Journal of Autoimmunity 96 (2019) 104–112

Contents lists available at ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

IL-17A is functionally relevant and a potential therapeutic target in bullous pemphigoid

Lenche Chakievska[a], Maike M. Holtsche[b], Axel Künstner[a], Stephanie Goletz[a], Britt-Sabina Petersen[c], Diamant Thaci[d], Saleh M. Ibrahim[a], Ralf J. Ludwig[a], Andre Franke[c], Christian D. Sadik[b], Detlef Zillikens[b], Christoph Hölscher[e], Hauke Busch[a], Enno Schmidt[a,b,*]

[a] Lübeck Institute for Experimental Dermatology (LIED), University of Lübeck, Lübeck, Germany
[b] Department of Dermatology, University of Lübeck, Lübeck, Germany
[c] Institute of Clinical Molecular Biology, University of Kiel, Kiel, Germany
[d] Institute for Inflammation Medicine, University of Lübeck, Lübeck, Germany
[e] Division of Infection Immunology, Research Center Borstel, Borstel, Germany

ARTICLE INFO

Keywords:
Autoantibody
BP180
Bullous pemphigoid
IL-17A

ABSTRACT

IL-17A has been identified as key regulatory molecule in several autoimmune and chronic inflammatory diseases followed by the successful use of anti-IL-17 therapy, e.g. in ankylosing spondylitis and psoriasis. Bullous pemphigoid (BP) is the most frequent autoimmune blistering disease with a high need for more specific, effective and safe treatment options. The aim of this study was to clarify the pathophysiological importance of IL-17A in BP. We found elevated numbers of IL-17A$^+$ CD4$^+$ lymphocytes in the peripheral blood of BP patients and identified CD3$^+$ cells as major source of IL-17A in early BP skin lesions. *IL17*A and related genes were upregulated in BP skin and exome sequencing of 51 BP patients revealed mutations in twelve IL-17-related genes in 18 patients. We have subsequently found several lines of evidence suggesting a significant role of IL-17A in the BP pathogenesis: (i) IL-17A activated human neutrophils in vitro, (ii) inhibition of dermal-epidermal separation in cryosections of human skin incubated with anti-BP180 IgG and subsequently with anti-IL-17A IgG-treated leukocytes, (iii) close correlation of serum IL-17A levels and diseases activity in a mouse model of BP, (iv) *IL17*A-deficient mice were protected against autoantibody-induced BP, and (v) pharmacological inhibition of lL-17A reduced the induction of BP in mice. Our data give evidence for a pivotal role of IL-17A in the pathophysiology of BP and advocate IL-17A inhibition as potential novel treatment for this disease.

1. Introduction

Bullous pemphigoid (BP) is a chronic subepidermal blistering autoimmune disease of the elderly associated with autoantibodies against two structural components of the dermal-epidermal junction, BP180 (type XVII collagen, Col17) and BP230 [1]. In BP, binding of anti-Col17 antibodies to the dermal-epidermal junction is a prerequisite for blister formation as shown in various experimental models [2–6]. These data are supported by the close correlation of anti-Col17 antibody serum levels with disease activity in BP patients [2,7–11]. The activation of complement and the subsequent influx of inflammatory cells into the upper dermis are crucial for the dermal-epidermal splitting that is finally mediated by reactive oxygen species and proteases such as matrix-metalloproteinase-9 (MMP-9) and neutrophil elastase [6,12–15]. The mechanisms and key mediators that lead to the infiltration of inflammatory cells and orchestrate the inflammatory milieu in the upper dermis are still largely elusive.

In contrast, in other chronic inflammatory diseases such as psoriasis, rheumatoid arthritis, and more recently, atopic dermatitis, the identification of central inflammatory mediators including TNFα, IL-6, IL-17A, IL-23, and IL-4/IL-13 have allowed the development of more specific, effective, and safe therapies [16–21]. In experimental pemphigoid diseases, inhibition of TNFα, IL-6, and IL-1β led to no or only modest reduction of diseases activity [22–24].

The aim of the present study was to (i) identify the cellular source of IL-17A in BP skin, (ii) analyze *IL17*A and related genes as risk factors for BP, and (iii) explore the functional relevance of IL-17A in experimental models of BP.

* Corresponding author. Lübeck Institute for Experimental Dermatology (LIED), University of Lübeck, Ratzeburger Allee 160, D-23538, Lübeck, Germany.
E-mail address: enno.schmidt@uksh.de (E. Schmidt).

https://doi.org/10.1016/j.jaut.2018.09.003
Received 23 July 2018; Received in revised form 4 September 2018; Accepted 7 September 2018
Available online 13 September 2018

2. Materials and methods

2.1. Human material

BP patients (n = 15) were diagnosed based on a compatible clinical picture, linear deposits of IgG and/or C3 at the dermal-epidermal junction by direct IF microscopy of a perilesional skin biopsy, and circulating autoantibodies by indirect IF microscopy on human salt-split skin (epidermal binding) and BP180 NC16A ELISA (Euroimmun, Lübeck, Germany). Whole blood and perilesional skin biopsies were taken at the time of diagnosis before therapy was initiated.

Whole blood was taken from patients with non-inflammatory dermatoses (mostly non-melanoma skin cancer) aged above 75 years (n = 12). Perilesional skin was used from patients with other inflammatory dermatoses (n = 7). Skin biopsies were stored at −80 °C. Peripheral blood cells were assayed on the same day when blood was taken. DNA from the peripheral blood of 51 BP patients diagnosed by the same criteria as outlined above, was isolated following the manufacturer's instruction (Qiagen, Hilden, Germany). DNA samples were stored at −80 °C until analyzed. The study was performed in accordance with the Declaration of Helsinki and was approved by the ethics committee of the University of Lübeck (08–156, 11–234, 12–178, 15–051). Blood from healthy volunteers for the in vitro and *ex vivo* studies was used on the same day. Latter studies were approved by the ethics committee of the University of Lübeck (09–140).

2.2. Cytokines in peripheral blood cells

1×10^6 cells from heparin-NH4-treated patient/control blood were suspended in 100 µl RPMI 1640 medium and stimulated with 250 ng/ml phorbol myristate acetate (PMA) plus 5 µg/ml ionomycin in the presence of brefeldin A (all Biolegend), a protein transport inhibitor, for 6 h at 37 °C with 5%CO_2. The cells were incubated with Brilliant Violet 510™ anti-CD3 (clone UCHT1), Alexa Fluor® 700 anti-CD8a (clone RPA-T8), Brilliant Violet 711™ anti-CD14 (clone M5E2), Brilliant Violet 421™ anti-CD25 (clone M-A251), Brilliant Violet 785™ anti-CD45 (clone HI30), and Brilliant Violet 605™ anti-CD127 antibodies (clone A019D5, all Biolegend) for 30 min at room temperature (RT) and subsequently subjected to live/dead staining with Zombie NIR™ Fixable Viability Kit (1:1,000, Biolegend). Using the True Nuclear Transcription Kit (Biolegend) cells were fixed and permeabilized at RT for 75 min. Subsequently, for intracellular staining the following antibodies were incubated for 30 min at RT; PerCP/Cy5.5 anti-CD4 (clone RPA-T4), PE/Dazzle™ 594 anti-IL-17A (clone BL168), Alexa Fluor® 647 anti-IL-21 (clone 3A3-N2), PE anti-IL-22 (clone BG/IL2), Alexa Fluor® 488 anti-FOXP3 (clone 259D; all Biolegend), and anti-IL-17F PerCP-eFluor® 710 (clone SHLR17, eBioscience, Waltham, USA). The following matched isotype antibodies were used as controls: Brilliant Violet 510™ mouse IgG1 (cloneMOPC-21, Biolegend), SAv-Brilliant Violet 711™ mouse IgG2a (clone MG2a-53; Biolegend), and PerCP-eFluor 710 rat IgG1κ (clone eBRG1, eBioscience). After stringent washes, samples were measured by flow cytometry using Benchtop analyzer LSRII (BD Biosciences, Franklin Lakes, USA). Data were analyzed by FlowJo (TriStar, Ashland, USA).

2.3. mRNA levels of IL-17A and related mediators in the skin

Total RNA was extracted from frozen perilesional skin biopsies using RNAse Mini Kit (Qiagen) according to the manufacturer's protocol. cDNA was amplified in concentration of 500 ng using First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, USA). RT-PCR was performed using SYBR Green-gene expression assay (BioRad, Munich, Germany). Results were calculated using the $2^{\Delta\Delta Ct}$ method. Primers used to detect *CCL2, CCL20, CCR6, CD4, CD8a, CSF2, CSF3, CXCL1, CXCL2, ELANE, ICAM1, IFNg, IL10, IL12B, IL12RB1, IL17A, IL17F, IL17RA, IL17RC, IL2, IL21, IL22, IL23A, IL23R, IL27, IL4, IL6, IRF4, KLRB1, RORc, SOCS3,* and *TNF* are detailed in Table S2.

2.4. Immunofluorescence microscopy and histopathology

H&E staining was performed according to standard protocols. For immunostaining of infiltrating cells in the perilesional skin, paraffin sections of perilesional skin biopsies were blocked with 3% bovine serum albumin (Carl Roth, Karlsruhe, Germany) in TBS. All washing steps were performed three times for 5 min in PBS-T (PBS + 0.05% Tween). The following primary antibodies were applied: rabbit anti-myeloperoxidase (1:150), mouse anti-CD3 (clone F7.2.38, 1:50), mouse anti-tryptase (clone AA1, 1:150), mouse anti-CD68 (clone PG-M1, 1:100; all Dako, Glostrup, Denmark), and rat anti-CD4 (clone YNB46.1.8, 1:300; Novusbio, Colorado, USA). As detection antibodies, donkey anti-mouse IgG Alexa Fluor® 594 (1:300; Abcam, Cambridge, USA) and chicken anti-rabbit IgG Alexa Fluor® 594 (1:200; Invitrogen, Carlsbad, USA) were used. IgG1 kappa (1:200; Biolegend) served as isotype control. DAPI staining was performed for all sections. Specimens were mounted in Fluoromount-G (Southern Biotech, Birmingham, USA) and kept at −20 °C until analyzed. Cell numbers were determined by counting fluorescent cells in relation to DAPI-positive cells in 5 visual fields of 2 sections using the BZ-9000 fluorescence microscope (Keyence, Frankfurt, Germany) and inverse confocal microscope FV 1000 (Olympus, Tokyo, Japan).

2.5. Exome sequencing

Samples were enriched using Illumina's TruSeq Rapid Exome Enrichment Kit. Sequencing of 2 × 75 bp paired-end reads was then performed on the Illumina HiSeq 4000. Whole exome sequencing raw data was quality filtered using Trimmomatic (version 0.36) [25]. Afterwards, trimmed reads were mapped to the human genome (hg19) using bwa mem (version 0.7.15) [26]. Resulting mappings were further improved by identifying duplicates (Picard tools, version 1.141), realignment around insertions and deletions (GATK, version 3.5.0) and base quality recalibration (GATK) [27]. Variant calling (single nucleotide polymorphisms and insertions/deletions) was performed using VarScan (version 2.4.3) and variants were annotated using Annovar (version 2017Jul16) and Combined Annotation Dependent Depletion (CADD, version 1.3) [28,29].

2.6. Stimulation of peripheral neutrophils with IL-17A

Neutrophils were isolated from peripheral blood of healthy adult volunteers by Polymorphprep™ (Axis-Shield, Dundee, UK) as described previously [30]. Neutrophils were left unstimulated or activated by 500 ng/ml recombinant IL-17A (Peprotech) and 10 ng/ml PMA (positive control), respectively, for 1h at 37 °C before stained with Brilliant Violet 510™ anti-CD45 (clone HI30), Pacific Blue™ anti-CD16 (clone 3G8), APC anti-CD66b (clone G10F5), PerCP/Cy5.5 anti-CD62L (clone DREG-56), PE/Cy7 anti-CD14 (clone HCD14), PE anti-CD193 (CCR3, clone 5E8), FITC annexin V, and Zombie NIR™ (all Biolegend). The activation status of neutrophils was determined by the expression of CD62L measured by MACS Quant Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach, Germany).

2.7. Cryosection assay

Separation of the dermal-epidermal junction in cryosections of normal human skin was evaluated using an *ex vivo* model as previously described [31–33]. Briefly, normal human skin was incubated with BP patient and normal human serum, respectively, for 1 h at 37 °C. Human leukocytes purified by a sedimentation gradient with dextran 500 (Carl Roth) were added together with 100 µg, 200 µg and 300 µg neutralizing anti-IL-17A antibody and an isotype control (Novartis), respectively, and incubated for 3 h at 37 °C before intense washing with PBS. Skin

sections were stained with hematoxylin and eosin (H&E), and dermal-epidermal separation was evaluated microscopically (Keyence).

2.8. Mice

C57BL/6J mice were bred and housed at 12-h light-dark cycle at the experimental animal facility in the University of Lübeck. IL17A$^{-/-}$ mice were kindly provided by Yoichiro Iwakura (Tokyo University of Science, Tokyo, Japan) and bred at the experimental animal facility of the Christian-Albrechts-Universität zu Kiel. All injections and bleedings were performed on eight to twelve-week old mice narcotized by intraperitoneal (i.p.) injection of a mixture of ketamine (100 µg/g) and xylazine (15 µg/g). Animal experiments were approved by the Animal Care and Use Committee of Schleswig-Holstein (Kiel, Germany; 21–2/11) and performed by certified personnel.

2.9. Antibody transfer-induced BP in adult mice

Experimental murine BP was performed as previously described [5]. Briefly, affinity-purified rabbit anti-murine BP180 IgG was injected subcutaneously into the neck of 6- to 8-week-old mice every other day over a period of 12 days at individual doses of 10 mg IgG/injection/mouse. At the time of IgG injections, mice were weighed and examined for their general condition and evidence of cutaneous lesions (i.e., erythema, blisters, erosions, and crusts). The percentage of affected body surface area was determined on days 4, 8, and 12. Cutaneous lesions were scored as involvement of the skin surface as previously described, assigning a certain percentage of body surface to particular body parts [5]. Calculating the total percentage of affected body surface area resulted in an overall clinical score. At day 12, the mice were sacrificed, sera and biopsies were taken and stored at −20 °C until used. The anti-IL-17A antibody (clone 17F3, BioXCell, West Lebanon, USA) and IgG1 isotype control antibody (clone MOPC-21, BioXCell) were injected at a dose of 200µg/injection as detailed below. Serum levels of IL-17A were determined by BioPlex (BioRad).

2.10. Statistics

The data are presented as the median or mean ± standard deviation. Statistical calculations were performed using GraphPad Prism version 6 (GraphPad Software Inc, San Diego, USA). For comparison of more than two groups Two-way ANOVA with Sidak's multiple comparisons test was used. When data were nonparametric Mann-Whitney-U test or Kruskal-Wallis test were applied followed by a Dunn posthoc or Bonferroni *t*-test for multiple comparisons. Pearson product moment correlation was used to test for correlations. In all tests, a p-value of 0.05 was considered to be statistically significant. The gene regulatory network was generated using NetworkAnalyst web server with "A list of proteins and genes" module [34]. In this analysis, significant genes identified by RT-PCR were provided as input. STRING database within web server with the medium threshold of 400 was used as background database for searching interacting partners [35]. We only considered zero degree or direct interaction. The output "SIF" files were visualized using Cytoscape software [36].

3. Results

3.1. CD4-positive lymphocytes are the major source of IL-17A in the peripheral blood of BP patients

When CD4$^+$ cells, CD8$^+$ cells, neutrophils, and monocytes in the peripheral blood of BP patients (n = 15) and age- and sex-matched controls (n = 12) were analyzed for their capacity to generate IL-17A, IL-17A was only found to be significantly increased in CD4$^+$ cells (p = 0.001) but not in CD8$^+$ cells, neutrophils, and monocytes (p = 0.14, p = 0.17, p = 0.19, respectively; Fig. 1A–D). In addition to

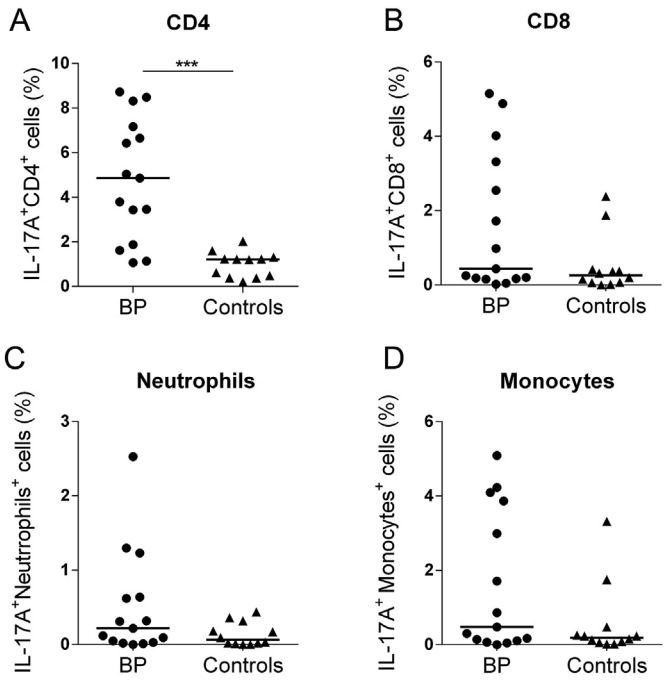

Fig. 1. CD4$^+$ cells are the main producers of IL-17A in the peripheral blood of patients with bullous pemphigoid (BP). A-D, When CD4$^+$ cells, CD8$^+$ cells, neutrophils, and monocytes of the peripheral blood from treatment-naïve BP patients and age-and sex-matched controls were analyzed by flow cytometry for their potential to produce IL-17A, significantly more IL-17A$^+$CD4$^+$ cells were found in BP patients compared to controls (**A**). No difference in the number of IL-17A producing cells between BP patients and controls were seen for CD8$^+$ cells, (**B**), neutrophils (**C**), and monocytes (**D**). ***, p < 0.001.

IL-17A, CD4$^+$ lymphocytes generated significantly higher amounts of IL-22 (p = 0.001; Fig. S1C) but not of IL-21 (p = 0.84; Fig. S1B) and IL-17F (p = 0.42; Fig. S1A).

3.2. mRNA levels of IL-17A and related mediators are upregulated in perilesional skin of BP patients

mRNA levels of IL-17A and selected related cytokines, chemokines, and regulatory factors (n = 32) were analyzed in perilesional skin biopsies from patients with BP and other inflammatory dermatoses, respectively (Table S2). Eighteen genes including *IL*-17A were significantly upregulated while one gene, *CD8*, was significantly less expressed in perilesional skin of BP patients compared to controls (Fig. 2A; Table S1). When the gene regulatory network was visualized IL-17A and related cytokines, e.g., IL-22 and the neutrophil-active chemokine CXCL1 were localized in the center (Fig. 2b).

3.3. CD3-positive T lymphocytes are the main source of IL-17A in perilesional skin of BP patients

Perilesional skin biopsies of 7 BP patients were stained with antibodies against IL-17A and CD3, CD4, CD68, tryptase, and myeloperoxidase (Fig. 3A). 41% of CD3$^+$ cells, 61% of CD4$^+$ cells, 45% of monocytes/macrophages, 45% of mast cells, and 35% of neutrophils showed IL-17A reactivity (Fig. 3B). Since on some cells, IL-17A staining only appeared on the cell surface, only those cells were counted as IL-17A producers that showed intracellular staining for IL-17A as determined by confocal microscopy (Fig. 3A). When the IL-17A producing cells of a certain cell population were analyzed in relation to the total number of IL-17A producing cells, CD3$^+$ cells appeared to be the main source of IL-17A representing 60% of the IL-17A producers followed by

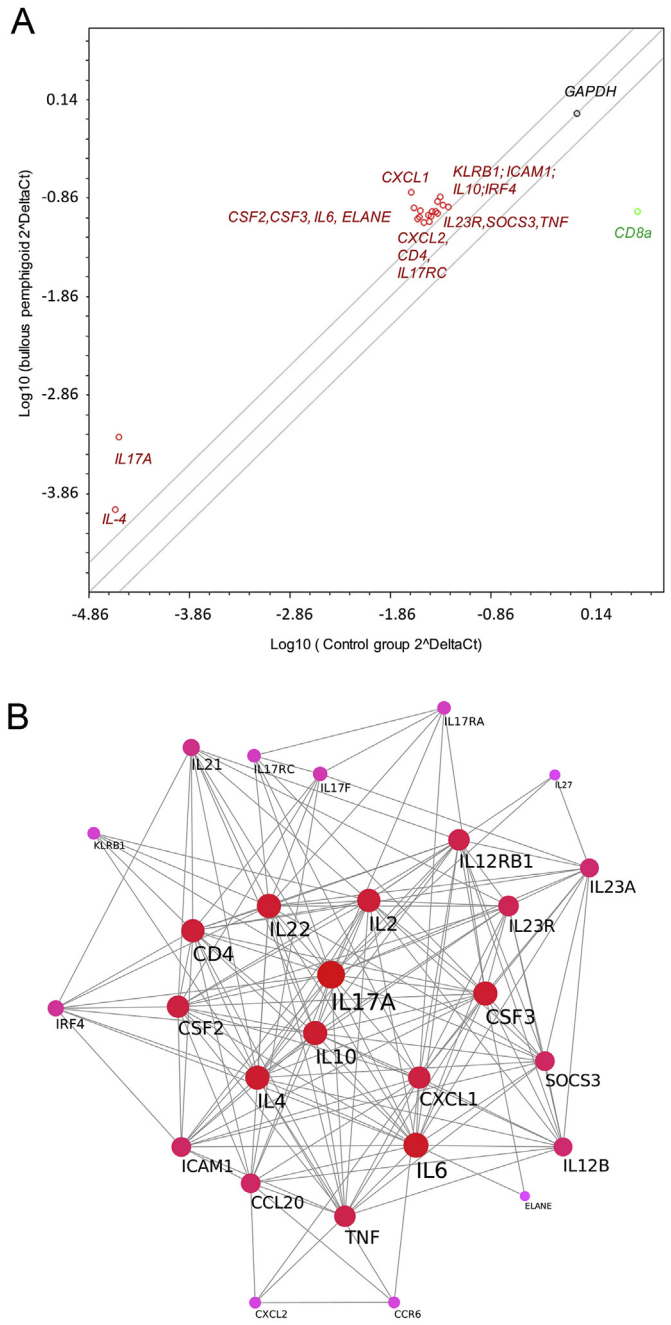

Fig. 2. mRNA levels of IL-17A and related mediators were upregulated in perilesional skin of bullous pemphigoid (BP) patients. A, In perilesional skin of BP patients, 18 of 32 analyzed IL-17-related genes were significantly upregulated (red), while *CD8* was significantly down regulated (green). **B,** A connectivity map of significantly regulated genes shows *IL17*A as a key inflammatory mediator. The complexity of connections of a mediator is indicated by the increasing redness, size and central position of the corresponding circle. The full list of analyzed genes and levels of significance are shown in Tables S1 and S2.

$CD68^+$ monocytes/macrophages (18%) and neutrophils (15%) (Fig. 3C).

3.4. BP is associated with mutations in IL17A and related genes

To evaluate whether dysregulation of the IL-17 pathway in BP might be caused by genetic predisposition, we performed whole exome sequencing of 51 patients. 157 genes of the IL-17 pathway, transcription factors of IL-17, IL-17 inhibitors, and related cytokines were analyzed for germline mutations, single nucleotide variants as well as small insertion and deletions (Table S3). Using this approach in 18 (35%) of the 51 BP patients, mutations in at least one of 12 IL-17-related genes were found (Fig. 4, Table S4). Only non-synonymous mutations having a CADD score > 5 as well as known rare mutations (minor allele frequency [MAF] < 0.01) are reported (Fig. 4, Table S4).

3.5. IL-17A activates human neutrophils in vitro

When neutrophils derived from peripheral blood of healthy volunteers (n = 3) were incubated with recombinant human IL-17A (500 ng/ml for 1 h) expression of CD62L on neutrophils was significantly decreased compared with unstimulated cells indicating a higher activation of IL-17A-treated cells (Figs. S2A and B). As expected, treatment of neutrophils with PMA also led to a significant reduced CD62L expression compared to untreated cells (Figs. S2A and C).

3.6. Anti-Col17 IgG-mediated dermal-epidermal splitting is prevented by inhibition of IL-17A

Incubation of cryosections of normal human skin with anti-Col17 IgG and subsequently with leukocytes from healthy volunteers pre-treated with an isotype control antibody resulted in dermal-epidermal splitting (Fig. 5D). When leukocytes were pre-treated with 100 µg anti-IL-17A antibody dermal-epidermal split formation occurred (Fig. 5A), whereas pre-treatment with 200 µg and 300 µg prevented splitting (Fig. 5 B, C.).

3.7. In the antibody transfer mouse model of bullous pemphigoid, IL-17A serum levels correlate with diseases severity

When sera of wildtype mice injected with anti-murine Col17 IgG (n = 34) were assayed for IL-17A, IL-17A levels significantly correlated with the extent of lesion formation in mice (r = 0.531; p = 0.001; Fig. S3).

3.8. IL-17A$^{-/-}$ mice are protected from the pathogenic effect of anti-Cil17 IgG

When IL-17A$^{-/-}$ (n = 8) and wildtype mice (n = 11) were injected with anti-Col17 IgG, wildtype mice developed significantly more erythema and erosions on the neck and ears compared to IL-17A$^{-/-}$ animals that were largely protected (day 4, p = 0.007; day 8, p = 0.0003; day 12, p= < 0.0001) (Fig. 6 A, B, E, F, I, J). No difference was seen in the deposition of IgG and C3 (data for C3 not shown) at the dermal-epidermal junction by direct IF microscopy of perilesional skin biopsies (Fig. 6C, G). While a dense inflammatory infiltrate was seen in the upper dermis of both IL-17A$^{-/-}$ and wildtype mice, dermal-epidermal splitting was only found in wildtype mice (Fig. 6 D, H).

3.9. Pharmacological inhibition of IL-17A significantly reduced anti-Col17 IgG-induced skin lesions in mice

Wildtype mice were injected with anti-Col17 IgG on days 0, 2, 4, 6, 8, and 10. On days −2, 0, 2, 4, and 6 mice were injected with anti-IL-17A and isotype control antibody, respectively (Fig. 6K, M). After day 8, skin lesions in anti-IL-17A-treated mice (n = 8) were reduced compared to mice that received the isotype control antibody (n = 8; day 14, p = 0.01; Fig. 6K). When the same experiment was repeated with 6 mice/group and a longer observation period, again after day 8 less skin lesions were observed in the anti-IL-17A-injected compared to isotype control antibody-injected mice antibody (day 14, p = 0.04; day 16, p = 0.004, day 18, p = 0.0002; day 8–18, p = 0.02; Fig. 6 M, N).

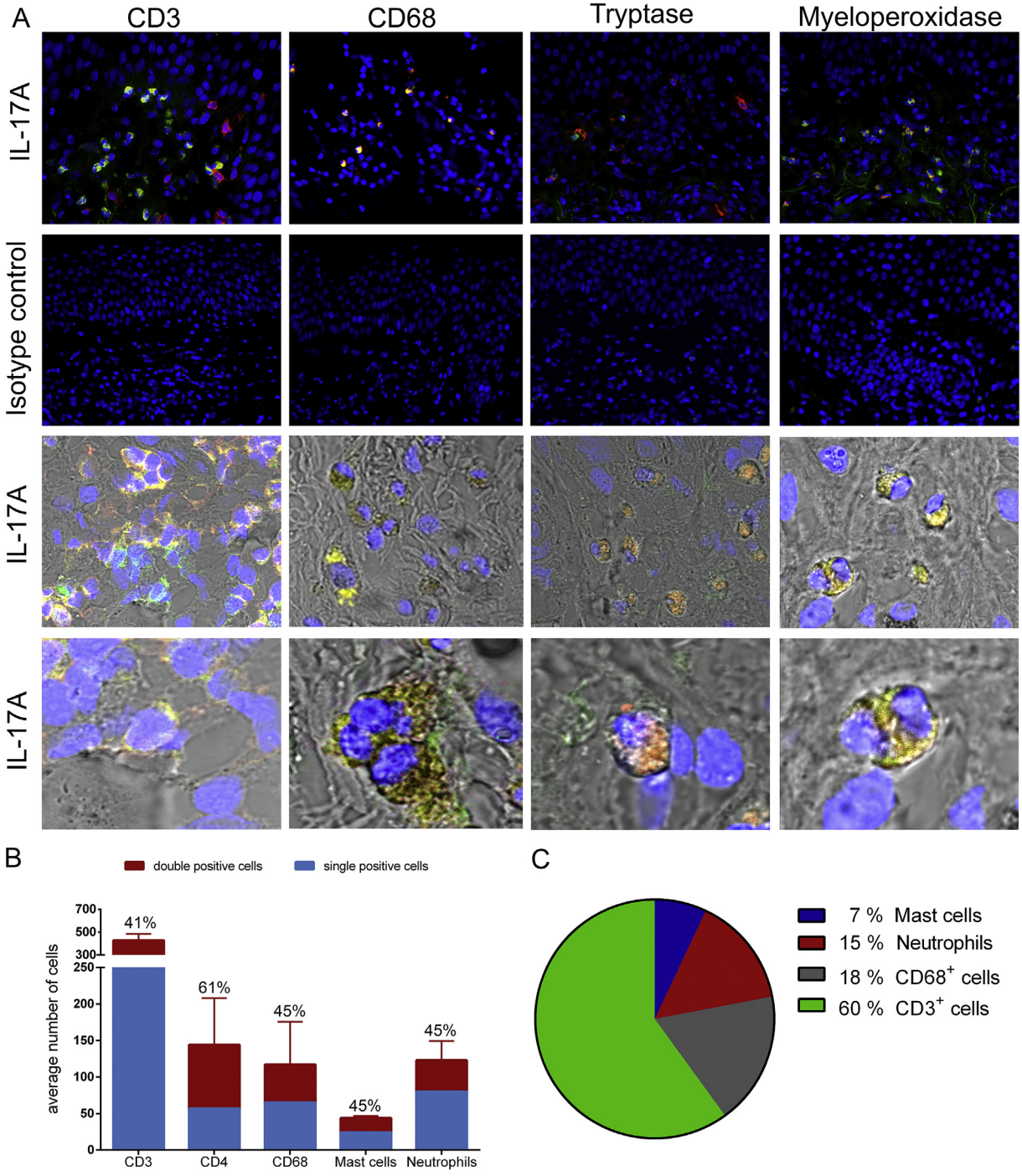

Fig. 3. CD3$^+$ lymphocytes are the main source of IL-17A in perilesional skin of bullous pemphigoid (BP) patients. A, Cryosections of perilesional skin were subjected to double staining with antibodies against IL-17A (green) and CD3, CD4 (not shown), CD68, tryptase, and myeloperoxidase, respectively (red; lanes 1, 3, and 4). Stainings with isotype antibodies was used as controls (lane 2). Confocal microscopic pictures show IL-17A staining both within the cell and on the cell surface of some cells (lanes 3 and 4). Counterstaining of nuclei was done with DAPI (blue). **B,** Statistical analyses of double-stained cells with intracellular fluorescence. Five representative pictures of 2 sections were analyzed. Bars show mean + SD. **C,** Diagram of the percentage of IL-17A-producing cells of a certain cell type in relation to the total number of IL-17A-producing cells.

4. Discussion

BP is the most frequent autoimmune blistering disease in Central Europe and North America with a prevalence of about 21.000 patients in Germany [37–39]. The only demonstrably effective therapies are systemic or superpotent topical corticosteroids that are, however, associated with considerable adverse events [40]. The number of severe adverse events and the increased mortality of BP patients are, at least in part, due to the corticosteroid treatment [1,41]. While recent randomized controlled clinical trials suggested some effect of doxycycline and dapsone [42,43], more specific, effective, and safe treatment options for BP are lacking.

IL-17A is a pleiotropic cytokine with particular significance at epithelial barrier sites including the skin and oral cavity. It has the ability to induce the expression of proinflammatory cytokines and chemokines in various stromal and innate immune cells [44,45]. IL-17A is of

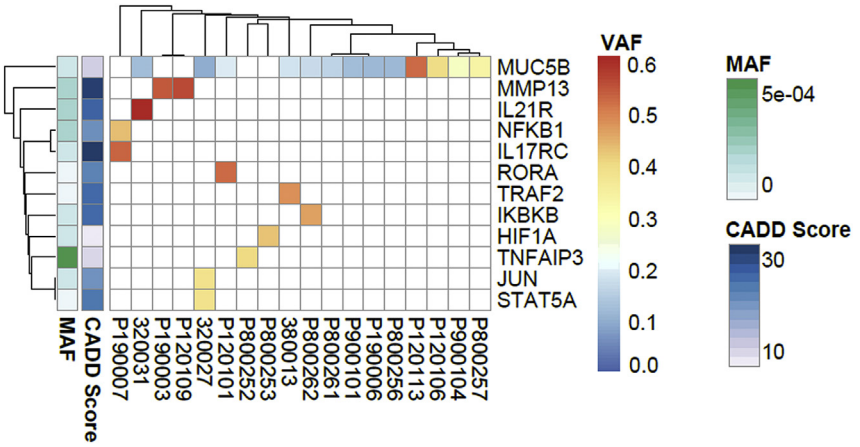

Fig. 4. Affected IL-17-related genes as identified by exome sequencing. The heatmap depicts rare and severe mutations related to IL-17 signaling and regulation found by whole exome sequencing in a cohort of 51 bullous pemphigoid patients. Heatmap colors correspond to the largest variant allele frequency (VAF) of the dominantly occurring mutation within one gene per patient. The two leftmost columns denote the corresponding population frequency (MAF, minor allele frequency) and the severity of the mutation as quantified by the combined annotation dependent depletion (CADD) score. The MAF score has been extracted from the maximum allele frequency of several population frequency databases, including the 1000 Genomes Project, ESP6500, ExAC, CG46. Only mutations with a CADD score of at least 5 were selected. The score quantifies the deleteriousness of variants in the human genome and is based on multiple annotations such as allelic diversity, pathogenicity, or experimentally measured regulatory effects. Rows and columns have been hierarchically clustered by complete linkage. Annotation of all mutations is detailed in Table S4.

particular importance for the initiation of the immune response by mediating the influx of neutrophils and monocytes and keeping these cells at the site of inflammation [45]. In addition to its role in host defense against microorganisms the importance of IL-17A in autoimmunity and chronic inflammatory disorders such as inflammatory bowel disease and psoriasis has previously been highlighted [45–47].

Here, we explored the cellular sources and functional relevance of IL-17A and related inflammatory mediators in the pathophysiology of BP and probed IL-17A inhibition in the treatment of this disease. In the peripheral blood of BP patients, $CD4^+$ lymphocytes, but not $CD8^+$ cells, neutrophils, and monocytes generated IL-17A. Peripheral $CD4^+$ cells from BP patients also produced elevated levels of IL-22. These data indicate that inflammation in BP is not confined to the skin but also involves circulating Th17 cells. To address the IL-17 network in the skin, mRNA levels of IL-17A and related inflammatory mediators in the perilesional skin of BP patients were compared to levels in patients with other inflammatory dermatoses. Upregulation of *IL17A, IL17RC, CCR6*, and *IL23R* point to an activated IL-17 network at the site of blister formation. IL-17A is a strong inducer of *CXCL1* and *CXCL2* expression [44] and the finding of upregulated *CXCL1* and *CXCL2,* encoding powerful chemoattractants for neutrophils, and upregulated *ELANE*, encoding for neutrophil elastase, validate previous data about the importance of neutrophils and neutrophil elastase in the pathogenesis of BP [13,48–50].

The central role of IL-17A, IL-22, and CXCL1 in the dermal immune network of BP was also highlighted by the gene regulatory network of significantly regulated genes in BP patient skin. Of interest, although not in the focus of this study, increased mRNA levels of anti-inflammatory mediators, such as IL-10 were found in BP skin indicating that mechanisms of resolution of inflammation maybe in place.

To identify the major producer of IL-17A in early skin lesions, the most abundant cell populations that are known to express IL-17A, i.e. neutrophils, macrophages, $CD3^+$ lymphocytes, and mast cells were analyzed for IL-17A expression. By confocal microscopy, we excluded cells that showed IL-17A staining on the cell surface [51] assuming that in these cells, IL-17A was bound to the ubiquitously expressed IL-17RA/IL-17RC e.g. on different T cell subsets and neutrophils [52,53]. By this method, we found $CD3^+$ lymphocytes to be the major source of IL-17A in BP skin accounting for 60% of the IL-17A-producers. Since here, early BP lesions without split formation were analyzed and cells bearing IL-17A on their surface were not counted our data may reconcile contradictory findings of previous studies that described lymphocytes [54], Th17 cells [55,56], and neutrophils [48] as major source of IL-17 in BP skin.

Since BP is a relatively rare disease with an incidence of about 14–42 new patients/million/year in Central Europe and the UK [1], we decided to perform exome sequencing of 51 BP patients to detect rare mutations associated with BP. One third of the patients showed rare, but mostly severe mutations in the coding regions of 12 IL-17-related genes. While none of the mutations was homozygous, they could have a modulatory effect on the IL-17 pathway activity. The most prevalent genes were related to NFκB signaling, such as IKBKB, NFKB1 and MUC5B. Incorrect activation of NFκB is associated with inflammatory diseases and constant inhibition of NFκB leads to incorrect immune cell development or delayed cell growth. MUC5B was most commonly mutated in our patients. It has been shown to play a role in the airway defense and its deficiency leads to chronic infection [57]. Further studies will need to validate these findings in other BP patient cohorts and analyze whether mutations in IL-17-related genes are associated with certain clinical or immunopathological characteristics.

We then explored the functional relevance of IL-17A for BP in five different approaches. We showed that (i) IL-17A activated normal human neutrophils in vitro, (ii) the dermal-epidermal splitting induced by anti-Col17 IgG was abrogated by incubation of leukocytes with anti-IL17A antibody, and (iii) serum levels of IL-17A closely correlate with the extent of skin lesion in experimental BP in adult mice. Our findings are in line with previous reports on the increased secretion of MMP-9 from neutrophils by IL-17A and CXCL10 [48,58]. MMP-9-deficient mice

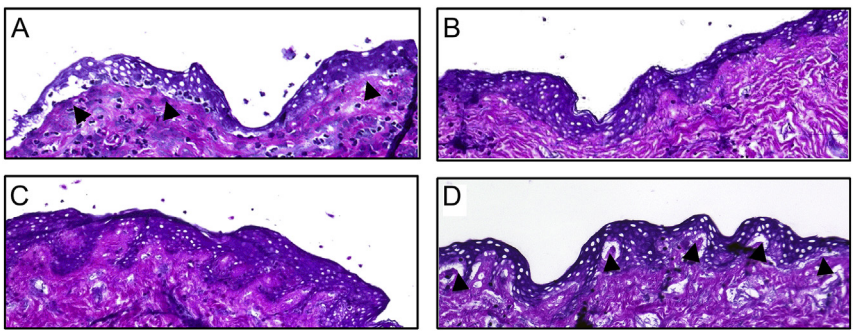

Fig. 5. IL-17A has functional relevance in experimental models of bullous pemphigoid (BP). A-D, Cryosection assay. **D,** Treatment of cryosections of human skin with BP serum and subsequently with leukocytes from healthy volunteers plus an isotype control (300 μg) resulted in dermal-epidermal separation (black triangles). **A-C,** Co-incubation of leukocytes with 200 μg (**B**) and 300 μg (**C**), but not with 100 μg anti-IL-17A IgG (**A**) prevented splitting. Representative pictures of 2 independent experiments with 3 blood donors are shown.

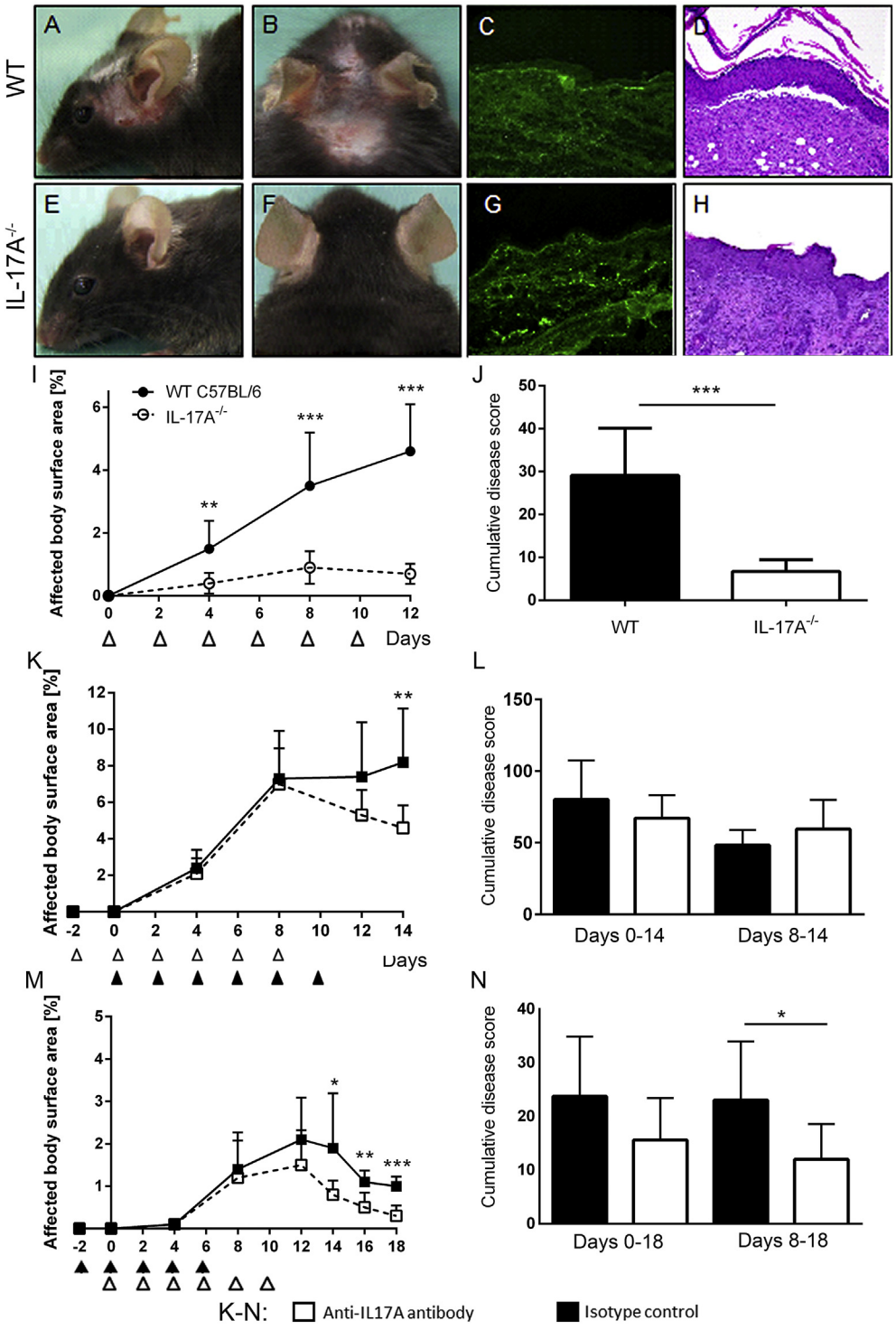

Fig. 6. In experimental murine bullous pemphigoid, IL17A$^{-/-}$ mice are protected and pharmacological inhibition of IL-17A significantly reduces skin lesions. **A-J,** When IL-17A$^{-/-}$ (**A-D**) and wildtype mice (**E-H**) were injected with anti-Col17 IgG (white triangles), wildtype animals developed erythema, erosions, and crusts on neck and ears (**A, B**), while IL-17A$^{-/-}$ mice hardly showed any skin lesions (**E, F**). Disease activity, as quantified by the affected body surface area, was significantly lower on day 4, 8, and 10 as well as during the entire experimental period in IL-17A$^{-/-}$ mice compared to wildtype animals (**I, J**). In both strains, no difference in IgG deposits at the dermal-epidermal junction was observed (**C, G**). By lesional histopathology a dense inflammatory infiltrate was seen in the upper dermis in both strains, while subepidermal splitting was only present in wildtype animals (**E, H**). **K-N,** when wildtype mice injected with anti-Col17 IgG (white triangles) were treated with anti-IL-17A and isotype control antibody, respectively (black triangles), anti-IL-17A-treated mice showed significantly less skin lesions compared to control antibody-treated mice. Two independent experiments are shown in **K** and **L** as well as in **M** and **N**. *p < 0.05, **p < 0.01, ***p < 0.001.

were resistant to anti-Col17 IgG-induced skin lesions and *CXCL10* expression in macrophages and keratinocytes was shown to be induced by IL-17A [58–60].

In additional two approaches (iv), and (v), further evidence for the importance of IL-17A in the pathophysiology of BP was generated. IL-17A$^{-/-}$ mice were nearly completely protected against the otherwise pathogenic effect of anti-Col17 IgG and anti-Col17 IgG-injected mice showed significantly reduced skin lesions when treated with an anti-IL-17A antibody compared to animals that received the isotype control antibody. Latter finding suggested pharmacological inhibition of IL-17A being a rational treatment approach in BP. Further studies in experimental murine BP will aim at identifying the main target cell of IL-17A in skin lesions to further explore how IL-17A exerts its effect in BP.

The presented data indicate that IL-17A has a functional role in BP and suggest anti-IL-17A therapy being a rational and promising therapeutic approach in patients with severe BP.

Conflicts of interest

D.T. has received honoraria or fees for serving on advisory boards, as a speaker, as a consultant for Lilly, Leo-Pharma, Novartis and UCB,

and grants from Novartis. D.Z. received honoraria from UCB and travel fees from Novartis and UCB. R.J.L. and E.S. received honoraria and travel fees from Novartis as well as honoraria from UCB.

Acknowledgement

We are grateful to Vanessa Krull and Ingrid Dahlke, Lübeck, for excellent technical support. We thank Franziska Schulze, Lübeck, for help with the animal experiments and supervision of L.C. and Yask Gupta, Lübeck, for generating the gene regulatory network. We also thank Yoichiro Iwakura for providing breeding pairs of IL-17A$^{-/-}$ mice and greatly appreciate the effort of Sarah Vieten, Gerhard Schultheiß, animal care takers at the Christian-Albrechts-Universität zu Kiel. The study was supported by Deutsche Forschungsgemeinschaft through the Research Training Group 1727 Modulation of Autoimmunity (to C.M.M.H., C.H., and E.S.), the Clinical Research Unit 303 *Pemphigoid Diseases* (to S.M.I., C.D.S., D.Z. and E.S), and structural funding through the Excellence Cluster Inflammation at Interfaces (to D.T., S.I., D.Z., R.L., and H.B.). The in vitro studies were funded in part by a research grant of Novartis (to E.S).

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.jaut.2018.09.003.

References

[1] E. Schmidt, D. Zillikens, Pemphigoid diseases, Lancet 381 (2013) 320–332.
[2] J.A. Fairley, C.T. Burnett, C.L. Fu, D.L. Larson, M.G. Fleming, G.J. Giudice, A pathogenic role for IgE in autoimmunity: bullous pemphigoid IgE reproduces the early phase of lesion development in human skin grafted to nu/nu mice, J. Invest. Dermatol. 127 (2007) 2605–2611.
[3] Z. Liu, L.A. Diaz, J.L. Troy, A.F. Taylor, D.J. Emery, J.A. Fairley, et al., A passive transfer model of the organ-specific autoimmune disease, bullous pemphigoid, using antibodies generated against the hemidesmosomal antigen, BP180, J. Clin. Invest. 92 (1993) 2480–2488.
[4] W. Nishie, D. Sawamura, M. Goto, K. Ito, A. Shibaki, J.R. McMillan, et al., Humanization of autoantigen, Nat. Med. 13 (2007) 378–383.
[5] F.S. Schulze, T. Beckmann, F. Nimmerjahn, A. Ishiko, M. Collin, J. Kohl, et al., Fcgamma receptors III and IV mediate tissue destruction in a novel adult mouse model of bullous pemphigoid, Am. J. Pathol. 184 (2014) 2185–2196.
[6] M. Hirose, A. Recke, T. Beckmann, A. Shimizu, A. Ishiko, K. Bieber, et al., Repetitive immunization breaks tolerance to type XVII collagen and leads to bullous pemphigoid in mice, J. Immunol. 187 (2011) 1176–1183.
[7] M.A. Sanjuan, D. Sagar, R. Kolbeck, Role of IgE in autoimmunity, J. Allergy Clin. Immunol. 137 (2016) 1651–1661.
[8] J.A. Fairley, C.L. Baum, D.S. Brandt, K.A. Messingham, Pathogenicity of IgE in autoimmunity: successful treatment of bullous pemphigoid with omalizumab, J. Allergy Clin. Immunol. 123 (2009) 704–705.
[9] F. Fichel, C. Barbe, P. Joly, C. Bedane, P. Vabres, F. Truchetet, et al., Clinical and immunologic factors associated with bullous pemphigoid relapse during the first year of treatment: a multicenter, prospective study, JAMA Dermatol. 150 (2014) 25–33.
[10] E. Schmidt, K. Obe, E.B. Brocker, D. Zillikens, Serum levels of autoantibodies to BP180 correlate with disease activity in patients with bullous pemphigoid, Arch. Dermatol. 136 (2000) 174–178.
[11] N. van Beek, N. Luttmann, F. Huebner, A. Recke, I. Karl, F.S. Schulze, et al., Correlation of serum levels of IgE autoantibodies against BP180 with bullous pemphigoid disease activity, JAMA Dermatol. 153 (2017) 30–38.
[12] Z. Liu, G.J. Giudice, S.J. Swartz, J.A. Fairley, G.O. Till, J.L. Troy, et al., The role of complement in experimental bullous pemphigoid, J. Clin. Invest. 95 (1995) 1539–1544.
[13] Z. Liu, S.D. Shapiro, X. Zhou, S.S. Twining, R.M. Senior, G.J. Giudice, et al., A critical role for neutrophil elastase in experimental bullous pemphigoid, J. Clin. Invest. 105 (2000) 113–123.
[14] Z. Liu, J.M. Shipley, T.H. Vu, X. Zhou, L.A. Diaz, Z. Werb, et al., Gelatinase B-deficient mice are resistant to experimental bullous pemphigoid, J. Exp. Med. 188 (1998) 475–482.
[15] K. Natsuga, W. Nishie, S. Shinkuma, H. Ujiie, M. Nishimura, D. Sawamura, et al., Antibodies to pathogenic epitopes on type XVII collagen cause skin fragility in a complement-dependent and -independent manner, J. Immunol. 188 (2012) 5792–5799.
[16] R.G. Langley, B.E. Elewski, M. Lebwohl, K. Reich, C.E. Griffiths, K. Papp, et al., Secukinumab in plaque psoriasis–results of two phase 3 trials, N. Engl. J. Med. 371 (2014) 326–338.
[17] K.A. Papp, A. Blauvelt, M. Bukhalo, M. Gooderham, J.G. Krueger, J.P. Lacour, et al., Risankizumab versus Ustekinumab for moderate-to-severe plaque psoriasis, N. Engl. J. Med. 376 (2017) 1551–1560.
[18] D. Thaci, E.L. Simpson, L.A. Beck, T. Bieber, A. Blauvelt, K. Papp, et al., Efficacy and safety of dupilumab in adults with moderate-to-severe atopic dermatitis inadequately controlled by topical treatments: a randomised, placebo-controlled, dose-ranging phase 2b trial, Lancet 387 (2016) 40–52.
[19] I.B. McInnes, G. Schett, Pathogenetic insights from the treatment of rheumatoid arthritis, Lancet 389 (2017) 2328–2337.
[20] J.E. Hawkes, T.C. Chan, J.G. Krueger, Psoriasis pathogenesis and the development of novel targeted immune therapies, J. Allergy Clin. Immunol. 140 (2017) 645–653.
[21] P.M. Brunner, E. Guttman-Yassky, D.Y. Leung, The immunology of atopic dermatitis and its reversibility with broad-spectrum and targeted therapies, J. Allergy Clin. Immunol. 139 (2017) S65–S76.
[22] U.K. Samavedam, K. Kalies, J. Scheller, H. Sadeghi, Y. Gupta, M.F. Jonkman, et al., Recombinant IL-6 treatment protects mice from organ specific autoimmune disease by IL-6 classical signalling-dependent IL-1ra induction, J. Autoimmun. 40 (2012) 74–85.
[23] H. Sadeghi, A. Lockmann, A.C. Hund, U.K. Samavedam, E. Pipi, K. Vafia, et al., Caspase-1-Independent IL-1 release mediates blister formation in autoantibody-induced tissue injury through modulation of endothelial adhesion molecules, J. Immunol. 194 (2015) 3656–3663.
[24] M. Hirose, A. Kasprick, F. Beltsiou, K. Dieckhoff Schulze, F.S. Schulze, U.K. Samavedam, et al., Reduced skin blistering in experimental epidermolysis bullosa acquisita after anti-TNF treatment, Mol. Med. 23 (2016).
[25] A.M. Bolger, M. Lohse, B. Usadel, Trimmomatic: a flexible trimmer for Illumina sequence data, Bioinformatics 30 (2014) 2114–2120.
[26] H. Li, R. Durbin, Fast and accurate long-read alignment with Burrows-Wheeler transform, Bioinformatics 26 (2010) 589–595.
[27] A. McKenna, M. Hanna, E. Banks, A. Sivachenko, K. Cibulskis, A. Kernytsky, et al., The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data, Genome Res. 20 (2010) 1297–1303.
[28] K. Wang, M. Li, H. Hakonarson, ANNOVAR: functional annotation of genetic variants from high-throughput sequencing data, Nucleic Acids Res. 38 (2010) e164.
[29] M. Kircher, D.M. Witten, P. Jain, B.J. O'Roak, G.M. Cooper, J. Shendure, A general framework for estimating the relative pathogenicity of human genetic variants, Nat. Genet. 46 (2014) 310–315.
[30] A. Recke, G. Vidarsson, R.J. Ludwig, M. Freitag, S. Moller, R. Vonthein, et al., Allelic and copy-number variations of FcgammaRs affect granulocyte function and susceptibility for autoimmune blistering diseases, J. Autoimmun. 61 (2015) 36–44.
[31] C. Sitaru, E. Schmidt, S. Petermann, L.S. Munteanu, E.B. Brocker, D. Zillikens, Autoantibodies to bullous pemphigoid antigen 180 induce dermal-epidermal separation in cryosections of human skin, J. Invest. Dermatol. 118 (2002) 664–671.
[32] E.N. Heppe, S. Tofern, F.S. Schulze, A. Ishiko, A. Shimizu, C. Sina, et al., Experimental laminin 332 mucous membrane pemphigoid critically involves C5aR1 and reflects clinical and immunopathological characteristics of the human disease, J. Invest. Dermatol. 137 (2017) 1709–1718.
[33] K. Vafia, S. Groth, T. Beckmann, M. Hirose, J. Dworschak, A. Recke, et al., Pathogenicity of autoantibodies in anti-p200 pemphigoid, PLoS One 7 (2012) e41769.
[34] J. Xia, E.E. Gill, R.E.W. Hancock, NetworkAnalyst for statistical, visual and network-based meta-analysis of gene expression data, Nat. Protoc. 10 (2015) 823–844.
[35] D. Szklarczyk, A. Franceschini, S. Wyder, K. Forslund, D. Heller, J. Huerta-Cepas, et al., STRING v10: protein–protein interaction networks, integrated over the tree of life, Nucleic Acids Res. 43 (2015) D447–D452.
[36] P. Shannon, A. Markiel, O. Ozier, N.S. Baliga, J.T. Wang, D. Ramage, et al., Cytoscape: a software environment for integrated models of biomolecular interaction networks, Genome Res. 13 (2003) 2498–2504.
[37] F. Hubner, A. Recke, D. Zillikens, R. Linder, E. Schmidt, Prevalence and age distribution of pemphigus and pemphigoid diseases in Germany, J. Invest. Dermatol. 136 (2016) 2495–2498.
[38] P. Joly, S. Baricault, A. Sparsa, P. Bernard, C. Bedane, S. Duvert-Lehembre, et al., Incidence and mortality of bullous pemphigoid in France, J. Invest. Dermatol. 132 (2012) 1998–2004.
[39] G. Marazza, H.C. Pham, L. Scharer, P.P. Pedrazzetti, T. Hunziker, R.M. Trueb, et al., Incidence of bullous pemphigoid and pemphigus in Switzerland: a 2-year prospective study, Br. J. Dermatol. 161 (2009) 861–868.
[40] P. Joly, J.C. Roujeau, J. Benichou, C. Picard, B. Dreno, E. Delaporte, et al., A comparison of oral and topical corticosteroids in patients with bullous pemphigoid, N. Engl. J. Med. 346 (2002) 321–327.
[41] B. Rzany, K. Partscht, M. Jung, W. Kippes, D. Mecking, B. Baima, et al., Risk factors for lethal outcome in patients with bullous pemphigoid: low serum albumin level, high dosage of glucocorticosteroids, and old age, Arch. Dermatol. 138 (2002) 903–908.
[42] H.C. Williams, F. Wojnarowska, G. Kirtschig, J. Mason, T.R. Godec, E. Schmidt, et al., Doxycycline versus prednisolone as an initial treatment strategy for bullous pemphigoid: a pragmatic, non-inferiority, randomised controlled trial, Lancet 389 (2017) 1630–1638.
[43] M. Sticherling, A. Franke, E. Aberer, R. Glaser, M. Hertl, C. Pfeiffer, et al., An open, multicentre, randomized clinical study in patients with bullous pemphigoid comparing methylprednisolone and azathioprine with methylprednisolone and dapsone, Br. J. Dermatol. 177 (2017) 1299–1305.
[44] T. Kuwabara, F. Ishikawa, M. Kondo, T. Kakiuchi, The role of IL-17 and related cytokines in inflammatory autoimmune diseases, Mediat. Inflamm. 2017 (2017) 3908061.
[45] M. Veldhoen, Interleukin 17 is a chief orchestrator of immunity, Nat. Immunol. 18 (2017) 612–621.

[46] W.H. Boehncke, M.P. Schon, Psoriasis. Lancet 386 (2015) 983–994.
[47] M. Kleinewietfeld, A. Manzel, J. Titze, H. Kvakan, N. Yosef, R.A. Linker, et al., Sodium chloride drives autoimmune disease by the induction of pathogenic TH17 cells, Nature 496 (2013) 518–522.
[48] S. Le Jan, J. Plee, D. Vallerand, A. Dupont, E. Delanez, A. Durlach, et al., Innate immune cell-produced IL-17 sustains inflammation in bullous pemphigoid, J. Invest. Dermatol. 134 (2014) 2908–2917.
[49] Z. Liu, X. Zhou, S.D. Shapiro, J.M. Shipley, S.S. Twining, L.A. Diaz, et al., The serpin alpha1-proteinase inhibitor is a critical substrate for gelatinase B/MMP-9 in vivo, Cell 102 (2000) 647–655.
[50] I. Shimanovich, S. Mihai, G.J. Oostingh, T.T. Ilenchuk, E.B. Brocker, G. Opdenakker, et al., Granulocyte-derived elastase and gelatinase B are required for dermal-epidermal separation induced by autoantibodies from patients with epidermolysis bullosa acquisita and bullous pemphigoid, J. Pathol. 204 (2004) 519–527.
[51] L. Monin, S.L. Gaffen, Interleukin 17 family cytokines: signaling mechanisms, biological activities, and therapeutic implications, Cold Spring Harbor Perspectives Biol. (2017).
[52] S.L. Gaffen, Structure and signalling in the IL-17 receptor family, Nat. Rev. Immunol. 9 (2009) 556–567.
[53] R. Lombard, E. Doz, F. Carreras, M. Epardaud, Y. Le Vern, D. Buzoni-Gatel, et al., IL-17RA in Non-Hematopoietic Cells Controls CXCL-1 and 5 Critical to Recruit Neutrophils to the Lung of Mycobacteria-Infected Mice during the Adaptive Immune Response, PLoS One 11 (2016) e0149455.
[54] A. Zebrowska, M. Wagrowska-Danilewicz, M. Danilewicz, O. Stasikowska-Kanicka, A. Cynkier, A. Sysa-Jedrzejowska, et al., IL-17 expression in dermatitis herpetiformis and bullous pemphigoid, Mediat. Inflamm. 2013 (2013) 967987.
[55] M. Arakawa, T. Dainichi, N. Ishii, T. Hamada, T. Karashima, T. Nakama, et al., Lesional Th17 cells and regulatory T cells in bullous pemphigoid, Exp. Dermatol. 20 (2011) 1022–1024.
[56] M. Fischer-Stabauer, A. Boehner, S. Eyerich, T. Carbone, C. Traidl-Hoffmann, C.B. Schmidt-Weber, et al., Differential in situ expression of IL-17 in skin diseases, Eur. J. Dermatol. 22 (2012) 781–784.
[57] M.G. Roy, A. Livraghi-Butrico, A.A. Fletcher, M.M. McElwee, S.E. Evans, R.M. Boerner, et al., Muc5b is required for airway defence, Nature 505 (2014) 412–416.
[58] M. Riani, S. Le Jan, J. Plee, A. Durlach, R. Le Naour, G. Haegeman, et al., Bullous pemphigoid outcome is associated with CXCL10-induced matrix metalloproteinase 9 secretion from monocytes and neutrophils but not lymphocytes, J. Allergy Clin. Immunol. 139 (2017) 863–872 e3.
[59] Z. Liu, N. Li, L.A. Diaz, M. Shipley, R.M. Senior, Z. Werb, Synergy between a plasminogen cascade and MMP-9 in autoimmune disease, J. Clin. Invest. 115 (2005) 879–887.
[60] C. Koga, K. Kabashima, N. Shiraishi, M. Kobayashi, Y. Tokura, Possible pathogenic role of Th17 cells for atopic dermatitis, J. Invest. Dermatol. 128 (2008) 2625–2630.