the monitoring of blister development. This model is addressed as “neonatal mouse model for PV”. We have already successfully used this model to address blister formation and test inhibitors to signaling molecules of interest. In the current study, we propose to use transgenic H2B-GFP mice (Jackson laboratories) to follow the fate of GFP-marked epidermal stem cells. The principle to mark epidermal stem cells is based on a timely limiting expression of GFP in dividing cells. Persistence of GFP in the stem cells is based on their intrinsic property to be slow cycling thus retaining the GFP marker once expression is turned off. To investigate Dsg3 antibody-induced signaling in the adult epidermis and epidermal stem cells at defined stages of the HF cycle (activity 4.6) but to also validate our results obtained in cultured keratinocytes in vivo (milestones M4.5 and M4.7), we first have established and characterized a model with passive transfer of the experimental PV antibody AK23 (pathogenic

mouse monoclonal anti-Dsg3 antibody) into adult 8-week-old C57Bl/6J mice (Schulze et al.) 2. Currently, most studies on PV involve passive transfer of pathogenic antibodies into neonatal mice. These mice have not finalized epidermal morphogenesis, and hence do not permit analyses of mature hair follicles (HFs) and stem cell niches. The model we established faithfully recapitulated major features described in PV patients and mouse models, such as widening of intercellular spaces between desmosomes, desmosomal Dsg3 depletion and predominant blistering in HFs and oral mucosa as well as EGFR activation, increased Myc expression and epidermal hyperproliferation (Milestone M4.5). According to this model of 8-week-old mice, several models for stem cell tracking, including the H2B-GFP mice have been validated (Schulze et al., in revision).

Activity 4.3: *PV patient biopsy sampling*
Paraffin embedded biopsies from seven PV patients and control paraffin embedded biopsies have already been collected and were used for analyses in our previous studies. They will initially be used in our study. Additional paraffin embedded biopsies as well as fresh material and cryosections have been obtained from partners in WP6 and are currently being used to validate our data (Partners 2, 7).

Activity 4.4: *Protein A affinity purified PV IgG and AK23*
During the funding period 900mg of pathogenic AK23 Ab were produced from hybridoma cells (gift Prof M. Amagai, University of Tokyo) and purified. 10 PV sera were also obtained from the consortium and affinity purification established in collaboration with Prof J. Schaller, University of Bern, using Affi-gel Blue column and HPLC (partner 5A).During the second funding period 350mg of AK23 antibody were produced from hybridoma cells and purified. This experimental monospecific PV antibody has proven in our hands to faithfully reproduce major findings in PV2. We therefore tested only a limited number of PV antibodies obtained from our partners. In addition, Dr Enno Schmidt (University Clinics Lübeck) provided us with a substantial amount of total human IgG isolated by immunoabsorption from two mucosal PV (containing Dsg3 but no Dsg1 antibodies) and two Pemphigus foliaceus (containing Dsg1 but no Dsg3 antibodies) patients to confirm our data.

Activity 4.5: *Dissecting the pathogenic signaling cascades downstream of Ab-targeted Dsg3 and cooperative EGF-R signaling in mouse keratinocytes at the transition between proliferation and terminal differentiation.*
Aiming for a comprehensive understanding of our results, data on signal pathway activation and inhibition in vitro and in vivo corresponding to activities 4.5 and 4.8 are discussed jointly in this section.

4.5.1 PI3K/Akt, EGFR, Notch and caspases-dependent Dsg3 antibodies-induced loss of cell adhesion.

We have demonstrated during the first founding period that non-junctional Dsg3 acts as an outside-in signaling receptor in keratinocytes at the transition between proliferation and terminal differentiation. Hence, AK23 binding disrupts Dsg3 function leading to continuing proliferation and loss of intercellular adhesion that involve PI3K/Akt inactivation and, conversely, EGFR activation.
In line with these results, we have now found that Akt1 knockdown and adenovirus-mediated Akt1 overexpression enhanced and prevented, respectively, AK23-induced loss of intercellular adhesion in our in vitro keratinocyte dissociation assay. In vivo, Dsg3/PI3K association was confirmed in 8-week-old C57Bl/6J mouse skin by co-

immunoprecipitation (Fig 1B, left panel). Moreover, already 30 minutes after AK23 injection in 8-week-old mice, Akt1 was inactivated (milestone M4.5) (Galichet, Berard et al., in preparation), and consistently, PI3K inhibition did not prevent blister formation in hair follicles (milestone M4.7) (Schulze et al., in revision).
Regarding EGFR, AK23 specifically induced a rapid receptor activation on Tyrosine 845, a Src kinase substrate known to promote mitogenesis, in cultured mouse keratinocytes (Fig 1C, 5 min) and in vivo (Sayar et al., unpublished), which, in the former, correlated with increased Src activation (milestone M4.5). In addition, involvement of EGFR in AK23-mediated loss of adhesion in cultured keratinocytes was further confirmed using several EGFR inhibitors (milestone M4.7) and EGFR/Dsg3 interaction was confirmed in mouse skin (Sayar et al., unpublished).
In our first funding period, we have reported on the enhancement of AK23-mediated loss of intercellular adhesion by a □-secretase inhibitor that was confirmed using another independent inhibitor (milestone M4.7). Conversely, expression of the active Notch intracellular domain NICD, produced by □-secretase cleavage of Notch, partially prevented AK23-mediated loss of adhesion (Fig 1A), suggesting that reduced Notch signaling, a crucial positive modulator of terminal differentiation in the skin4, is involved in AK23-induced loss of intercellular adhesion. Such Dsg3/Notch crosstalk in keratinocytes and impaired Notch function in PV was further implied by Dsg3 interaction with Notch extracellular truncated domain NEXT in mouse keratinocytes as well as decreased expression of Hes1, a Notch target gene, in AK23-injected 8-week-old C57Bl/6J (milestone M4.5) (Luyet et al., unpublished).
Lastly, it has been controversially discussed whether apoptosis is involved in PV5. Based on the absence of TUNEL positive cells and lack of biochemical markers of apoptosis in AK23-treated keratinocyte, in AK23-injected mice as well as in PV patient epidermis, we can now say with confidence that apoptosis is not involved in PV (milestone M4.5). However, caspase 3 inhibitors prevented AK23-induced loss of adhesion (please refer to the precedent report), which was confirmed using several independent inhibitors (milestone M4.7). This suggested a non-apoptotic role of caspase 3 in PV. Indeed, Dsg3 cleavage in AK23-treated keratinocytes was dependent of caspases 3 (Luyet et al., in preparation).

4.5.2 EGFR and PI3K-dependent Dsg3 antibodies-induced cell migration

Hyperproliferation in association with PV, which we were first to report, has long been overlooked due to absence of hyperplasia in the epidermis of PV patients and PV mouse models 3,6. We have now demonstrated that this phenomenon is due to enhanced keratinocyte outward-migration and hence, accelerated epidermal turnover in non-lesional epidermis in response to Dsg3 antibody-induced signal activation. Enhanced adhesion-dependent and -independent migration of AK23-treated keratinocytes was confirmed at three levels: i) in vitro by scratch wounding assays, ii) in an in vivo wound healing assay, and finally iii) in vivo in unwounded skin. The contribution of impaired Dsg3 and PG7,8 function in keratinocyte migration was further confirmed by both Dsg3-/- and PG-/- keratinocytes, which, like AK23-treated cells, demonstrated an accelerated scratch closure in vitro and, as expected, did not respond to AK23. Furthermore, enhanced migration of keratinocytes devoid of PG and Dsg3 or treated with AK23 or PV IgG involved both PI3K/Akt and EGFR signal activation as shown by abrogation of this effect by specific inhibitors (milestone M4.7). Accelerated re-epithelialization was also confirmed in an in vivo wound healing assay in AK23-treated mice (Schulze et al., in preparation). Comparably, in unwounded multilayered epidermis of 10 days-old mice, AK23-induced hyperproliferation correlated with more numerous actively proliferating cells that had

left the basal cell layer in presence of AK23, as demonstrated by an increased number of BrdUbright cells in the suprabasal cell layers in AK23-treated epidermis after a short BrdU pulse (Schulze et al., in preparation). AK23-induced enhanced outward-migration further correlated with EGFR activation on P-Tyr1173 linked to PI3K/Akt signal activation, also known as survival signal2. Enhanced phosphorylation of Akt1 and the consequent stabilization of c-Myc (Schulze et al., in revision,) was further associated with integrin remodeling, characterized by decreased steady state levels of β1 integrin and increased activation of integrin-linked kinase 1 (ILK1) (Schulze et al., in preparation). In agreement with the involvement of PI3K in this event, inhibition of PI3K in AK23-treated 8-week-old C57Bl/6J mice prevented AK23-induced modulations of Akt1, c-Myc, β1 integrin and ILK1 (milestone M4.5) (Schulze et al, in revision, Schulze et al., in preparation).

Activity 4.6: *Dissecting the pathogenic signalling cascades downstream of Ab-targeted Dsg3 and cooperative EGF-R signalling in the epidermal stem cell compartment.*

Using the PV mouse model, we first demonstrated that PV Ab alter epidermal stem cells dynamics and functions (milestone M4-5). The observation that subcutaneously injected AK23 homed to the best described niche for hair follicle stem cells (hsSC) in mice, known as the bulge, indicated that bulge stem cells (buSC) are a target of the Ab's harmful attack. In agreement, stem cell depletion was demonstrated using the widely validated clonogenic assay which allows in vivo quantification of cells with high growth potential (stem cells). A significant reduction in the colony forming efficiency (CFE) was observed following injection of AK23 into neonatal C57bl/6J mice (Schulze et al, unpublished). Consistently, CD34+/α6integrin+ buSC were significantly depleted as demonstrated by immunofluorescence microscopy as well as by multicolour flow cytometry (figure 4C). In parallel to observations made in human PV patients, the proliferation marker Ki67 was further upregulated by 10% in the adult PV mouse model. Collectively these results demonstrate that epidermal stem cells dynamics is altered in a Dsg3-dependent manner in PV. Furthermore, NFATc1, a repressor of proliferation in buSC was also reduced, in line with a relive of the proliferation block and the role of Dsg3 in driving cell cycle exit in epidermal keratinocytes. Using various 8-week-old mouse models, we have investigated the effect of pathogenic anti-Dsg3 antibodies AK23 on the best characterized epidermal stem cell niche, the hair follicle stem cells (HF-SC) niche known as the bulge (Schulze et al., in revision). Combining functional analyses with SC marker expression, label retention and tracing experiments, we have now demonstrated that functional Dsg3-mediated adhesion in the resting (telogen) hair follicle is required for SC quiescence (Schulze et al., in revision). When adhesion was compromised by AK23 binding, bulge SCs became activated as revealed by decreased expression of CD34, a marker characterizing mouse HF-SCs, but also of stemness signature markers such as NFATc1, Sox9, Lhx2 and Lgr5. Consistent with hyperproliferation in PV patients' epidermis and hair follicles3, HF-SC activation in lesional areas of AK23-injected tetOFF H2BGFP mice correlated with loss of their slow cycling nature and increased proliferation, shown by a marked reduction in the number of GFPbright label retaining cells in the telogen bulge. However, AK23-induced HF-SC activation and proliferation did not lead to loss of SCs or premature anagen onset and did not alter normal hair cycling or multipotency in grafting and reconstitution assays. This indicates that the observed profound SC alterations allowed for niche repair after injury to preserve the multipotent HF-SC niche. At the molecular level, drastic repression of Hedgehog (Hh) signaling supported lack of premature anagen

induction. In contrast, decreased Bone morphogenic protein (BMP) expression and late PI3K/Akt signal activation correlated with increased proliferation and niche repair. Furthermore, the EGFR inhibitor Erlotinib, used in clinics, prevented AK23-mediated downregulation of CD34 protein steady-state levels and of the quiescent marker NAFTc1 (milestone M4.5). Taken together, the necessity of Dsg3-mediated adhesion in HF-SC quiescence and the discovery of a hitherto unreported repair mechanism identify PV as the first human disease where HF-SC homeostasis and repair can be addressed.

Activity 4.7: *Confirming results obtained in vitro and in the animal model in PV patients.*
Investigations regarding analysis of SC alterations in human PV patients are not finalized. Several immnunostaining protocols like for P-Akt were established on frozen biopsy sections (Schulze et al., in revision) and hence need to be set-up on paraffin sections. Furthermore, several experimental obstacles related to modulation of PI3K/Akt, caspases and EGFR in AK23-injected mice biopsies need to be solved prior to analysis of human PV patients' biopsies. For instance, activated caspase 3 was not efficiently detected or specific P-EGFR antibodies tested on EGFR-/- mice skin biopsies (gift Pr. M. Sibilia, Vienna) showed unspecific staining. However other signaling pathways have been validated. In addition to increased c-Myc expression and hyperproliferation3, for example the absence of apoptosis was confirmed in non- and peri-lesional skin and oral mucosa of PV human patients using TUNEL assay (Luyet et al., in preparation) (milestone M4-6).

Activity 4.8: *Testing of inhibitors in cultured mouse keratinocytes and animal models (initiated earlier than projected).*
A functional keratinocyte dissociation assay confirmed the involvement of PI3K and underscored the relevance of EGFR activation including potential downstream effectors such as JNK kinase in PV pathogenesis. This assay further allowed us to comprehensively address the involvement of various other signalling pathways in PV (milestone M4-7). The findings are consistent with our previous results on the key role of c-Myc in PV and reports by other investigators on an involvement of p38, caspases and Src kinase. Furthermore, the results demonstrate that TGFβ-R and γ-secretase/Notch are other pathways to be taken into consideration in PV (Luyet et al., unpublished). Together these results provide the basis to test therapeutic drugs in the PV mouse model (M4-5). Although broad variety of inhibitors prevented AK23-induced loss of adhesion in cultured keratinocytes, in vivo, PI3K inhibition was inefficient in this regards (Schulze et al., in revision). Erlotinib, an EGFR inhibitor used in clinics, is currently being tested in AK23- and PV IgG-injected 8-week-old mice and, in contrast to neonatal mice, preliminary results indicate that the efficacy depends on the antibody titer.

Activity 4.9: *Using the in vitro and in vivo models established to test the signaling activity of epitope-specific Ab, human anti-Dsg3, Dsg1 Mabs, and define the beneficial effect of anti-CD20 antibody treatment.*
This activity has started but is not finalized. Having recognized the important gain of knowledge on epidermal stem cell quiescence, activation and repair using Dsg3-function disrupting antibodies, we have put major effort into the stem cell project, and have postponed this activity.

Objective 4 (work package 5)

Activity 5.1: *Pemphigus patients sampling.*
Biological samples (peripheral blood mononuclear cells, serum, skin biopsies) have been collected from 15 pemphigus patients: (i) 3 at diagnosis before treatment initiation, (ii) 11 therapy-resistant, (iii) and 1 in clinical remission. The 11 pemphigus patients with severe therapy-resistant disease have been treated with rituximab and followed up for 18 months after rituximab administration (partner 2, 5B and 6). The samples from these patients have been used for activities 5.2, 5.3, 5.5 and 5.6. In the second part of the project serum samples from additional 30 pemphigus patients have been collected (partner 2, 5B and 6). The samples from these patients have been used for activity 5.3 as well.

Activity 5.2: *Characterization of memory B cell repertoire.*
To characterise the autoreactive epithelium-specific memory B cell repertoire in pemphigus patients, a previously developed method for the efficient immortalization of human memory B cells using EBV in the presence of a TLR agonist has been employed (Traggiai et al, Nat Med 2004; 10: 871-875). IgG+ memory B cells have been isolated by a combination of magnetic and fluorescence activated cell sorting, seeded at a density of 1-30 cells/well in 96-well microplates and immortalized with EBV in the presence of irradiated mononuclear cells and a TLR9 agonist, a CpG oligonucleotide that acts as a polyclonal activator of memory B cells (partner 8). The frequency and specificity of autoreactive memory B cells have been determined 14 days later by screening culture supernatants for their autoAb specificity using: (i) ELISAs based on the ectodomain of Dsg1 and Dsg3, as the main target antigens of pemphigus autoAb (partner 2); (ii) staining of cell substrates (epithelial HaCaT cell line and human keratinocyte) and detection by a confocal microscopy system for cellular high-throughput screening (partner 8); (iii) staining of different epithelial tissues: monkey and guinea pig oesophagus and human skin (partner 2). The anti-Dsg3 memory B cell precursors in pemphigus patients showed a frequency ranging from 0.01 to 2.12%, while in normal donors anti-Dsg3 B cell precursors were undetectable (partner 2 and 8). Interestingly, the frequency of anti-Dsg3 memory B cells appeared to vary from patient to patient and was not related with treatment and disease activity. In addition, the study of the specificity of autoreactive memory B cells by immunofluorescence (IF) on epithelial cells has shown reactivity against non-Dsgs membrane antigens and other intracellular and membrane-associated proteins (partner 8).

Activity 5.3: *Isolation and characterization of autoreactive Mabs.*
Peripheral blood samples from three patients with pemphigus vulgaris (PV), one untreated at the time of diagnosis (PVC) and two corticosteroid-resistant (PVA, PVB), were used. IgG+ memory B cells were isolated and immortalized as described in activity 5.2 (partner 8). Polyclonal cultures were screened by ELISA based on Dsg1/3 and by IF on live and permeabilized keratinocytes (partner 2 and 8) and the cultures producing Abs of interest were cloned by limiting dilution (partner 8). The specificity of isolated hMabs was determined by IF on different substrata (human skin, guinea pig and monkey oesophagus) (partner 2). Sixteen Dsg-reactive hMabs were isolated and cloned from patients PVA and PVC, who presented a higher frequency of anti-Dsg3 memory B cell precursors (0.17% and 2.1%, respectively) than PVB (0.012%). Three hMabs that reacted with both Dsg1 and 3 were also isolated from patient PVA. To characterize the epitope recognized by hMabs, subdomains of Dsg3 ectodomain

(EC1-5) produced as recombinant proteins in baculovirus expression system have been used. These constructs have been recently shown to retain only non-conformational epitopes (Muller et al, Exp Dermatol 2008; 17: 35-43). By ELISAs based on these constructs only one (PVC45) of 16 Dsg3-reactive hMabs reacted with a non-conformational epitope (partner 2). Furthermore, EDTA treatment abolished the reactivity of most hMabs against the entire Dsg3 ectodomain, indicating the calcium-sensitivity of the recognized epitopes. Similar results were also obtained by IF. In addition, the majority of calcium sensitive hMabs reacted with epitopes also sensitive to denaturation with urea (partner 2). These findings define three types of Dsg3 epitopes: 1) calcium- and urea-dependent (11/16); 2) calcium-dependent and urea-independent (PVC16, PVC22); 3) calcium- and urea-independent (PVA3, PVC45, PVA172). In the second part of the project, domain-swapped molecules based on Dsg3 and Dsg1 ectodomains on the Dsg2 backbone (available through a collaboration with Dr. M. Amagai) will be used to map the epitopes recognised by isolated hMabs.

Further studies have been aimed to genetically characterize the isolated hMabs (partner 8). The isolation of 16 independent hMabs in 2 PV patients bearing distinct combinations of variable heavy and light chains suggested an anti-Dsg3 polyclonal response (figure 4). Of note, the isotypes more represented were IgG1 (8/16) and IgG4 (5/16), similar to the isotype distribution in pemphigus. In addition, the sequence comparison of VH and VL genes to germline was consistent with an antigen-induced somatic hypermutation process. Finally, the findings of VH and VL chain sequencing were suggestive of a restricted pattern of heavy chain usage (3 hMabs V4-39*07 and 3 V3-23*01; 7/16 with V3 heavy chain; 5/16 with V4 heavy chain) (partner 8). Thus, we successfully isolated for the first time anti-Dsg hMabs from IgG+ memory B cells of PV patients. Of note, our strategy has allowed retrieval of patients Abs in their natural combinations of heavy and light chains.

In parallel, to identify novel epithelial target antigens in PV, polyclonal cultures derived from immortalized memory B cells were screened by IF on live and permeabilized keratinocytes (partner 8). Twenty hMabs reacting with intracellular membrane-associated epithelial antigens (staining permeabilized keratinocytes) and 1 hMab recognizing a membrane antigen (labelling live keratinocytes) were cloned. Immunoblotting and immunoprecipitation studies on selected non-Dsg reactive hMabs indicated periplakin, envoplakin and a 100 kDa protein as possible targets. In addition, a non-Dsg hMab that bound keratinocyte membranes was able to immunoprecipitate a 200 kDa antigen (partner 2). The 37 isolated hMabs recognizing Dsg1/3 (16 hMabs) or other epithelial antigens (21 hMabs) represent valuable tools for diagnosis and for investigating mechanisms of blister formation and disease evolution in pemphigus.

During the second part of the project the previously isolated anti-Dsg hMabs have been fully characterized. To map conformation-dependent epitopes, 15 hMabs have been tested using an ELISA competition assay based on domain-swapped molecules containing Dsg3 subdomains in the context of Dsg2. Interestingly, 6 hMabs isolated from patient PVA reacted against different Dsg3 subdomains (EC1, EC2, EC3, EC5), while 9 hMabs isolated from patient PVC prevalently recognized (7/9, 77%) the EC2 subdomain. In line with these data, the same strategy applied to the whole PVC serum showed that it mainly reacts with the EC2 subdomain (partner 2). Of note, 9 of the 15 antibodies reacted against the N-terminal region of Dsg3 ectodomain (EC1 and EC2) that is known to represent the major target region of PV autoantibodies. To identify immunodominant pathogenic epitopes within EC1 and EC2, Dsg3 coated-wells were incubated with a fixed concentration of PV and control sera followed by

the addition of biotinylated hMabs: 3 pathogenic hMabs (PVA224, PVC28 and PVC124) (see activity 5.4) and controls (PVC16, AK23, RD monoclonal antibody). All PV sera, but not control sera, strongly inhibited the binding of PVA224 consistent with the presence in all patients of antibodies against the PVA224 site on EC1. In contrast, only 7 and 8 out 10 sera inhibited, respectively, AK23 and PVC16 binding and the inhibition in some cases was partial. Similarly, only some sera were able to inhibit the binding of PVC28 and PVC124. The extremely high prevalence of PVA224-like antibodies in the PV population and the lack of such antibodies in sera from healthy individuals and patients with an unrelated autoimmune blistering diseases, bullous pemphigoid (BP), was further supported by analysis of a large cohort PV sera (n=57) (partner 2). These results indicate that the PVA224-site on Dgs3 EC1 is immunodominant in PV patients, while sites defined by AK23 on EC1 and by the other EC2 specific antibodies are not targeted by all individuals. To determine the proportion in PV sera of antibodies targeting the same or nearby epitopes recognized by PVA224 and AK23, we have then performed a competition ELISA in which Mabs, in the opposite way than before, were used as competitors on a Dsg3 ELISA plate. The reactivity of all PV sera was largely inhibited by PVA224, while AK23 was able to reduce the reactivity of only 5 PV sera (partner 2). These findings further support the notion that antibodies PVA224-like are present in most PV sera. The mouse pathogenic antibody AK23 has been shown to disrupt the trans-adhesive EC1-EC1 interface of Dsg3 (Sekiguchi et al, J Immunol 2001; 167: 5439-48). Strikingly however, no significant cross-competition between AK23 and PVA224 was observed, indicating that PVA224 recognizes a distinct site on EC1 Dsg3. Consistent with this notion, the PVA224 antibody immunoprecipitated both the mature form with unmasked trans-adhesive interface and the higher molecular weight precursor where the trans-adhesive interface is not exposed, while AK23 immunoprecipitated only the mature form of Dsg3 (partner 2). To finely map pathogenic hMabs PEPSCAN technology (Geysen et al, J Immunol Methods 1987; 102: 259-74) has been employed. The PVA224 epitope has been mapped by the using 3 synthetic peptide libraries designed on the EC1 amino acid sequence of Dsg3 (Figure 4A). One library comprised linear epitopes (15-mers linear peptide library) and the other 2 were constrained-peptide libraries: (i) a library of 17-mers peptides looped by a disulfide bond, and (ii) a double CLIPS (chemical linkage of peptides onto scaffolds) library based on peptides locked into double loops by 3 disulfide bonds. The peptides recognized by PVA224 were: i) a linear peptide from the 15 mers library (KITYRISGVGIDQPP) and ii) a conformation-dependent peptide from the double CLIPS library (RALNAQGLDVEKPLI). Since structural information of the Dsg3 EC1 and EC2 domains is not available we aligned the Dsg3 with Dsg2 and C-cadherin for which structural information is known. The positioning of the homologous peptides on Dsg2 EC1 NMR structure (PDB entry 2YQG) and on the C-cadherin crystal structure (Boggon et al, Science 2002; 296: 1308-13) defined the PVA224 epitope as a discontinuous epitope which is located in the EC1 region on the opposite side of the trans-adhesive interface recognized by AK23 (partner 8). The same approach was used to map the epitope recognized by pathogenic antibodies PVC28 and PVC124 which bind the EC2 subdomain of Dsg3. The peptides recognized by PVC28 and PVC124 were comprised in the double CLIPS library. Both antibodies reacted with a CLIPS-peptide (SKIAFKIVSQEPAGT). In addition, PVC124 also bound two amino acid stretches located at the beginning (DINDNPPV) and end (ADKDGEGLSTQCECN) of EC2 subdomain. The location of the homologous peptides on the C-cadherin ectodomain defines discontinuous largely overlapping epitopes located in the EC2 region that interacts in cis with the EC1 of the

neighboring molecule (Boggon et al, Science 2002; 296: 1308-13) (partner 2 and 8). These data highlight for the first time the existence of pathogenic autoantibodies in PV patients that target novel regions of the Dsg3 ectodomain. Specifically, pathogenic hMabs recognize overlapping epitopes located in the EC2 subdomain (PVC28, PVC124) and an epitope in the EC1 subdomain on the opposite side of the trans-adhesive interface recognized by other anti-EC1 pathogenic Mabs described so far (Tsunoda et al, Immunol 2003; 170: 2170-8, Yamagami et al, J Clin Invest 2010; 120: 4111-7). Thus, our data indicate that pathogenetic antibodies PVA224, PVC28 and PVC124 could directly interfere with Dsg3-Dsg3 cis-interaction. Since these pathogenic antibodies alter the adhesive function of cultured human keratinocytes and can induce in mice blisters clinically and histologically similar to those observed in PV patients, we hypothesize that disruption of Dsg3-Dsg3 cis-interaction could represent an additional, novel mechanism contributing to loss of keratinocyte adhesion and thus to blister formation in pemphigus.
The isolated antibodies used different VH and VL genes with a slight bias towards IGHV3 and IGHV4 and carried a variable load of somatic mutations. The H-CDR3 varied from 10 to 20 amino acids and the L-CDR3 from 4 to 10. None of the clones carried the H-CDR3 D/ExxxW consensus motif described by Yamagami and co-workers (Yamagami et al, J Clin Invest 2010; 120: 4111-7) (partner 8).
As for hMabs reacting with membrane-associated epithelial antigens, the target antigen of PVC144 has been identified as the α-catenin. Immunoblotting studies with multiple PV sera on immunoprecipitated envoplakin (recognized by PVB1) and α-catenin (recognized by PVC144) have shown that envoplakin was bound by 2 of 8 PV sera, while no PV sera reacted with α-catenin (data not shown). These findings suggest a secondary role of these antigens in PV disease.
In parallel, IgG+ memory B cells were isolated from 2 additional patients with pemphigus vulgaris (PV), and immortalized as previously described (partner 8). Polyclonal cultures were screened by ELISA on Dsg3 and Dsg1 and IF on live and permeabilized keratinocytes (partner 2 and 8) and the cultures producing Abs of interest were cloned by limiting dilution (partner 8). The specificity of isolated hMabs was determined by IF on different substrata (human skin, guinea pig and monkey oesophagus) (partner 2). One hMab was isolated and cloned from each patient (PVE and PVI). PVE1, a Dsg3 reactive hMab, reacted against a non conformational epitope mapped in the EC5 subdomain of Dsg3 and PVI4 reacted against an extracellular non-Dsg antigen so far unknown (partner 2 and 8).
The isolated hMabs represent valuable tools for diagnosis and to get further insight into the mechanisms of blister formation and disease evolution in pemphigus.

Activity 5.4: *Determination of pathogenic activity of generated Mabs.*
The ability of AK23, a murine anti-Dsg3 Mab, to dissociate a monolayer of normal human keratinocytes has been associated with its pathogenicity (Ishii et al, J Invest Dermatol 2005; 124: 939-946). The pathogenic activity of 4 of 16 hMabs had been assessed (at 1 µg/ml) using this in vitro assay in the first part of the project. Interestingly, testing all hMabs at a concentration of 10 µg/ml has allowed to show that also PVA224 is able to dissociate the keratinocyte monolayer, while the recently isolated PVE1 was not. In addition, all the previously isolated hMabs specific for non-Dsg epithelial antigens as well as the recently isolated PVI4 did not show any pathogenic activity by the in vitro assay (partner 2). To corroborate the in vitro results, we selected two pathogenic antibodies that cross-react with murine Dsg3 (PVA224 and PVC28, data not shown) and tested them in a well-established passive transfer mouse model of pemphigus. When subcutaneously injected, together with

exfoliative toxin A (ETA), in newborn mice PVA224 and PVC28 induced: (i) macroscopic blisters, (ii) intraepidermal suprabasal blistering and acantholysis, and (iii) intercellular deposition of human IgG in the epidermis. In contrast, antibodies PVC16 and PVC124, which do not recognize murine skin by IF, did not cause macroscopic or microscopic blisters or IgG deposition in the epidermis (partner 2). Taken together the above findings provide new knowledge on the role of specific B cells in pemphigus. Specifically, the identification of pathogenic hMabs that do not react against the putative trans-adhesive interface of Dsg3 but react against the putative cis-adhesive interface of Dsg3 provides a novel insight into the mechanism of blister formation. Furthermore, increased knowledge about the requirements that make anti-Dsg3 Mabs able to induce blister formation can allow to develop novel disease-specific therapeutic approaches based on the direct inhibition of pathogenic autoantibodies by using blocking peptides and/or anti-idiotypic antibodies.

Activity 5.5: *Characterization of B cell dynamics and Ab response in rituximab treated patients.*

In order to characterize the dynamics of B-cell and antibody response after rituximab treatment (Zambruno and Borradori, J Invest Dermatol 2008; 128: 2745-7), peripheral blood samples were collected at selected time points from patients presenting with refractory pemphigus undergoing rituximab therapy. Rituximab was administered according to the schedule designed for the treatment of rheumatoid arthritis (two administrations of 750 mg/m2 body surface area at 2 week interval) and blood samples were obtained at day 0, 15, month 1, 3, 6, 9, 12 and 18. 11 patients were regularly followed-up and disease severity monitored by evaluating disease extent and activity parameters (partner 2). The anti-Dsg1 and 3 Ab response in time in rituximab-treated patients was determined by ELISA and reactivity against Dsg appeared roughly related with disease activity (partner 2). After rituximab infusion the number of B cell dramatically decreased while Ab response, probably due to the contribution of short and/or long lived plasma cells, remained significantly high. For this reason, the present activity has been slightly modified and the characterization of B cell dynamics has been substituted by the monitoring of disease activity in rituximab treated patients, with a focus on the detection of autoantibody pathogenic activity during patient long-term follow-up. Ab response in time was determined by Dsg3 ELISA in 11 rituximab-treated pemphigus patients (partner 2). Although in the majority of PV cases anti-Dsg3 ELISA scores fluctuated in parallel with disease activity, in a subset of rituximab-treated patients high ELISA index values were found during remission. Six of the 11 rituximab-treated PV patients were selected to investigate this discrepancy. All the investigated patients had an Ikeda disease severity score of 6 or more and in all the cases the prednisone dosage before the beginning of the rituximab infusions was 40 mg/day or more. Soon after the first rituximab infusion, tapering of prednisone dose was started. All the patients improved following treatment: three were in complete remission at month 3 (PV1, PV2, PV6) and one at month 6 (PV4), while the remaining two patients achieved remission 6 months after a single additional rituximab infusion. During the 18-24 month follow-up, all patients presented relapses which regressed following adjunctive rituximab administration (500 mg, red arrow), without the need for steroids. Disease severity at relapse was mild, exceeding the Ikeda score of 2 in only one patient (PV4). No serious side effects occurred after the infusions of rituximab. Two years after the end of infusions all the patients were in complete remission without lesions and with prednisone 5 mg or less per day (partner 2). Anti-Dsg3 and anti-Dsg1 autoantibody levels and pathogenic in vitro activity were measured in the 6 patients. Five of 6

patients (PV1, PV3, PV4, PV5, PV6) showed a relative decrease of Dsg3-reactive IgG titers (measured by diluting sera which presented saturating score values) to 10–50% of the pretreatment values within 3 or 9 months from the initiation of rituximab treatment (Figure 8A). In parallel, in two representative patients (PV1, PV5), the number of peripheral B lymphocytes dramatically decreased after treatment (red and blue arrows), while the overall count of T lymphocytes remained unaffected (partner 2). In 4 (PV1, PV3, PV4, PV5) of 6 cases the ELISA scores of anti-Dsg3 autoantibodies and their in vitro pathogenic activity fluctuated in parallel with disease activity. In contrast, in the remaining two cases (PV2, PV6) anti-Dsg3 titers were elevated during remission. In particular, patient PV2, who was in remission at month 9, showed a high anti-Dsg3 index value at this time point, while the dissociation index was near the cut off value and thus correlated well with lack of disease activity. The same patient presented a mild relapse at month 18 which related well with dissociation index, while anti-Dsg3 levels showed a huge increase reaching values higher than those detected at diagnosis. The remission state of patient PV6 at month 18 did not correlate with the elevated anti-Dsg3 titers and was better paralleled by IgG pathogenic activity measured by the dissociation index. Of note, these two patients had high anti-Dsg3 titers at diagnosis suggesting that the elevated levels of Dsg3 ELISA in remission could depend on non-pathogenic autoantibodies that were probably also elevated at diagnosis. On the other hand, although both ELISA and keratinocyte dissociation assays were not able to predict relapses in the course of the disease, in the majority of PV patients a rapid increase of disease activity was paralleled by a raise of dissociation index values (PV3, PV4, PV5, PV6) and anti-Dgs3 levels (PV4, PV5). Two PV patients (PV4, PV5), who showed both mucosal and cutaneous involvement at diagnosis, presented as expected circulating IgG against both Dsg1 and Dsg3 which fluctuated consistently with the clinical course. The mucosal relapse in patient PV5 was characterized by a concomitant increase of anti-Dsg3 and not of anti-Dsg1 antibodies (partner 2).

Furthermore, anti-Dsg3 titers and dissociation indexes were not absolute indicators for the severity of the disease among patients. In fact, patients with the same disease activity score (PV1, PV3, PV6) at diagnosis possessed IgG titers and dissociation indexes ranging from 56 U/ml to 754 U/ml and from 222 to 651, respectively. These data strongly suggest a role for other susceptibility factors (genetic factors, T cells and autoantibodies against other antigens) in determining the patient clinical phenotype.

In parallel, the effect of rituximab on the profile of IgG against distinct regions of the Dsg3 ectodomain has been studied in 22 PV patients (partner 1). Specifically, 14/22 showed IgG reactivity against the Dsg3EC1 subdomain, 5/22 patients against Dsg3EC2, 7/22 against Dsg3EC3, 11/22 against Dsg3EC4, and 2/22 against Dsg3EC5. Within 6 months after rituximab treatment, all the patients showed significant clinical improvement and reduced IgG against Dsg3 (5/22) and the various subdomains, that is, Dsg3EC1 (7/22), Dsg3EC2 (3/22), Dsg3EC3 (2/22), Dsg3EC4 (2/22), and Dsg3EC5 (0/22). During the entire observation period, 6/22 PV patients experienced a clinical relapse which was associated with the reappearance of IgG against previously recognized Dsg3 subdomains, particularly against the Dsg3EC1. Thus, in PV, rituximab only temporarily depletes pathogenic B cell responses against distinct subdomains of Dsg3 which reappear upon clinical relapse (Muller et al, Dermatol Res Pract. 2010; 2010:321950) (partner 1).

Activity 5.6: *Characterization of memory B cell repertoire following rituximab treatment.*

Characterization of memory B cell repertoire following rituximab treatment was not performed due to almost undetectable memory B cells. This activity has been substituted by the above described study of monitoring of disease activity by specifically detecting autoantibody pathogenic activity.

Objective 5 (work package 6)

Activity 6.1: *Enrolment of patients with PV and PF.*

Approval of the study by local Ethic's Committees
Ethics approval has been obtained for the participation of 36 centers. The draft of the study has been approved by the following Ethic's committees: Rouen (partner 7 - for all the 30 French centers), Marburg (partner 1), Freiburg (partner 4), Tübingen (partner 6), Roma (partner 2) and Bern (partner 5B) (Appendix 4). Approval from additional German centres Ethic's committees have still to be obtained. All but one Ethics committees (France) confirmed that this non-interventional study does not require an insurance. Owing to delayed Ethics approval in most centres, enrolment of patients has begun in July 2009 and it has been closed in February 20011. One hundred and one patients have been included in 18 months, in accordance with the recruitment which was expected (64 patients from France, 17 from Germany, 15 from Italy and 6 from Switzerland). The24 month follow-up time of the last patient included will be achieved by February 2013.

Editing the investigator's brochure.
The CRFs have been made, validated by the statistic department on the first 3 patients included in the study, and then translated in different languages (French, German, Italian). They have been edited and have been circulated among investigators.

Activity 6.2: *Assessment of disease severity and patients´ quality of life.*
Several administrative procedures for precise evaluation of clinical activity were part of the clinical study: Two quality of live indices have been chosen: the DLQI and the Skindex. These 2 questionnaires have been translated and have been validated in the different European languages (French, Italian and German translations were obtained). The two scoring systems, which are tested, are the ABSIS and the PDAI scores. Both scores have been published but their reliability and reproducibility has not been extensively evaluated, yet. The results of this study comparing precision of ABSIS and PDAI scoring systems will allow to define clinical read-out parameters, which could form a basis for prospective studies in order to advance monitoring of clinical trials.

Investigators meeting to check quality of life indices, clinical scoring system and standard therapeutic options.
Three investigator meetings have been organized to check the scoring methods; the first one was held in Frankfurt in June 2008. It was mainly devoted to discuss various aspects of the study (number of visits, treatments, transportation of samples, etc.) The second one was held in Paris with the French investigators in May 13, 2009. The third one was held in Frankfurt with German, Italian and Swiss investigators in October, 2nd, 2009. These 2 latter meetings were devoted to training of investigators and clarifying some points.

Agreement on standard therapeutic options in the different centers depending on pemphigus severity.
The standard therapeutic options and treatment endpoints have been determined in accordance with the "Consensus statement on disease end points and therapeutic response for pemphigus". However, in order to keep the status of the study as non interventional, partners have wished that treatments would not be standardized. It is only suggested to treat patients according with the recommendations of the Consensus statement.

Organization of serum samples transportation to the partner 7 laboratory for storage before ELISA assays
Collection and transportation of blood samples has been organized in 2 different ways:
Blood samples which are collected for lymphocyte analyses are sent to Marburg University.(samples are delivered at Marburg University less than 24 hours after having been collected). Blood samples which are collected for serum analyses are frozen and conserved on site. Frozen samples will be collected in each center twice a year and sent to Rouen University. Serum samples will then be re-circulated to Bern, Roma, Marburg and Freiburg. The first collection of frozen samples has been organized in December 2010. A last transportation will be organized at the end of the study in February 2013.

Appointment of 2 investigators in each center
In each centre, two investigators have been appointed to independently perform the severity score assessment.

Activity 6.3: *Evolution of anti-Dsg Ab.*
Collected serum samples for analyses were frozen, conserved on site and have been sent to Rouen University twice a year. Serum samples will be recirculated to Bern, Roma, Marburg and Freiburg. ELISA assays for the assessment of the evolution of anti-Dsg1 and -Dsg3 Ab have been partly bought. The first collection of frozen samples has been organized in December 2010. A last transportation will be organized at the end of the study in February 2013. Analysis of sera has been standardized and PCR analyses are currently available in the immunology department of Rouen University hospital.

Activity 6.4: *Treatment of patients and follow-up.*
The standard therapeutic options and treatment endpoints have been determined in accordance with the "Consensus statement on disease endpoints and therapeutic response for pemphigus". In order to keep this study as non-interventional, treatment regimens are only suggested. The consequence is, that only 50 to 75% of patients will be treated with the same regimen, whereas about 25% will be treated with other therapeutic regimens using either a fixed low dose of corticosteroid for the 1rst year (patients with a mild type of pemphigus), or a short low dose corticosteroid regimen either alone, or in association with rituximab (for patients with a contraindication to high doses of corticosteroids).
Study inclusion is restricted to newly diagnosed patients and clinical and serological monitoring is defined to perform at particular time points. A reminder of the next visit is systematically sent to investigators by the sponsor (Rouen University hospital) one week before the next visit.

Activity 6.5: *Statistical analysis*
Statistical data analysis will be performed at the end of the study in February 2013.

Activity 6.6**:** *Develop and validate clinical read-out parameters for prospective studies in pemphigus.*
A first serum analysis will be performed in the in Immunology laboratory of the Rouen University hospital in January 2012 when all serum samples from the 47 patients who have completed their 2 years of follow-up, will be available (after the second transportation of sera organized in December 2011). A second serum analysis will be performed in March 2013 at the end of the study when all sera from the most recently included patients will be available (after the last transportation of sera which will be organized in February 2013).
On October 2011, 47 CRF from the patients who have completed their 2 years of follow-up have been entirely captured in the database. Additionally, 26 CRF have been partially captured. 89% of cases correspond to PV and 11% to PF.

Description of the potential impact, the main dissemination activities and the exploitation of results.

The project “Pemphigus - from autoimmunity to disease” represents a successfully joined effort of European scientists and clinicians who managed to establish a consortium of groups providing new basic insights into the underlying disease mechanisms, research tools and new diagnostic assays in the field of pemphigus. The vast majority of the different scientific objectives of the project were successfully achieved. The promising results obtained in the various projects are going to have a tremendous impact on the future research in pemphigus.

As an important part of the experimental work, constructs for recombinant plakins, collagen VII and XVII, in particular protein fragments encompassing the epitopes targeted by patients' autoAb were generated. After full protein chemical characterization, these recombinant antigens were used to map epitopes targeted by pemphigus patient autoAb during the disease course. Further, these antigens - together with recombinant forms of desmogleins and several hemidesmosomal proteins already available to the consortium – will be employed to evaluate epitope spreading during the disease course and to correlate it with the effects of therapy. The characterization of the antigenic determinants recognized by pemphigus sera and of epitope spreading phenomena during the course of the disease will allow for developing improved diagnostic tools for this severe autoimmune disease. These tools will also enable clinicians to better adapt the therapeutic regimens and therefore improve quality of life and life expectancy in pemphigus. In addition this approach may be translated to other rare autoimmune and inflammatory diseases facilitating improvements in diagnosis and treatment possibly resulting in reduction of the associated medical costs.

Moreover, in collaboration with the Functional Genomics Centre of Zürich (FGCZ) a protein microarray for the detection of autoantibodies in skin blistering diseases has been developed. This technique allows the simultaneous detection in patient sera of autoantibodies against all the spotted antigens, the identification of the immunogenic domains recognized by the autoantibodies and is easily expandable. This tool should enable clinicians to better understand the mechanisms of skin blistering diseases, correlate the symptoms with the targeted antigens by the autoantibodies and analyze the impact of medical treatments. Finally, these results should facilitate the diagnosis and treatment of skin blistering diseases, possibly resulting in improvement of quality of life and life expectancy and reduction of the associated medical costs.

Molecular events following autoAb binding to the target autoantigen in pemphigus have been extensively characterized. With the ultimate goal to identify novel therapeutic targets in pemphigus, molecular events were comprehensively addressed in cultured keratinocytes, the epidermis as well as the epidermal stem cell compartments. Pathogenic signaling cascades activated at the plasma membrane upon PV autoAb binding have been identified and a variety of novel potential therapeutic targets have been identified. To address the efficacy of these drugs in adult skin and epidermal stem cell compartments, an 8-week-old adult mouse model to be used in addition to or instead of the classical neonatal model has been established.

Specific signaling cascades identified to be involved in loss of intercellular adhesion in PV are for example EGFR and Notch signaling. In the stem cell compartment enhanced proliferation further impacts on functional marker expression which in turn results, as a good outcome for PV patients, in a repair mechanism to prevent the loss of epidermal stem cells. In conclusion, the investigations on Dsg3 receptor-mediated signaling have revealed that this adhesion molecule functions as a signaling node in keratinocytes both in the stem cell and proliferative compartment and has allowed us to pinpoint a variety of novel testable therapeutic targets.

The advance in the molecular understanding of PV pathogenesis will be of major relevance to develop new targeted therapeutic approaches for PV patients. The finding on the necessity of Dsg3-mediated adhesion in hair follicle stem cell quiescence and the discovery of a hitherto unreported repair mechanism further identify PV as the first human disease where epidermal stem cell homeostasis and repair can be addressed. Hence, the in-depth understanding of Dsg3 function is of broad significance and equally impacts on epidermal homeostasis as well as other epidermal disorders than PV.

Novel pathogenic Dsg3 epitopes have been characterized using newly generated monoclonal Dsg3-reactive antibodies. The results obtained in this project point for the first time to the disruption of Dsg3 cis-interaction as an additional mechanism involved in blister formation in PV and provides further information and tools to understand the basic molecular mechanism of Dsg-mediated cell–cell adhesion. Finally, knowledge of pathogenic epitopes and idiotypes may result in more specific diagnosis, monitoring of disease and allow the development of novel therapeutic approaches in this life-threatening disease.

The prospective randomized multicenter clinical trial in pemphigus will provide very important information on the future use of disease activity parameters. The validation of the two scoring systems PDAI and ABSIS is going to be a crucial step towards establishing a generally accepted outcome measurement to be applied in future multicenter clinical trials in pemphigus.