Reduced Skin Blistering in Experimental Epidermolysis Bullosa Acquisita After Anti-TNF Treatment

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Epidermolysis bullosa acquisita (EBA) is a difficult-to-treat subepidermal autoimmune blistering skin disease (AIBD) with circulating and tissue-bound anti-type VII collagen antibodies. Different reports have indicated increased concentration of tumor necrosis factor α (TNF) in the serum and blister fluid of patients with subepidermal AIBD. Furthermore, successful anti-TNF treatment has been reported for individual patients with AIBD. Here we show that in mice, induction of experimental EBA by repeated injections of rabbit anti-mouse type VII collagen antibodies led to increased expression of TNF in skin, as determined by real-time polymerase chain reaction (PCR) and immunohistochemistry. To investigate whether the increased TNF expression is of functional relevance in experimental EBA, we inhibited TNF function using the soluble TNF receptor fusion protein etanercept (Enbrel) or a monoclonal antibody to murine TNF. Interestingly, mice that received either of these treatments showed significantly milder disease progression than controls. In addition, immunohistochemical staining demonstrated reduced numbers of macrophages in lesional skin in mice treated with TNF inhibitors compared with controls. Furthermore, etanercept treatment significantly reduced disease progression in immunization-induced EBA. In conclusion, increased expression of TNF in experimental EBA is of functional relevance, as both the prophylactic blockade of TNF and the therapeutic use of etanercept impaired induction and progression of experimental EBA. Thus, TNF is likely to serve as a new therapeutic target for EBA and AIBDs with a similar pathogenesis.

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and confirmed the concept of a common pathophysiology among immune-mediated inflammatory diseases, with TNF having a predominant role. Five TNF inhibitors have currently been approved for treatment of 1 or more immune-mediated diseases: adalimumab, etanercept (ETA), golimumab, infliximab and certolizumab pegol (6). Because of the role of TNF blockade in many inflammatory diseases, we evaluated its contribution to another immune-mediated inflammatory disease, epidermolysis bullosa acquisita (EBA), a prototypic antibody-dependent and organ-specific autoimmune blistering skin disease (AIBD) (14–17).

The pathogenic relevance of autoantibodies in EBA has been clearly demonstrated (18,19). These autoantibodies are directed against type VII collagen (COL7), a major component of anchoring fibrils. After binding to its target in the skin, a proinflammatory milieu is generated. In different experimental models of EBA, it has been demonstrated that proinflammatory cytokines such as IL-1, granulocyte macrophage colony-stimulating factor (GM-CSF) and antiinflammatory cytokines such as IL-6 are involved in the pathogenesis of EBA (20,21). Together with complement activation (22), this milieu leads to neutrophil extravasation and activation (23) in an FcγR-dependent manner (24). Reactive oxygen species and proteolytic enzymes are released by neutrophils, resulting in subepidermal blister formation (23). Increased expression of TNF in skin and/or serum of AIBD patients has long been noted (25). Individual case reports show clinical remission of pemphigoid disease after second-line therapy with etanercept (26,27) and effects in patients with pemphigus vulgaris (PV) (28–31). Functional data demonstrated that after transfer of pemphigus patient serum into neonatal mice, TNFR1-/TNFR2-deficient mice developed fewer blisters than wild-type mice (32). In contrast, no data concerning the role of TNF in the pathogenesis of EBA have been published so far. Therefore, we analyzed the effects of TNF blockade on the effector phase of this disease.

**MATERIALS AND METHODS**

**Experiments with Human Biomaterial**

For determination of TNF expression and cryosections assays, normal human skin as well as lesional and nonlesional skin from EBA and bullous pemphigoid (BP) patients was obtained. Diagnosis was established based on clinical presentation, detection of IgG and/or C3 deposits in direct IF microscopy of perilesional skin biopsies, detection of the corresponding circulating autoantibodies or detection of a u-serrated pattern in direct IF microscopy in the case of EBA patients. All experiments using human samples were approved by the local ethics committee (University of Lübeck, Germany, and University of Groningen, the Netherlands) and were performed according to the Declaration of Helsinki. Blood donors and patients provided written informed consent prior to study participation.

**Animal Experiments**

C57BL/6j mice (Charles River) were used for the antibody transfer-induced EBA model with prophylactic application of ETA and monoclonal antibody. B6.SJL-H2s C3c/1CyJ (B6.S) animals were provided by Jackson Laboratories and bred at the University of Lübeck, Germany. Mice were used for experimental EBA models at the age of 8 to 10 wks. Animals were examined once per week, and body surface areas affected by erythema, blisters, erosions, crust or alopecia were observed. Relative clinical scores were calculated by normalizing the weekly score to the initial clinical score when allocated into treatment groups (weekly clinical score/initial clinical score). Disease severity was defined as the integrated relative clinical score over time (area under the curve).

Local antibody transfer–induced BP: Rabbits were immunized with a fragment of murine type XVII collagen and IgG from immune serum was isolated as previously described (36). Mice were injected once with 1 mg rabbit anti-mCOL7C IgG per mouse into the ear base. ETA (Enbrel) was administered i.p. (2.5 mg/kg body weight) 2 d prior to the initial anti-mCOL7C IgG injection and then every day (4× total). Ear thickness and scoring were performed at d 2 after IgG injection.

**Histopathology and Direct Immunofluorescence Staining**

Biopsies of lesional and perilesional skin were obtained at d 12 of antibody transfer–induced EBA and prepared for
examination by histopathology and IF microscopy, as described previously (33). In brief, the biopsies collected from mice were fixed in 4% phosphate-buffered saline (PBS) and buffered formalin, and subsequently sections from paraffin-embedded tissues were stained with hematoxylin and eosin. IgG and C3 deposits were detected by direct IF microscopy on frozen sections prepared from tissue biopsies using fluorescein isothiocyanate-labeled antibodies specific to rabbit IgG (Dako) and murine C3 (MP Biomedicals).

**Immunohistochemistry and Immunofluorescence**

Immunohistochemical analysis was performed as previously described, with minor modifications (21). Monoclonal antibodies specific for murine TNF (clone MP6-XT22) were purchased from AbD Serotec, anti-human TNF antibody (clone 52B83) was purchased from Acris Antibodies. Serial cryosections (6 μm) of organ tissue samples were fixed in absolute acetone for 10 min at 4°C and air-dried before blocking of nonspecific immunoreactive sites with 3% bovine serum albumin or 5% normal goat serum (Sigma) in PBS for 20 min at room temperature. The sections were subsequently incubated with primary antibodies at the appropriate dilution at 4°C overnight. Staining was visualized by addition of secondary antibodies (peroxidase-conjugated goat anti-rat IgG, Dianova) and Histogreen Chromogen (Linaris), and the reaction was terminated by washing in tap water. Samples were counterstained with Meyer’s hematoxylin and mounted with VectaMount (Vector Laboratories). Alternatively, fluorescent-labeled secondary antibodies (Cy3-conjugated goat anti-mouse IgG [H + L] polyclonal antibody, 1/100 in PBS-T, Jackson Laboratories) were used and the sections were embedded using DAPI and Mowiol 4-88 (Sigma).

**Real-time PCR**

Real-time PCR of skin sections was performed as previously described (37). RNA from lesional (n = 4) and nonlesional (n = 3) skin from corresponding anatomical sites was obtained using the innuPREP RNA Mini Kit (Analytik Jena). After reverse transcription, cDNA was added to the qPCR MasterMix Plus (Eurogentec) and amplified using an SDS ABI 7000 or SDS ABI 7900 system (Applied Biosystems). TaqMan probes and forward and reverse primers were designed with CloneManager (SciEd); MLN-51 was used as a housekeeping gene. The primers used were mMLN-51for (5′ CCA AGC CAG CCT TCA TTC TG), mMLN-51rev (5′ TAA CCG TTA GCT CGA CCA CTC TG), mTNFfor (5′ CCC TCA CAC TCA GAT CAT CTT CTC) and mTNFrev (5′ TGG CTC AGC AGC TCC AG).

**Bio-Plex**

On d 12 of antibody transfer–induced EBA, serum was obtained from the mice and analyzed for concentrations of cytokines (TNF, IL-6, G-CSF and keratinocyte chemoattractant [KC]). This was performed by a commercial supplier using the Bio-Plex system (Bioglobe GmbH).

**Reactive Oxygen Species Release Assay**

A Lumitrack high-binding 96-well plate (Thermo Fisher Scientific) was coated with immune complexes (ICs) consisting of human Col7E-F antigen at a final concentration of 2.5 μg/mL and anti-human Col7-IgG1 or anti-human Col7-IgA2 antibody at a final concentration of 1.8 μg/mL as described previously (38); 2 × 10⁴ cells were added per well. As controls, antigens or antibodies alone were added to the wells. Just before measurement, luminol (Sigma) was added to the wells and chemiluminescence resulting from reactive oxygen species (ROS) production was measured immediately in a luminescence reader (Wallac 1420 Manager, Victor3). The ROS release was measured for 1 s per well 66× for a period of ~3 h at a constant temperature of 37°C (38).

**Human Monocytes and Polymorphonuclear Purification**

Human polymorphonuclear (PMN) leukocytes and peripheral blood mononuclear cells (PBMCs) were isolated from whole blood samples using the PolymorphPrep (Progen) gradient according to the manufacturer’s instructions. In the next step, human monocytes were purified from the isolated PBMCs by the magnetic cell separation method using a Monocyte Isolation Kit II, human (Miltenyi), according to the manufacturer’s instructions. The purity of monocytes and neutrophils was evaluated by fluorescent staining with PE/Cy7 anti-human CD14 antibody (clone HCD14, Biolegend) and fluorescein isothiocyanate anti-human CD16 antibody (BD Biosciences) in a flow cytometer (MACSQuant Analyzer 10, Miltenyi).

**Human Cryosection Assay**

Human breast skin biopsies from healthy donors were cut into 6 μm sections. The slides were incubated in a humidified air incubator containing 5% CO₂ (Membert) for 1 h at 37°C with 200 μg/mL anti-human Col7-IgA2 antibody or 1 × PBS for the negative controls. Cryosection assay was performed as previously described (38). In short, a chamber of approximately 0.3 mm thickness and 0.25 mL volume was created by covering a slide containing the tissue section with a second slide carrying transparent adhesive tape around its ends and taping them together with Parafilm (Pechiney). Next, 5 × 10⁶ monocytes or PMNs were added to the chamber at a final volume of 150 μL. After incubation for 2.5 h at 37°C, the chamber was disassembled. A washing step with 1 × PBS for 15 min followed, and the sections were fixed in formalin and stained with hematoxylin and eosin. Images in bright-field mode were captured using a Keyence BZ-9000 fluorescence microscope and BZ-II Viewer software (Keyence). The length of the split formation was assessed by 2 independent and blinded observers.
Statistical Analysis

All data are presented as the mean ± standard error of the mean, unless otherwise indicated. GraphPad Prism 5 and 7 were used to determine significant differences. For comparison of differences between two groups, t test was used, and for analysis of more than two groups, one-way analysis of variance with Bonferroni post-test was applied. A p value < 0.05 was considered statistically significant.

RESULTS

TNF Levels Are Increased in the Skin of Experimental and Human AIBDs

To investigate a possible effect of TNF during EBA and other AIBDs, we stained three human lesional and nonlesional skin specimens from EBA and BP patients and three normal human skin specimens using anti-TNF antibody. As expected, only inflamed regions (around the blister) showed clear staining for TNF, whereas normal human skin and nonlesional regions were negative for TNF (Figure 1A). To verify these findings in experimental models of EBA, we performed repeated injections of rabbit anti-mCOL7C IgG in mice (39). To verify these findings in mouse models of AIBD, ear samples were collected from mice injected with anti-mCOL7C IgG and with normal rabbit IgG 12 d after the first injection. By immunohistochemistry staining of the ear skin with rat anti-mouse TNF antibody, we demonstrated increased staining of TNF in skin lesions of diseased mice, while less staining was found in the control skin (Figures 1B, C). Furthermore, TNF mRNA expression in lesional skin of mice with antibody transfer–induced EBA was significantly increased compared to that in healthy skin from the same mice (p = 0.0382, Figure 1D). In contrast, the level of TNF in the serum of mice after antibody transfer–induced EBA was not different from that of control mice (p = 0.7075, Figure 1E).

Prophylactic TNF Inhibition Reduces Blistering in Antibody Transfer–Induced EBA and BP

Mice treated with TNF blocking antibody (TNF mAb) and subsequently injected with anti-mCOL7C IgG showed a significant difference regarding disease progression at d 8 and 12 when compared with mice treated with an isotype-control antibody (Figures 2A–D) (d 8, p = 0.029; d 12, p = 0.009). Immunohistochemistry staining revealed reduced monocytes/macrophages (anti–MOMA-2) and TNF in lesional skin of the TNF mAb–treated group, while blister formation and IgG/C3 deposition at the dermal-epidermal junction were comparable in both groups (Figures 2F–O).

Currently, the contribution of monocytes/macrophages to the development of skin lesions in EBA, in both patients and experimental models, has not been studied in detail. Here, we show that macrophages constitute a substantial percentage
with either isotype or ETA and TNF mAb. Although serum levels of TNF were unaltered in the TNF intervention groups compared with untreated mice (Figure 1A), levels of IL-6, G-CSF and KC were reduced after TNF inhibition (Figure 4). To verify the effect of TNF blockage in another mouse model for autoimmune blistering disease, we also injected mice with ETA and subsequently with anti-mCOL17 IgG as an experimental model for BP (40). Again, treatment with ETA showed a significant difference regarding disease progression at d 2 ($p = 0.04$) (Figures 2F–G).

**Therapeutic Treatment with ETA Diminishes the Extent of Blistering in Immunization-Induced EBA**

We next evaluated the therapeutic potential of ETA in mice with already established clinical EBA. B6.S mice were immunized with the vWFA2 domain of COL7C for induction of experimental EBA. Mice that presented at least 2% of body surface area affected by blistering were randomly allocated to either the ETA or PBS treatment group. Compared to PBS-treated controls, mice that received ETA showed a clinically significantly reduced extent of blistering (Figure 5), indicating the important role of TNF during the pathogenesis of experimental EBA.

**DISCUSSION**

In this study, we provide evidence for a proinflammatory role of TNF in experimental models of a prototypic organ-specific autoantibody-mediated disease. First, we observed increased TNF expression in the skin of mice that developed experimental EBA and in human BP and EBA patients. Second, pharmacological blockade of TNF by ETA or a murine TNF-blocking mAb, if applied before the onset of skin blisters, led to significantly milder disease progression in anti-mCOL7C IgG-treated mice compared with control mice. Third, in addition to this prophylactic treatment, we further demonstrated that TNF inhibition effectively improved already established...
disease activity in mice as a therapeutic approach. Taken together, these observations suggest that TNF is significantly involved in the effector phase of experimental EBA. Previously, different experimental studies demonstrated that TNF treatment was also effective in other AIBDs. More specifically, TNF plays a role in the acantholytic process in PV (32,41), and its expression in serum and skin of patients with PV (32,41) and BP (42) was increased. Furthermore, case reports documented the successful use of infliximab and ETA in PV patients (28–31), and in mucous membrane pemphigoid, the use of ETA was suggested for patients not responsive to high-dose intravenous immunoglobulins (26,43). Interestingly, here we observed increased levels of TNF only at the site of inflammation (i.e., the skin), while TNF concentrations in the serum were identical in mice with or without experimental EBA. The reason for unchanged serum TNF expression in experimental EBA could be either too low concentration in the skin to affect serum expression or retention in the skin by binding to TNF receptors.

For TNF blockade, we employed MP6-XT22, a rat TNF-blocking IgG1 mAb that neutralizes murine TNF and is the standard TNF blocking antibody used in murine studies (44). The human reagent ETA is a TNFR2-Fc fusion protein (soluble p75-TNF receptor-human IgG1) that is also effective at neutralizing TNF in mice and humans (44). Both compounds exhibited the same inhibitory effect on disease progression when applied in our model of antibody transfer–induced EBA. TNF signals via two receptors, TNFR1 and TNFR2. In contrast to TNFR1, which is ubiquitously expressed and mediates proinflammatory processes and cell death, TNFR2 is also involved in several immune diseases and inflammatory processes (10), 

Figure 3. Prophylactic inhibition of TNF using ETA in antibody transfer–induced EBA and BP model reduced disease severity. (A–E) Representative clinical pictures on d 12 after injection of mice with rabbit anti-mCOL7C IgG and treatment with (A,B) isotype control (shared with Figure 2) and (C,D) ETA every other day until d 10 starting 2 d prior to the initial anti-mCOL7C IgG injection. Both groups developed erythema and erosions. (E) Clinical disease score, shown as the percentage of body surface area affected by blistering, was reduced in ETA-treated mice (n = 6) compared to isotype-treated mice with EBA (n = 8) at d 8 and d 12. (F) Representative pictures of mouse ears on d 2 after injection with rabbit anti-mCOL17 IgG and treatment with PBS or ETA every other day starting 2 d prior to anti-mCOL17 IgG injection. (E) Clinical disease score, shown as the percentage of ear surface area affected by blistering, was reduced in ETA-treated mice (n = 6) compared to isotype-treated mice with EBA (n = 8) at d 2 (**p < 0.01).
which makes it likely to function as the major signaling receptor in EBA, thus is interesting for therapeutic modulation. However, which receptor primarily contributes to EBA development and progression is a subject of future research.

Our data indicate a reduced inflammatory milieu after TNF blockade that may contribute to improvement of skin disease. After use of TNF-blocking antibodies in antibody transfer–induced EBA, we observed strongly reduced TNF expression and reduced numbers of macrophages (MOMA-2–positive cells) in lesional skin. To date, the contribution of macrophages to the development of skin lesions in EBA, in both patients and experimental models, has been unclear. Here, we show that macrophages constitute a substantial percentage of infiltrated leukocytes in lesional skin obtained from mice with experimental EBA. In addition, serum cytokine levels of macrophage-released cytokines such as IL-6, G-CSF and the murine IL-8 equivalent keratinocyte-derived chemokine (46,47) were reduced in mice with antibody transfer–induced EBA after therapy with ETA and/or TNF blockade, indicating a disease-related effect of macrophages in experimental EBA. To address the question of whether this increased number of macrophages in the skin of human and experimental AIBDs represents a bystander effect of tissue inflammation or actively contributes to disease expression, we analyzed the role of monocytes/macrophages during ROS release and split formation using human isolated monocytes. Indeed, here we show that isolated monocytes directly contribute to mechanisms important in the pathogenesis of EBA and other pemphigoid diseases.

In accordance with our data, it was previously shown that TNF blockade reduces cell infiltration in different autoimmune diseases, such as rheumatoid arthritis (48). In addition, it had been demonstrated that ETA, in contrast to other TNF inhibitors (such as infliximab), exerts its effect not by inducing apoptosis of macrophages (49) but by phosphorylating p38 MAPK and STAT3 and reducing complement-dependent lysis of macrophages (50). The reduced number of macrophages observed after use of TNF-blocking antibodies in antibody transfer–induced EBA may therefore be due to reduced infiltration of these cells.

CONCLUSION

In conclusion, increased TNF expression in the skin of mice with experimental EBA suggests that this cytokine may be a new potential therapeutic target. Furthermore, both ETA and TNF-blocking antibody significantly impaired skin blistering in a prophylactic setting of the antibody transfer–induced model of EBA. Moreover, therapeutic intervention of TNF activity was able to reduce disease activity in mice with fully developed skin lesions. Our results suggest blockade of TNF function as a new therapeutic approach for the treatment of EBA. In line with the fact that antibodies like sCD32, anti-Flightless-1 homolog, anti–GM-CSF and anti-FcgRIV have promising inhibitory effects in model systems of pemphigoid diseases, we believe that the therapy options for these difficult-to-treat autoimmune disorders will improve in coming years (20,24,51,52).

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