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Untersuchungen zur Bedeutung reaktiver Sauerstoffradikale für die Blasenbildung der Epidermolysis bullosa acquisita

Pathogenicity of Reactive Oxygen Species for Blister Formation in Epidermolysis Bullosa Acquisita

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Abbreviations

ADCC,	antibody-dependent cellular cytotoxicity
BMZ,	bazal membrane zone
BP,	bullous pemphigoid
BP180,	bullous pemphigoid antigen 2
BP230,	bullous pemphigoid antigen 1
CGD,	chronic granulomatous disease
DEJ,	dermal-epidermal junction
DH,	dermatitis herpetiformis
Dsc,	desmocollins
Dsg,	desmogleins
EAE,	autoimmune encephalomyelitis
EBA,	epidermolysis bullosa acquisita
ELISA,	Enzyme-Linked ImmunoSorbent Assay
FAD,	flavin adenine dinucleotide
IF,	immunofluorescence
LAD,	Linear IgA disease
mCVII,	murine type VII collagen
MMP,	mucous membrane pemphigoid
MOG,	myelin oligodendroglial glycoprotein
MPO,	myeloperoxidase

NCF1, neutrophil cytosolic factor 1($p47^{phox}$); NCF2, neutrophil cytosolic factor 2

NRIgG, normal rabbit IgG

PBS, phosphate buffered saline

PF, Pemphigus foliaceus

PNP, Paraneoplastic pemphigus

PV, Pemphigus vulgaris

ROS, reactive oxygen species

$O_2^{\cdot -}$, superoxide anion

WT, wild type

Introduction

Autoimmune diseases are characterized by defined self antigens, organ specificity, and autoantibodies and/or autoreactive T cells that can transfer the disease. Autoimmune bullous diseases represent a heterogeneous, organ specific, group of diseases in which the immune response is directed against structural proteins of the skin and/or mucous membranes. Based on their clinical localization and pathological phenotype the bullous diseases can be classified in two major groups **(Table 1)**. The first group (pemphigus diseases) is characterized by the presence of autoantibodies directed against structural proteins mediating the cell-cell adhesion of keratinocytes. The latter includes the subepidermal blistering diseases caused by the loss of the attachment between basal keratinocytes and the underlying basement membrane, with autoantibodies directed against the dermal-epidermal junction components. A schematic representation of numerous molecules that can act as autoantigens in bullous skin diseases is depicted in **Figure 1A** and **1D**. The sustained research efforts over the past 25 years contributed to the large body of evidence that elucidated most of the molecules acting as autoantigens and some of the pathomechanisms involved in the progression of these diseases. Although autoantibodies' pathogenicity is generally accepted and strongly supported by clinical observations and experimental data for most autoimmune bullous diseases, the fine cellular and molecular effectors as well as the mechanisms of initiation of the autoimmune response are far from being fully understood and need further investigation. Nevertheless, the blister-inducing potential of autoantibodies in dermatitis herpetiformis, usually classified as an autoimmune subepidermal blistering disease, has not yet been demonstrated (Sitaru and Zillikens, 2005).

Table 1. Autoimmune blistering diseases

Bullous disease	Target autoantigens
I. INTRAEPITHELIAL (Pemphigus group)	
Pemphigus vulgaris	Dsg1, Dsg3, Dsg4, pemphaxin, (acetylcholine R), annexins
Pemphigus foliaceus	Dsg1
IgA pemphigus	Dsc1, Dsg3
Paraneoplastic pemphigus	Dsg1, Dsg3, desmoplakin I, II, BP230, envoplakin, periplakin, HD1-plectin, 170kD protein
Pemphigus herpetiformis	Dsg1, Dsg3
II. SUBEPIDERMAL BLISTERING DISEASES	
Pemphigoid group	
Bullous pemphigoid	BP180, BP230
Pemphigoid (Herpes) gestationis	BP180, BP230
Mucous membrane pemphigoid (Cicatricial pemphigoid)	BP180, Laminin 5 (epiligrin) and 6, α6β4 integrin
Linear IgA disease	LAD-1
Lichen planus pemphigoides	BP180
Bullous systemic lupus erythematosus	Type VII collagen
Anti p-200 pemphigoid	p200
Epidermolysis bullosa acquisita	Type VII collagen
Dermatitis herpetiformis	Transglutaminases

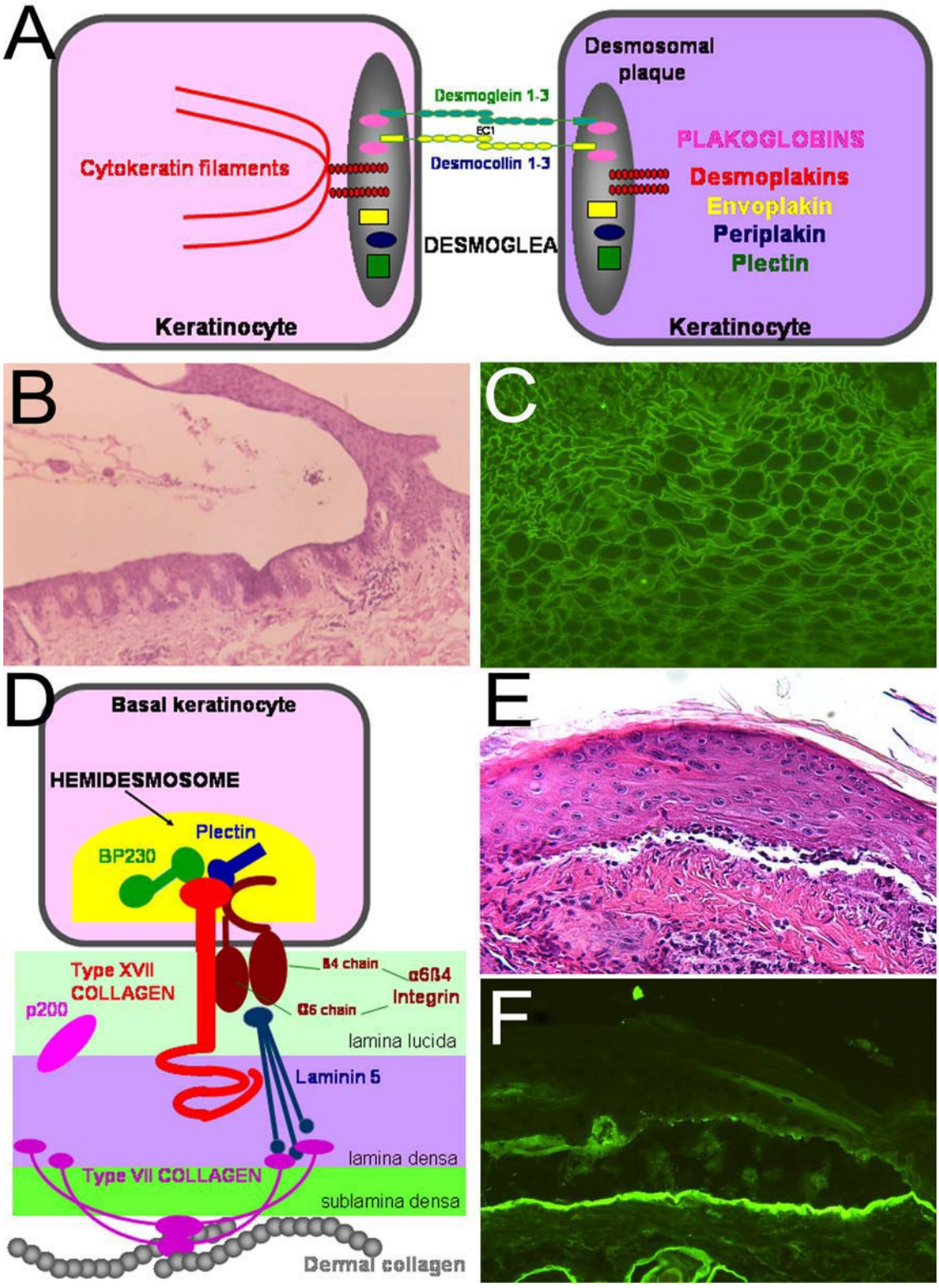

Figure 1. Autoimmune bullous skin diseases. (A) Structure of the desmosome. Desmosomes, the main adhesion structures of the epidermis, are composed of an intracellular region, i.e. the desmosomal plaque and an extracellular region, i.e. the desmoglea. The desmosomal plaque is a complex structure comprising two different classes of proteins: 1) plakoglobins and plakophilins binding to the intracellular domain of the cadherins and 2) the plakins (desmoplakin,

envoplakin, periplakin and plectin) who make contact with the cytokeratin filaments of the cytoskeleton. The desmoglea is composed of the extracellular domains of the cadherins (desmocollins and desmogleins) which interact with their 1st extracellular domains (EC1) on neighbouring keratinocytes. The extracellular domains of Dsg1 and Dsg3 represent the major autoantigens in PF and PV, respectively. Intradesmosomal proteins as well as some of the proteins of hemidesmosomes are antigens in PNP. **(B)** Histopathology in a PF patient's skin shows the loss of cell-cell adhesion (acantholysis) within the epidermis. **(C)** By indirect immunofluorescence (IF) microscopy, the autoantibodies from a PV patient stain the intercellular spaces. **(D)** Structure of the hemidesmosome. The adhesion of basal keratinocytes to the underlying basal membrane is mediated by multiple anchoring proteins, most of them being recognized as targets by autoantibodies in patients with autoimmune skin blistering diseases. The main autoantigens in BP are BP180 and BP230. Anti-p200 pemphigoid sera react with a 200kD protein of unknown origin localized at the lamina lucida-lamina densa interface. Type VII collagen, the autoantigen in EBA, makes contact in the upper dermis with the dermal collagen. **(E)** By histology, in the skin of mice with experimental EBA, the blister formation occurs subepidermal and is associated with a massive inflammatory infiltrate. **(F)** Typical direct IF microscopy aspect of lesional skin in experimental EBA shows linear deposits of IgG on the dermal side of the blister.

Classification of autoimmune blistering diseases

I. Pemphigus group

Pemphigus designates a rare group of life-threatening-autoimmune blistering diseases characterized by intraepithelial blister formation resulting from the loss of cell-cell adhesions (acantholysis) at the desmosomal level (Payne *et al.*, 2004; Stanley and Amagai, 2006). Autoantibodies in pemphigus vulgaris (PV) and pemphigus foliaceus (PF) patients target different keratinocyte proteins including desmosomal cadherins like desmoglein 1 (Eyre and Stanley, 1987), desmoglein 3 (Eyre and Stanley, 1988; Amagai *et al.*, 1991) **(Figure 1A)** and desmoglein 4 (Kljuic *et al.*, 2003), annexins (Nguyen *et al.*, 2000b) and acetylcholine receptors (Nguyen *et al.*, 2000a). The autoantibodies belong mainly to the IgG4 and to a lesser extent to the IgG1 subclass. Human IgG4 antibodies are non-complement fixing possessing only a reduced phagocyte-activation capacity. It has been therefore proposed that in pemphigus, autoantibodies mediate their pathogenic functions independently of their Fc portions (Liu *et al.*, 2004; Sitaru and Zillikens, 2005). Actually, compelling evidence from both *ex vivo* and *in vivo* experiments gathered over the past 25 years sustains the major role of IgG Fc-independent functions in the pemphigus group. However, the mechanism by which acantholysis is induced by pemphigus autoantibodies is intensely debated. It is believed that autoantibodies induce acantholysis and blister formation by directly interacting with desmosomal functions. In addition, recently it has been shown that pemphigus autoantibodies may induce apoptosis in keratinocytes leading to acantholysis (Wang *et al.*, 2004). The typical pathological findings in a pemphigus patients are presented in **Figure 1B,C**.

II. SUBEPIDERMAL BLISTERING DISEASES

Pemphigoid designates a group of autoimmune diseases characterized by vesicles and bullae on the skin and mucous membranes. Bullous pemphigoid (BP) and pemphigoid gestationis (PG) are characterized by the presence of autoantibody production against two major components of the hemidesmosomes, namely BP180 and BP230. The major epitopes are clustered within the 16th immunodominant noncollagenous (NC) domain of type XVII collagen (Morrison *et al.*, 1988; Zillikens *et al.*, 1997; Sitaru *et al.*, 2002b; Sitaru *et al.*, 2003; Herrero-Gonzalez *et al.*, 2006). Circulating and tissue-bound IgG subclasses seen in patients are IgG4 and IgG1 (Bird *et al.*, 1986; Brooks *et al.*, 1989; Yamada *et al.*, 1989; Shirakata *et al.*, 1990) and the mechanism proposed for the blister formation is thought to be initiated by the binding of IgG1 autoantibodies to the DEJ followed by the activation of complement and recruitment and activation of leukocytes. IgG4 autoantibodies, which were shown to stimulate leukocytes directly (Holland *et al.*, 2004; Mihai *et al.*, 2005), may thus activate the inflammatory cells already recruited in the upper dermis by the IgG 1 antibodies (Sitaru *et al.*, 2007). Compelling evidence from *in vivo* (Liu *et al.*, 1993; Liu *et al.*, 1995; Liu *et al.*, 1997; Liu *et al.*, 1998; Liu *et al.*, 2000) and *ex vivo* (Gammon *et al.*, 1982; Sitaru *et al.*, 2002b; Shimanovich *et al.*, 2004; Herrero-Gonzalez *et al.*, 2006) experiments contributed to the large body of evidence sustaining the pathogenic role of autoantibodies, complement, and cell-released proteases in BP and PG. In spite of the advances in our understanding of the disease and the use of corticosteroids, the mortality rate found by Lever in 1953, namely 24%, could not be significantly decreased. Recent studies report a mortality form BP of 15-20% (Venning and Wojnarowska, 1992) and 40% (Joly *et al.*, 2002), respectively.

Epidermolysis bullosa acquisita (EBA) is a chronic autoimmune bullous disease of the skin and mucous membranes characterized by subepidermal blisters and both tissue bound and circulating autoantibodies to the dermal-epidermal junction (**Figure 1D**). Although the first cases were reported more than one century ago by Elliott (Elliott, 1895), its fine characterization is due to research efforts of recent date (Sitaru *et al.*, 2002a; Sitaru *et al.*, 2005; Woodley *et al.*, 2005; Sitaru *et al.*, 2006). Patients' autoantibodies recognize the 290kD type VII collagen, the major component of anchoring fibrils (Sitaru and Zillikens, 2005). The pathogenic relevance of these autoantibodies is supported by compelling evidence:

1) EBA autoantibodies were shown to recruit and activate leukocytes resulting in dermal-epidermal separation in cryosections of human skin (Sitaru *et al.*, 2002a; Shimanovich *et al.*, 2004);

2) Antibodies against type VII collagen induce subepidermal blisters when passively transferred into mice (Sitaru *et al.*, 2005; Woodley *et al.*, 2005; Woodley *et al.*, 2006);

3) Immunization of mice with autologous type VII collagen induces a specific autoimmune response and a blistering phenotype reproducing human EBA (Sitaru *et al.*, 2006);

In experimental EBA, tissue damage is independent of T cells since blistering can be induced in nude mice and *ex vivo* with purified granulocytes (Sitaru *et al.*, 2002a; Sitaru *et al.*, 2005; Sitaru, 2007). Tissue bound and circulating antibodies in human EBA mainly belong to the IgG1 and IgG4 subclasses (Mooney and Gammon, 1990; Bernard *et al.*, 1991; Gandhi *et al.*, 2000; Sitaru *et al.*, 2007). A similar distribution with complement fixing IgG2a and 2b and non-complement fixing IgG1 has also been shown to occur in mice immunized with recombinant forms of type VII collagen

(Sitaru *et al.*, 2006). The strong bias toward production of IgG4 in these organ-specific autoimmune diseases suggests a chronic antigenic stimulation (Sitaru *et al.*, 2007). We hypothesized that after antibody binding to type VII collagen at the dermal-epidermal junction, as detected by IF microscopy, bound IgG activates the complement system and recruits and activates granulocytes in the upper dermis, thus directing the release of inflammatory mediators and proteases resulting in subepidermal blistering *in vivo*. Clinical features in EBA are heterogeneous: lesions may either arise on a non-inflammatory base (the classical/mechanobullous form of EBA resembling the inherited forms of dystrophic epidermolysis bullosa) with increased skin fragility of the trauma-prone sites or on an inflammatory (rich neutrophil infiltrate with or without eosinophils) background showing clinical features resembling BP (Gammon *et al.*, 1984), LAD or MMP (Dahl, 1979; Lang and Tapert, 1987; Caux *et al.*, 1997). Typical pathology in the lesional skin of experimental EBA mice are presented in **Figure 1E,F**.

Dermatitis herpetiformis (DH)

Although classified as an autoimmune disease, the direct evidence for its autoimmune nature is still missing. Despite *ex vivo* (Hendrix *et al.*, 1990) and recent *in vivo* (Marietta *et al.*, 2004) data sustaining a pathogenic role of IgA autoantibodies, the mechanism of granular IgA deposition targeting tissue transglutaminasein at the DEJ of the skin and its correlation with the circulating IgA autoantibodies are largely unknown.

The phagocytes

Recognition of phagocytes' major role in innate immunity belongs to scientific knowledge. These cells respond to the perturbation of their plasma membrane (induced by various microorganisms) with increased oxygen consumption, a process known as the 'respiratory burst'. The products of oxygen reduction (superoxide anion, hydrogen peroxide, hydroxyl radicals, hypochlorite radicals) and excitation (singlet oxygen) (Klebanoff, 1980) and other cytotoxic products (lysozyme, peroxidases, elastase) released by phagocytes (neutrophils, eosinophils and mononuclear phagocytes), have been implicated in their toxic properties.

Although it is accepted that many of these products posses direct cytotoxic potential, understanding is limited of how they interact to initiate and modulate cytotoxic responses *in vivo* (Rosen *et al.*, 1995). The prompt, nonspecific response of phagocytes makes them the perfect effectors in defense against a multitude of daily encountered invaders. However, lacking the specificity of T cells, phagocytes activated against self structures may also damage 'innocent bystanders' (surrounding structures) (Dahlgren and Karlsson, 1999) and induce apoptosis in other cells of the immune system contributing thus to the pathogenesis of some autoimmune diseases (Liu *et al.*, 1997; Wipke and Allen, 2001; Girardi *et al.*, 2003; Looney *et al.*, 2006). In many inflammatory conditions, the ability of phagocytes to produce ROS is thought to be crucially involved in host defense and tissue damage (Weiss, 1989).

Because of their high toxicity, the process of reactive oxygen species production is tightly regulated involving phosphorylation, translocation, and multiple conformational

changes. The enzyme responsible for the initiation of the respiratory burst is a reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase. The system is dormant in resting phagocytes being rapidly activated when needs arise. NADPH oxidase is a multiprotein complex comprising a membrane-bound flavocytochrome b_{558} and water soluble proteins of cytosolic origin. The structure of the NADPH oxidase complex is depicted in **Figure 2**.

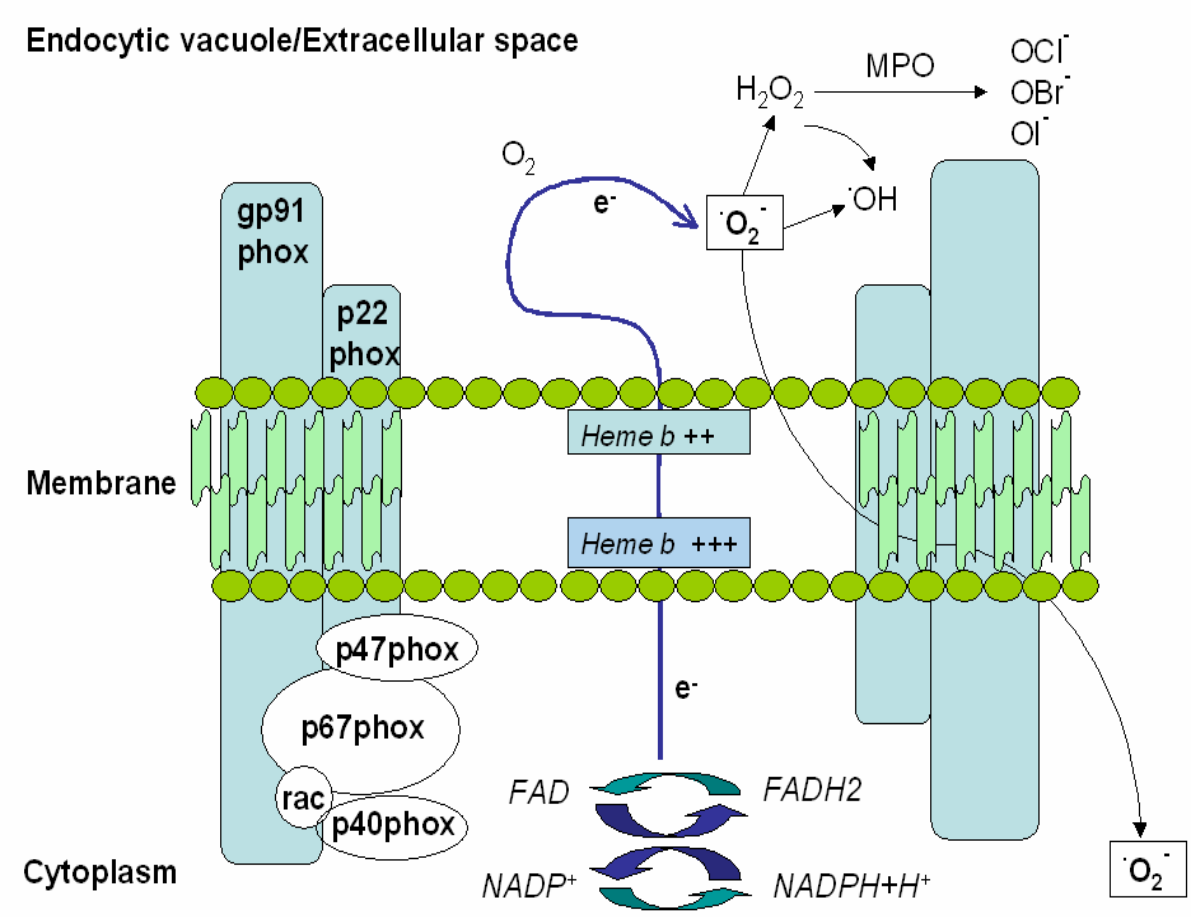

Figure 2. Structure of the granulocytic NADPH oxidase complex. The enzyme in its unactivated form has two distinct groups of components: a membrane-bound heterodimer gp91-p22phox (flavocytochrome b_{558}) and cytosolic proteins represented by $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and rac proteins. Upon cell activation, the cytosolic subunits translocate to the membrane and assemble with the flavocytochrome b_{558}, to transfer electrons from the NADPH and FAD through the two catalytic hemes of the flavocytochrome b_{558} to the molecular oxygen giving rise to the superoxide anion ($O_2^{\cdot -}$), which is further converted to other ROS such as hydrogen peroxide.

Structure of the NADPH oxidase

Flavocytochrome b_{558} is a membrane bound heterodimer that consists of a smaller alpha subunit ($p22^{phox}$) and a larger ß subunit ($gp91^{phox}$). It is the catalytic core of the enzyme having two hemes responsible for transferring the electrons from the flavin adenine dinucleotide (FAD) through the membrane to the molecular oxygen giving rise to the superoxide anion ($O_2^{\cdot -}$), which will be further converted to other ROS including hydrogen peroxide (Babior, 2004).

$p47^{phox}$ (NCF1) is the first protein to interact with the flavocytochrome b during oxidase assembly. Its association with the membrane-bound flavocytochrome b was shown to be a prerequisite for translocation of the other two cytosolic proteins, namely for $p67^{phox}$ and $p40^{phox}$ (Kleinberg *et al.*, 1990; Heyworth *et al.*, 1991). Some mechanisms have been proposed among which the phosphorylation of $p47^{phox}$ seems to be a key molecular event associated with NADAPH oxidase. Following the phosphorylation, a conformational change or the neutralization of the cationic domain of the protein make possible its interaction with the other NADPH-oxidase components (Ago *et al.*, 1999; Nauseef, 1999).

$p67^{phox}$ (NCF2). Even though *in vivo* the binding of $p47^{phox}$ to the membrane-bound flavocytochrome b_{558} is a prerequisite for the complex assembly, there are sufficient data to support a direct interaction of $p67^{phox}$ with the flavocytochrome b_{558} in *ex vivo* $p47^{phox}$-free systems (Freeman and Lambeth, 1996; Koshkin *et al.*, 1996; Miyano *et al.*, 2001). As it is the case with $p47^{phox}$, phosphorylation of $p67^{phox}$ seems to have an important role in its assembling properties.

p40^{phox} is a small cytoplasmatic protein that immunoprecipitates with p67^{phox}. It can also be phosphorylated. Its functions are far from being understood. However, it may facilitate the oxidase assembly. The recent development of a knock-out mouse for this protein (Ellson *et al.*, 2006) should help to characterize the precise role this component is playing.

Rac1 and **rac2** are cytosolic guanosine triphosphate-binding factors, whose distribution depends on the cell type and species (Bokoch and Diebold, 2002). Whereas Rac1 seems to be implicated mainly in other neutrophil functions (Glogauer *et al.*, 2003; Gu *et al.*, 2003), numerous studies showed a critical role for Rac2 in correct assembly and function of the NADPH oxidase *in vivo*. It interacts with both the p67^{phox} and gp91^{phox} subunits and it is thought to have an additional role in regulating cytoskeletal structure (Burridge and Wennerberg, 2004).

The biological relevance of NADPH oxidase is evident from inherited or targeted disruptions in the genes that encode its subunits. These mutations result in chronic granulomatous disease (CGD), characterized by defective destruction of pathogens by the phagocytes resulting in recurrent bacterial and fungal infections and granulomas (Baehner and Karnovsky, 1968; Clark, 1990; Jackson *et al.*, 1995; Pollock *et al.*, 1995; Huang *et al.*, 2000). The complex function of NADPH oxidase is further emphasized by the observation that a naturally occurring polymorphism of the neutrophil cytosolic factor (Ncf)1 gene (encoding neutrophil cytosolic factor 1 also known as p47^{phox}, a component of the NADPH oxidase complex) regulates disease severity in experimental arthritis and autoimmune encephalomyelitis (EAE) (Olofsson *et al.*, 2003; Becanovic *et al.*, 2006).

It is widely believed that NADPH oxidase and neutrophil-derived ROS are relevant for the pathogenesis of autoimmune inflammatory diseases. However, the precise contribution of NADPH oxidase to both the initiation of the autoimmune response and tissue injury is still unclear. Depending on the experimental setting, inhibition of ROS production by pharmacological agents or gene defects resulted in diminution (van der Veen *et al.*, 2000; Ross *et al.*, 2004) or augmented severity of experimental autoimmune conditions (Hultqvist *et al.*, 2004). Therefore, the capacity of ROS to mediate tissue injury in autoimmune inflammatory diseases still remains an unresolved question. An additional problem is to separate this effect from ROS influences on the afferent autoimmune response (e.g., by activation/inhibition of autoreactive T helper cells).

Aim of the study

We have previously demonstrated that incubation of normal human skin cryo-sections with antibodies from BP or EBA patients and subsequently with granulocytes from healthy donors results in subepidermal blistering *ex vivo* (Sitaru *et al.*, 2002a; Sitaru *et al.*, 2002b). The blistering was associated with a massive respiratory burst (production of reactive oxygen species) of granulocytes. Moreover, we recently showed that rabbit antibodies generated against recombinant forms of murine type VII collagen induce *in vivo* a subepidermal blistering disease, closely resembling human EBA, when passively transferred into mice (Sitaru *et al.*, 2005). In the skin of the diseased mice we found a massive inflammatory infiltrate dominated by neutrophils. An important aspect in the pathogenesis of blistering that has not yet been addressed in either our or other models of antibody-induced blistering diseases is weather there is an association between the granulocytes' respiratory burst and the blister formation. It was therefore the aim of the present study to reveal the contribution of ROS for blistering induced by the passive transfer of antibodies against type VII collagen using both *ex vivo* and *in vivo* experimental models.

Materials and methods

Patients

Serum samples were collected from patients with EBA (n=5) and BP (n=5), before the initiation of treatment, as well as from healthy donors (n=10). Criteria for inclusion of patients with EBA and BP in the study have been previously published. (Sitaru *et al.*, 2002a; Sitaru *et al.*, 2002b; Sitaru *et al.*, 2005). Leukocytes were obtained from the peripheral blood of healthy donors (n=10) or from patients with chronic granulomatous disease (n=8). Characteristics of CGD patients included in this study are summarized in **Table 2**. For the experiments conducted, we obtained institutional approval issued by the ethics committee at the Medical Faculty of the University of Lübeck (Institutional Board Projects 04-061, 04-144 and 05-056). In adherence to the Helsinki Principles, we obtained informed consent from all patients whose material was used in this study.

Mice

Six to eight weeks old mice with a body weight of approximately 20 g were used. Female BALB/c were obtained form Charles River (Sulzfeld, Germany) and male and female C57BL/6J and C57BL/6J-*Ncf1*^{m1J}/J (Ncf1$^{-/-}$)(Huang *et al.*, 2000) from Jackson Laboratory (Bar Harbor, Maine). CD18$^{-/-}$ mice were previously described (Scharffetter-Kochanek *et al.*, 1998). All injections and bleedings were performed on mice narcotised using either inhalation of isoflurane or intraperitoneal administration of a mixture of ketamine (100 µg/g) and xylazine (15 µg/g). All i*n vivo* experiments were approved by the local authorities of the Animal Care and Use Committee (Kiel, Germany; no. 6/g/04 and 6/k/06) and performed by certified personnel.

Table 2. Patients with chronic granulomatous disease included in this study

No.	Patient ID	Age years, male, female	Defective gene (affected protein)	Residual NADPH oxidase activity	Mutation	Remark	Reference
1	AA	49, f	*CYBB* (gp91-phox)	98% none, 2% normal[A]	del.A409[B]	X-linked carrier[C]	i)
2	CO	18, m	*CYBB* (gp91-phox)	none	del.G161		ii) pat. 2
3	JL	17, m	*CYBB* (gp91-phox)	none	del.G1025, T1029G	2 muta-tions[D]	
4	SM	26, f	*NCF1* (p47-phox)	traces of H_2O_2 production	del.GT ex2, homozygous	frequent mutation[E]	iii)
5	FK	23, m	*CYBB* (gp91-phox)	approx. 5% in all cells	dupl. of 9bp 585-593	dupl. of 3 aa[F]	
6	AK	56, f	*CYBB* (gp91-phox)	95% none, 5% normal[A]	960delC[B]	X-linked carrier[C]	iv)
7	SD	37, f	*NCF1* (p47-phox)	traces of H_2O_2 production	del.GT ex2, homozygous	frequent mutation[E]	ii) pat. 5
8	SK	11, m	*CYBB* (gp91-phox)	none	del.Gly533		ii) pat. 3

[A]percent of neutrophils in peripheral blood; [B]on one allele; [C]skewed X-inactivation; [D]the two mutations are likely to result from one loop forming mutational mechanism; [E]caused by recombination with pseudogene, see ref. B); [F]in frame duplication (dupl.) of 3 amino acids; numbering of mutations according to ENSEMBL.

i) Lun A, Roesler J, Renz H. Unusual late onset of X-linked chronic granulomatous disease in an adult woman after unsuspicious childhood. Clin Chem. 2002; 48:780-1.

ii) Roesler J, Koch A, Porksen G, von Bernuth H, Brenner S, Hahn G, Fischer R, Lorenz N, Gahr M, Rosen-Wolff A. Benefit assessment of preventive medical check-ups in patients suffering from chronic granulomatous disease (CGD). J Eval Clin Pract. 2005;11:513-21.

iii) Roesler J, Curnutte JT, Rae J, Barrett D, Patino P, Chanock SJ, Goerlach A. Recombination events between the p47-phox gene and its highly homologous pseudogenes are the main cause of autosomal recessive chronic granulomatous disease. Blood. 2000 15;95:2150-6.

iv) Rosen-Wolff A, Soldan W, Heyne K, Bickhardt J, Gahr M, Roesler J. Increased susceptibility of a carrier of X-linked chronic granulomatous disease (CGD) to Aspergillus fumigatus infection associated with age-related skewing of lyonization. Ann Hematol. 2001; 80:113-5.

Antibodies against the dermal-epidermal junction

Rabbits were immunized with recombinant forms of murine type VII collagen as described (Sitaru *et al.*, 2005). IgG from serum of patients as well as immune and preimmune rabbits was purified by affinity chromatography using Protein G and analyzed as described (Sitaru *et al.*, 2002a; Sitaru *et al.*, 2005). Preparations of rabbit IgG against murine type VII collagen were adjusted to an end-point titre of 1:128,000 by IF microscopy on murine skin sections. Patients autoantibody preparations were used at a titre of 1:80 by IF microscopy on human skin sections. Control IgG preparations were adjusted to the same protein concentration.

Leukocytes

Total human leukocytes and granulocytes were isolated from peripheral blood of healthy donors and CGD patients and tested for viability and purity as described (Sitaru *et al.*, 2002a; Shimanovich *et al.*, 2004).

Murine granulocytes were isolated from peripheral blood and bone marrow of donor mice by 3% dextran sedimentation followed by density gradient centrifugation using Ficoll-Paque (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and hypotonic lysis in 0.2% NaCl. Cell viability and purity were assessed by trypan blue exclusion, Giemsa stain, and flow cytometry using mAb specific to Gr-1 (clone RB6-8C5) and CD11b (clone M1/70) (BD-Biosciences). All flow cytometry analyses were performed on 10^4 events using a FACSCalibur™ (BectonDickinson-Biosciences, San Jose, CA) and CellQuest software (Becton Dickinson). Data were analyzed using WinMDI version 2.8 software (Scripps Research Institute, http://facs.scripps.edu/software.html).

Neutrophils were depleted in mice using the rat monoclonal antibody RB6-8C5 (Hestdal *et al.*, 1991) that binds to Gr-1 and depletes *in vivo* mature murine neutrophils and eosinophils, but not lymphocytes and macrophages. The RB6-8C5 hybridoma cell line was cultured in HL-1 FCS-free medium (Cambrex Bio Science, Verviers, Belgium) supplemented with 4 mM L-glutamine, 100 U penicillin/ml, and 100 µg streptomycine/ml (CCPro, Neustadt, Germany) and the antibody purified by 33% ammonium sulphate precipitation, tested by SDS-PAGE analysis, sterile-filtered ([pore size, 0.22µm], Millipore, Bradford, MA) and stored at -80°C until used. 0.25 mg of the RB6-8C5 or a mock antibody (normal rat IgG; Sigma) was injected intraperitoneally 24 h before the first s.c. injection of rabbit antibody against type VII collagen followed by subsequent injections at 3 days interval. Neutrophil depletion was monitored by flow cytometry analysis of Gr-1^{high}CD11b^{high} cells and Giemsa stains of peripheral blood samples taken every second day during the observation period. In line with previous observations (Stephens-Romero *et al.*, 2005), in our study, depletion of neutrophils in mice could not be maintained for longer than 7 days probably due to generation of blocking mouse anti-rat antibodies.

Autoantibody-induced dermal-epidermal separation

Blister-inducing potential of IgG preparations from patients was assessed *ex vivo* using a model of autoantibody-induced leukocyte-dependent dermal-epidermal separation in cryosections of normal human skin (Sitaru *et al.*, 2002a; Shimanovich *et al.*, 2004). Preparation of cryosections and incubations with antibody and cell preparations followed published protocols (Sitaru *et al.*, 2002a). NADPH oxidase activity was inhibited using the specific flavoprotein inhibitor diphenylene iodonium (Alexis Biochemicals, Grünberg, Germany), that was dissolved in dimethylsulfoxide

(Sigma) and added to the leukocyte suspensions at a final concentration lower than 0.1% dimethylsulfoxide to avoid toxic effects on neutrophils. A modified nitroblue tetrazolium reduction slide test was used as a non-quantitative test for the presence of reactive oxygen products (Sitaru *et al.*, 2002a). All slides were scored by 2 investigators blind to both the aim of the experiment and the identity of the antibody preparations used. In general, the results were reproducible in at least two independent experiments. However, availability of fresh granulocytes from CGD patients was limited. Therefore, we were able to perform the experiments using granulocytes from CGD patients 2 and 3 twice, whereas granulocytes from the remainder of CGD patients were used in only one experiment.

Induction of blistering *in vivo* and phenotype assessment

Passive transfer studies followed published protocols with minor modifications (Sitaru *et al.*, 2005). Briefly, if not stated otherwise, 7.5 µl/g body weight of IgG was administered subcutaneously into the back every second day. Blisters or erosions were counted and the extent of skin disease (disease activity) was scored as follows: 0, no lesions; 1, < 10 lesions or < 1% of the skin surface; 2, > 10 lesions or 1-5% of the skin surface; 3, 5-10%; 4, 10-20%; and 5, >20% involvement of the skin surface. Sera were obtained from mice at different time points and assayed for antibody reactivity by indirect IF microscopy on cryosections of mouse skin and ELISA using recombinant protein. Biopsies of lesional and perilesional skin were obtained 2 days after the last injection of IgG and prepared for examination by histopathology, and IF microscopy as described (Sitaru *et al.*, 2005). The staining intensity of immunoreactants in the skin of injected mice was assessed semiquantitatively using a score comprising 0, for no staining; 1, focal, faint staining; 2, faint staining, 3,

medium; and 4, intense staining. Neutrophil infiltration of murine skin was assayed as described (Bradley *et al.*, 1982), with minor modifications. Briefly, in both clinically diseased and not diseased mice, the left ear was removed after killing the mice and skin samples (approximately 10 x 5 mm in size) were extracted by homogenization in a buffer containing 0.1 M Tris-Cl, pH 7.6, 0.15 M NaCl, 0.5% hexadecyltrimethylammoniumbromide (Sigma). MPO activity in the supernatant fraction was measured by the change in optical density at 460 nm resulting from decomposition of H_2O_2 in the presence of o-dianisidine (Sigma). A standard reference curve was established using known concentrations of purified MPO (Sigma). MPO content was expressed as units of MPO activity per mg of protein. Protein concentrations were determined by the Bradford dye binding assay (Bio-Rad Laboratories, Richmond, CA). For the *in vivo* reconstitution with leukocytes, neutrophils were purified from donor C57BL/6J mice as described above. Mice were injected with 2 x 10^6 cells in 50 µl medium s.c. in the ears. The animals were examined clinically after 12 and 24 h, subsequently killed and samples prepared and analyzed as described above.

Detection of antibody levels by ELISA

ELISA using recombinant murine type VII collagen was performed at room temperature on 96-well microtitre plates as previously reported (Sitaru *et al.*, 2006) with minor modifications. Briefly, each well was coated with 500 ng of the recombinant protein glutathione-S-transferase (GST)-mCOL7C or with an equimolar amount of GST in 0.1 M bicarbonate buffer, pH 9.6 and incubated with 200-fold dilutions of mouse serum for 60 min. Bound antibodies were detected using a 10,000-fold dilution of an HRP-labelled goat anti rabbit IgG antibody (DAKO) and

ortho-phenylene diamine (Sigma). The colour reaction was read at 490 nm using a multilabel counter (Victor 3; Perkin Elmer, Wellesley, MA).

Measurement of superoxide production by cytochrome c reduction

Production of superoxide anion by leukocytes stimulated with phorbol 12-myristate 13-acetate (Sigma) was measured as the rate of reduction of ferricytochrome c to its ferrous form as described (Tan *et al.*, 1998) with minor modifications. Briefly, 10^6 granulocytes were equilibrated in 1 ml PBS, pH 7,4 with 20 mM glucose, 1 mM MgCl2, and 0.05 mM cytochrome c (Sigma) for 10 min at 37°C with continuous gentle mixing. Because various reductants other than superoxide may reduce ferricytochrome c, specificity for the superoxide responsible reduction was achieved by adding 50 µg superoxide dismutase (Sigma) in the reference cuvettes. 100 ng phorbol 12-myristate 13-acetate were added and incubation was carried out at 37°C, absorbance at 550 nm being monitored every second minute until a plateau was reached, using a Victor 3 multilabel counter. Diphenylene iodonium at the concentrations used in the cryosection experiments was added to the samples. The rate of cytochrome c reduction in the reference was subtracted from the one in the samples to obtain the values of the superoxide dismutase-inhibitable reduction of cytochrome c which reflects the superoxide production. Control samples also included blanks containing only buffer and cytochrome c and unstimulated cells with buffer and cytochrome c. Results were expressed as percent against the blank tube containing all reagents except the cells. All samples were assayed in triplicate. Data presented are representative of 5 experiments.

Statistical analysis

Statistic tests were computed using the OpenStat2 free software for Linux (http://www.agrivisser.com/cgibin/English/OpenStat2.htm). To compare the IF staining intensity at the DEJ and MPO activity in skin biopsies, the independent-samples Stundent's *t* test was used. Differences in disease severity were calculated using the Chi-square test. To estimate the correlation between inhibition of dermal-epidermal separation and superoxide production by diphenylene iodonium, the Spearman's rank correlation test was applied. Means are presented ± s.e.m.; $p < 0.05$ was considered statistically significant.

Results

Ncf1$^{-/-}$ mice are resistant to blistering induced by antibodies against type VII collagen

In initial *in vivo* experiments, we injected Ncf1$^{-/-}$ (n=18) and Ncf1$^{+/+}$ (n=14) mice with IgG against murine type VII collagen. All Ncf1$^{+/+}$ mice developed single blisters 4-5 days after the first injection. Widespread lesions, including blisters, erosions and crusts, occurred 5-6 days after the first injection (**Figure 3A**). There was no evidence of clinical lesions in 17 of 18 Ncf1$^{-/-}$ mice (**Figure 3B**) treated with antibody against type VII collagen or both Ncf1$^{-/-}$ (n=2) and Ncf1$^{+/+}$ mice (n=2) (**Figure 3C**) that received control antibody at any time during the observation period (**Figure 4A**). In serum of Ncf1$^{-/-}$ and Ncf1$^{+/+}$mice injected with IgG against type VII collagen, levels of specific antibodies were comparable (**Figure 4B**). Immunofluorescence (IF) microscopy of mouse skin revealed linear deposits of rabbit IgG (**Figure 3D,E**) and murine complement C3 (**Figure 3G,H**) at the DEJ in mice that received IgG specific to type VII collagen, but not in mice injected with control IgG (**Figure 3F,I**). Histological examination of lesional skin biopsies from diseased Ncf1$^{+/+}$ mice injected with IgG against type VII collagen revealed extensive dermal-epidermal separation accompanied by dense inflammatory infiltrates that were dominated by neutrophils (**Figure 3J**). In contrast, skin biopsies from all non-diseased Ncf1$^{-/-}$ animals injected with IgG against type VII collagen demonstrated moderately dense inflammatory infiltrates, but no dermal-epidermal separation (**Figure 3K**). In one Ncf1$^{-/-}$ mouse that developed limited erosions on the ears, the histopathological analysis confirmed these lesions, but no frank dermal-epidermal separation was observed. In Ncf1$^{+/+}$ mice injected with control IgG, we found no subepidermal cleavage and no

inflammatory infiltrate (**Figure 3L**). The neutrophil infiltration in murine skin was assessed using a myeloperoxidase (MPO) activity assay. In mice injected with IgG against type VII collagen, a significantly lower MPO activity was found in the skin of $Ncf1^{-/-}$ compared with $Ncf1^{+/+}$ mice (**Figure 4C**).

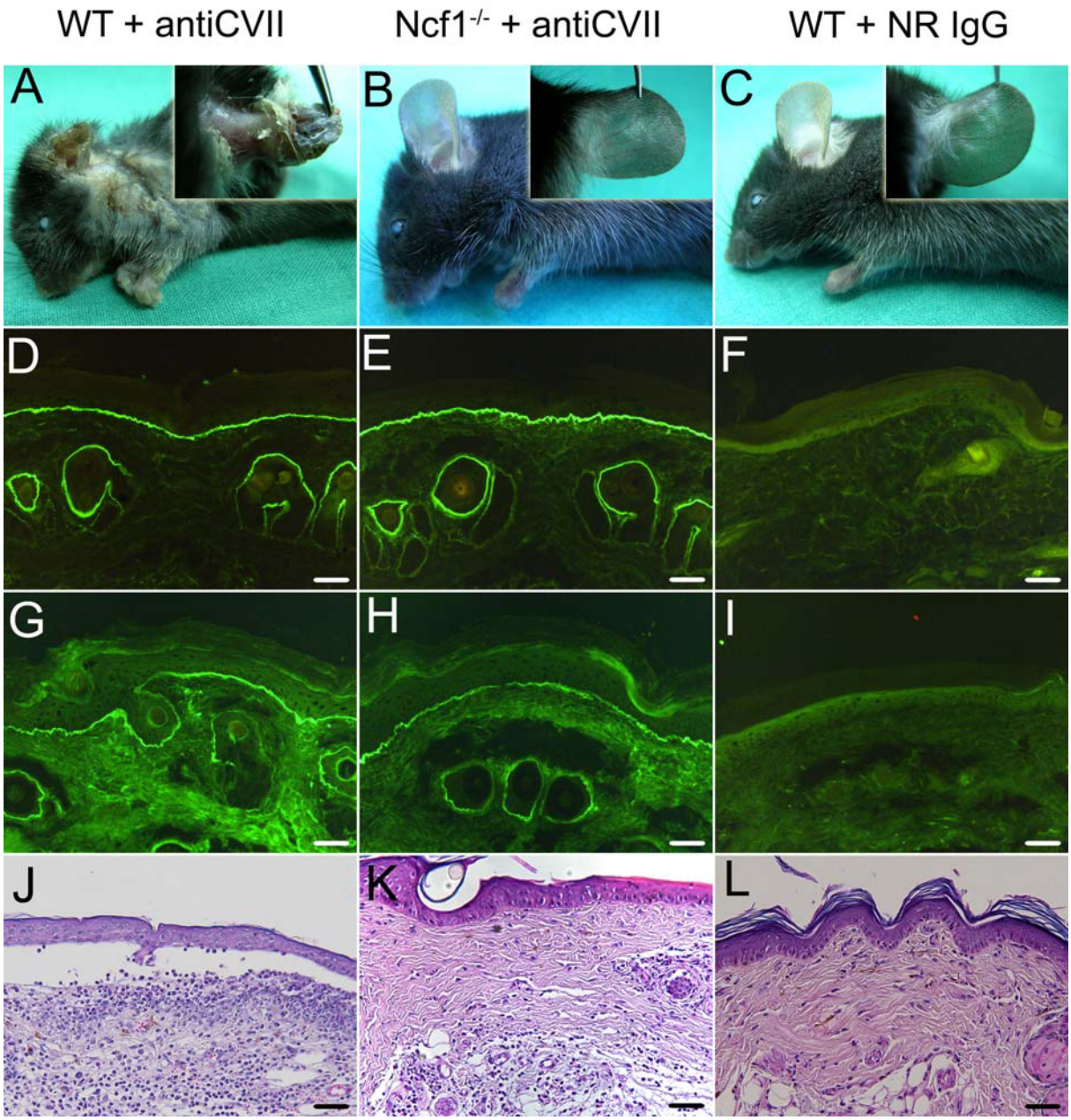

Figure 3 $Ncf1^{-/-}$ mice are resistant to blisters induced by antibodies against type VII collagen. (**A**) Injection of a total dose of 45 µl/g body weight of IgG against type VII collagen (antiCVII) results in extensive skin lesions, including blisters, erosions, partly covered by crusts and epidermal detachment on the trunk and (**inset**) ears in a C57BL/6J mouse. (**B**) A $Ncf1^{-/-}$ mouse injected with the same

dose of pathogenic IgG against type VII collagen, and (**C**) a C57BL/6J mouse injected with normal rabbit (NR) IgG, did not develop skin lesions. Immunofluorescence microscopy of murine skin reveals linear deposits of rabbit IgG at the dermal-epidermal junction in mice injected with (**D,E**) antibodies to type VII collagen, but not with (**F**) control antibody. Complement deposits are found at the dermal-epidermal junction in mice injected with (**G,H**) antibodies to type VII collagen, but not with (**I**) control antibody. Histological analysis of lesional murine skin demonstrates subepidermal cleavage and a neutrophil-rich inflammatory infiltrate in a (**J**) C57BL/6J, but only a moderate infiltrate of neutrophils in (**K**) a Ncf1$^{-/-}$ mouse, receiving antibodies to type VII collagen. Normal histological appearance in a (**L**) C57BL/6J mouse treated with control antibody. Scale bars, all 50 µm.

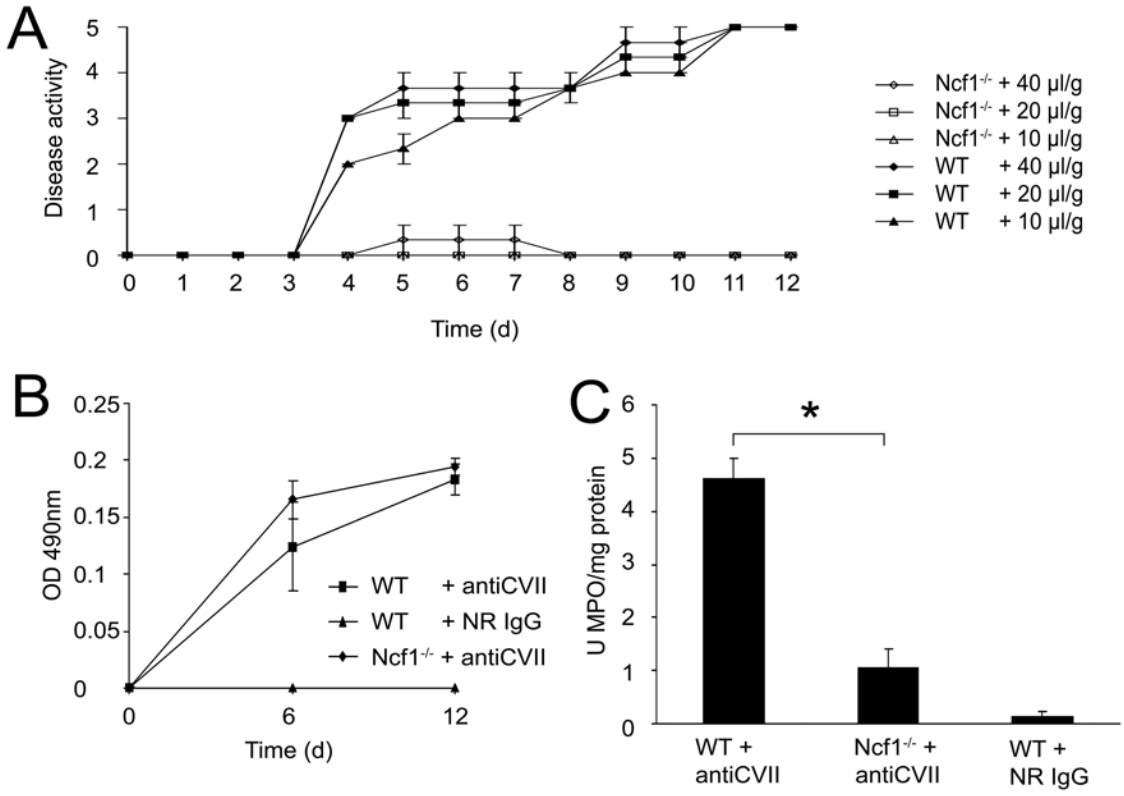

Figure 4 Clinical disease severity, antibody levels and granulocytic infiltrates in Ncf1$^{-/-}$ mice injected with rabbit IgG against type VII collagen (antiCVII) or normal rabbit IgG (NR IgG). (**A**) Disease activity in Ncf1$^{-/-}$ and wild type (WT) mice (n=3 per group) treated with different doses of the rabbit antibody against type VII collagen was assessed as described in Methods. (**B**) Serum levels of antibodies to type VII collagen in Ncf1$^{-/-}$ and wild type mice (n=5 per group) injected with 10 µl/g body weight rabbit IgG as detected by ELISA using recombinant antigen. (**C**) Significantly less neutrophil infiltrates in the skin of Ncf1$^{-/-}$ compared with wild type (WT) mice as assessed by measuring myeloperoxidase (MPO) activity in skin biopsies (*, p, < 0.05). All values are means ± s.e.m..

Inhibition of NADPH oxidase abolishes dermal-epidermal separation induced by autoantibodies *ex vivo*

Autoantibodies from patients with autoimmune blistering diseases such as EBA and bullous pemphigoid recruit and activate granulocytes at the DEJ by an Fc-dependent mechanism and cause dermal-epidermal separation (Sitaru *et al.*, 2002a; Sitaru *et al.*, 2002b). Therefore, we addressed the question whether the autoantibody-induced tissue damage in this *ex vivo* human system is depending on granulocytic NADPH oxidase. Cryosections of human skin were incubated with IgG from patients with EBA (n=5) and bullous pemphigoid (n=5) and, with leukocytes from healthy donors. IgG from patients' sera (**Figure 5A,B**), but not from healthy controls (**Figure 5C**), bound to the DEJ as revealed by indirect IF microscopy. Addition of leukocytes resulted in their recruitment at the DEJ in cryosections that had been incubated with patients' autoantibodies both in the absence (**Figure 5D**) and presence (**Figure 5E**) of 75 µM diphenylene iodonium, but not in those incubated with control IgG (**Figure 5F**). When leukocyte suspensions were supplemented with nitroblue tetrazolium, deposits of formazan were observed at the DEJ in cryosections incubated with patients' IgG and leukocytes (**Figure 5G**), but not in those incubated with patients' IgG, leukocytes and diphenylene iodonium (**Figure 5H**) or with control IgG and leukocytes (**Figure 5I**). Dermal-epidermal separation was seen in cryosections incubated with patients' IgG and leukocytes (**Figure 5J**), but not in those incubated with patients' IgG, leukocytes and diphenylene iodonium (**Figure 5K**) or with control IgG and leukocytes (**Figure 5L**). Incubation of cryosections with autoantibodies and leukocytes in the presence of diphenylene iodonium revealed a dose-dependent inhibition of both superoxide production from phorbol ester-stimulated human granulocytes and autoantibody-induced dermal-epidermal separation (**Figure 6**). In the presence of different

concentrations of diphenylene iodonium, superoxide production from granulocytes highly correlates with the extent of dermal-epidermal separation (r, 0.99; p<0.001).

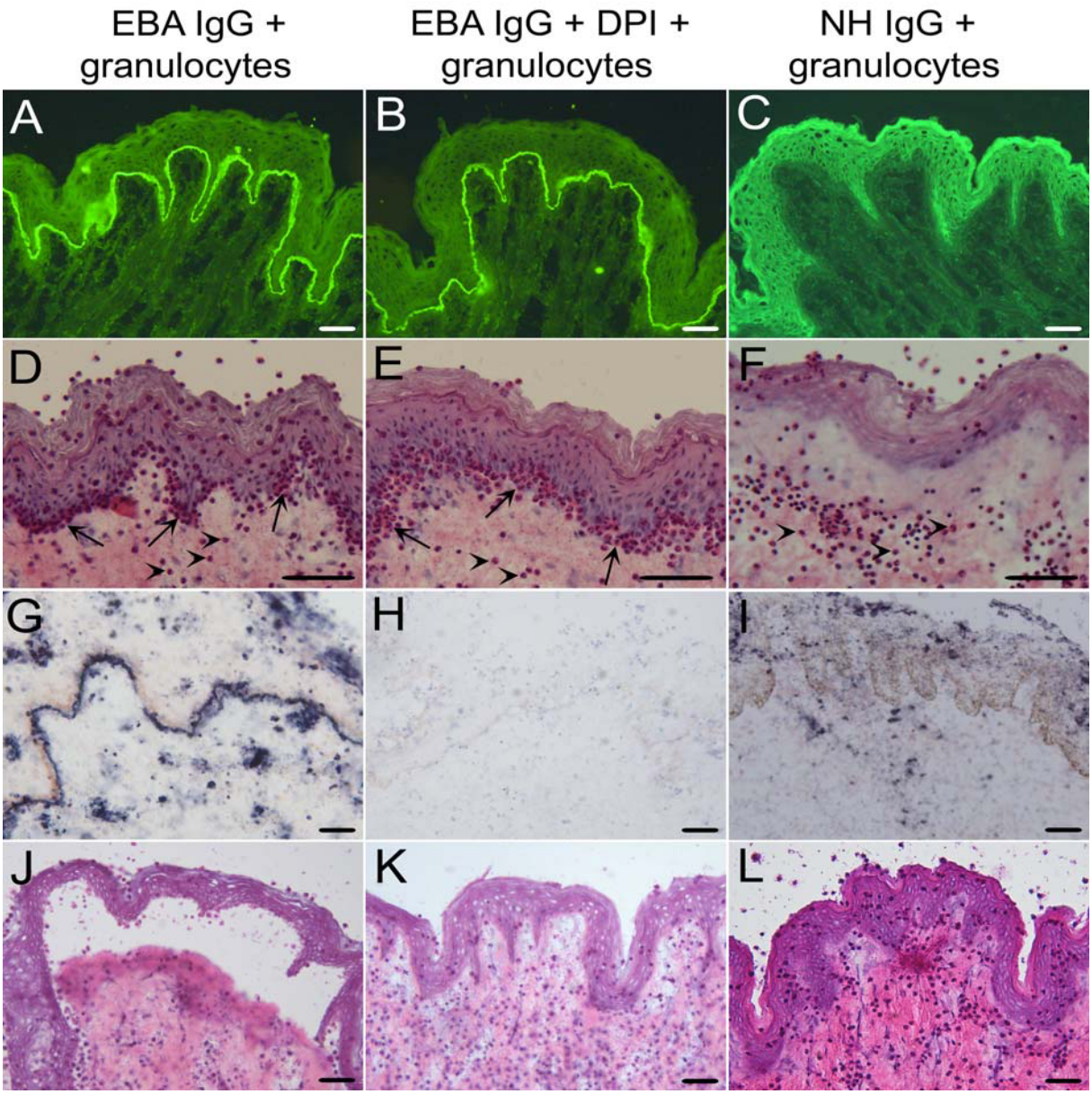

Figure 5 Inhibition of NADPH oxidase abolishes dermal-epidermal separation induced by autoantibodies in cryosections of human skin. Frozen sections of normal human skin were incubated with 100 µl IgG from a patient with epidermolysis bullosa acquisita (EBA1) or a healthy control. (**A,B**) EBA IgG autoantibodies, in contrast to (**C**) normal human (NH) IgG, bind to the dermal-epidermal junction as revealed by immunofluorescence microscopy. One h incubation with 1.5 x 10^7 granulocytes purified from the peripheral blood of healthy donors results in their recruitment at the dermal-epidermal junction (arrows) in cryosections incubated with IgG from the EBA patient both in the (**D**) absence and (**E**) presence of the NADPH oxidase inhibitor diphenylene iodonium. (**F**) In cryosections incubated with IgG from the healthy control no recruitment of leukocytes

at the dermal-epidermal junction are observed. In all sections, leukocytes, randomly scattered over the entire epidermis and dermis (arrow heads), are seen (hematoxylin and eosin). In the presence of 0.05% nitroblue tetrazolium, formazan precipitates are present at the dermal-epidermal junction in cryosections treated with (**G**) EBA IgG and granulocytes, but not with (**H**) EBA IgG, granulocytes, and diphenylene iodonium or with (**I**) normal IgG and granulocytes. Two h incubation with granulocytes results in dermal-epidermal separation in cryosections treated with IgG from our EBA patient in the (**J**) absence of diphenylene iodonium. In contrast, no subepidermal splits develop in (**K**) cryosections incubated with autoantibodies and leukocytes when diphenylene iodonium was added or (**L**) in cryosections incubated with IgG from the healthy control and leukocytes. Scale bars, all 50 µm.

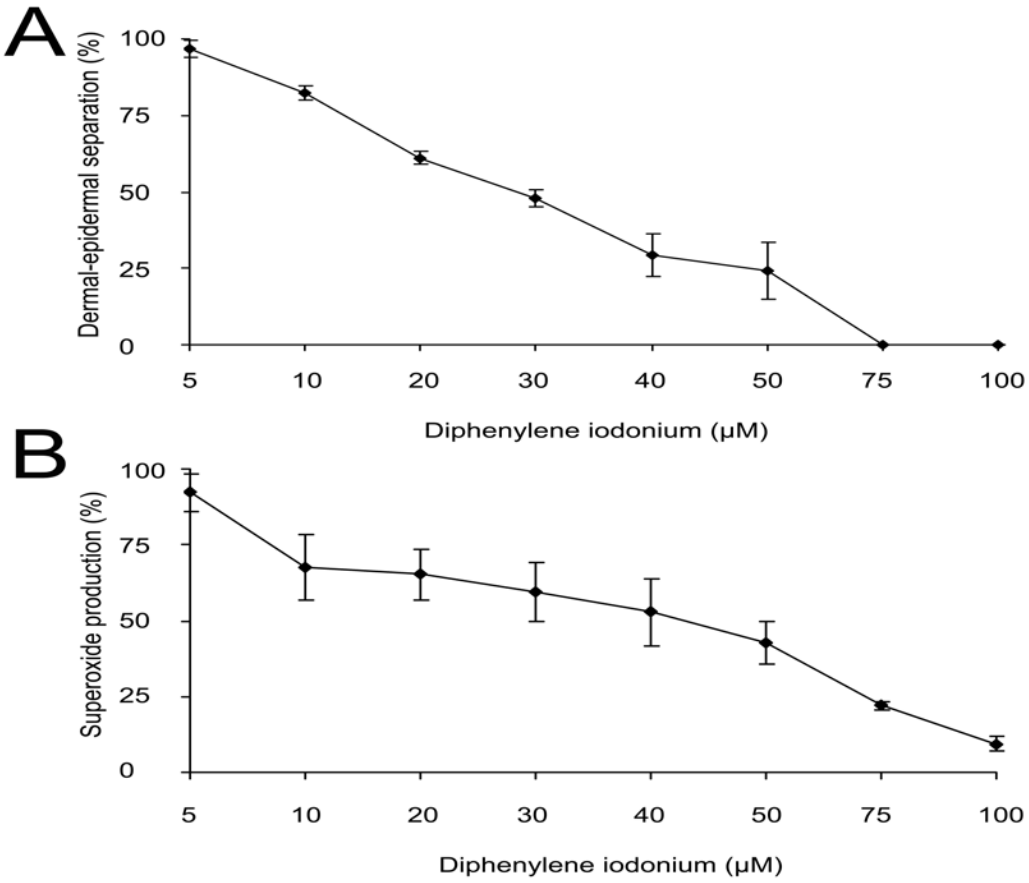

Figure 6 Diphenylene iodonium inhibits both production of superoxide by granulocytes and autoantibody-induced granulocyte-dependent dermal-epidermal separation. (**A**) Cryosections of normal human skin were incubated with IgG purified from patients with epidermolysis bullosa acquisita (n=5) and, subsequently, with leukocytes from healthy donors in the presence of different concentrations of diphenylene iodonium. (**B**) Superoxide production was measured as described in Methods in 10^6 granulocytes stimulated with phorbol 12-myristate 13-acetate in the presence of different concentrations of diphenylene iodonium. All values are means ± s.e.m..

Granulocytes from chronic granulomatous disease (CGD) patients do not mediate autoantibody-induced tissue damage *ex vivo*

Human skin cryosections were incubated with IgG purified from patients with EBA (n=2) and bullous pemphigoid (n=2) and, subsequently, with granulocytes from patients with CGD (n=8) or from healthy donors (n=5) (**Figure 7**). IgG from patients' sera (**Figure 8A,B**), but not from healthy controls (**Figure 8C**), bound to the DEJ as revealed by indirect IF microscopy. Addition of granulocytes from both healthy donors (**Figure 8D**) and CGD patients (**Figure 8E**) resulted in their recruitment at the DEJ in cryosections that had been incubated with patients' autoantibodies. No attachment of leukocytes at the DEJ was found in cryosections incubated with control IgG and granulocytes from healthy donors (**Figure 8F**). When leukocyte suspensions were supplemented with nitroblue tetrazolium, deposits of formazan were observed in cryosections incubated with patients' IgG and granulocytes from healthy donors (**Figure 8G**), but not in those incubated with patients' IgG and CGD granulocytes (**Figure 8H**) or with control IgG and leukocytes from healthy donors (**Figure 8I**). Dermal-epidermal separation was only seen in cryosections incubated with patients' IgG and granulocytes from healthy donors (**Figure 8J**). In contrast, no subepidermal splits were observed in cryosections treated with patients' IgG and CGD granulocytes (**Figure 8K**) or with control IgG and leukocytes from healthy donors (**Figure 8L**).

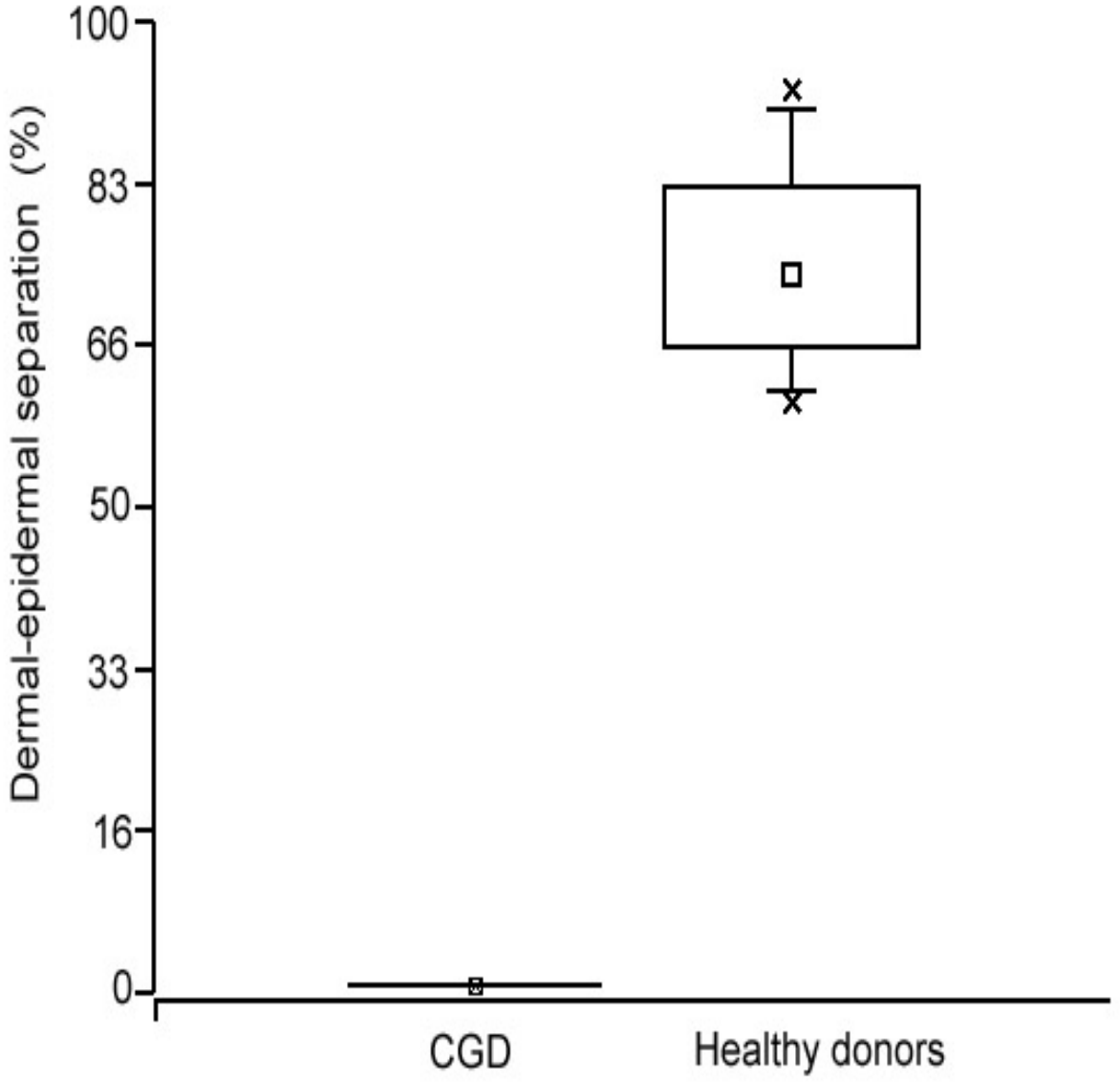

Figure 7 Neutrophils from patients with chronic granulomatous disease (CGD) do not mediate autoantibody-induced dermal-epidermal separation of cryosections. Cryosections of normal human skin were incubated with IgG from patients with EBA (n=2) and bullous pemphigoid (n=2) and subsequently, with $1.5x10^7$ neutrophils from CGD patients or healthy donors for 2 h at 37°C. Dermal-epidermal separation was scored for each section as described in Methods. All values are means ± s.e.m..

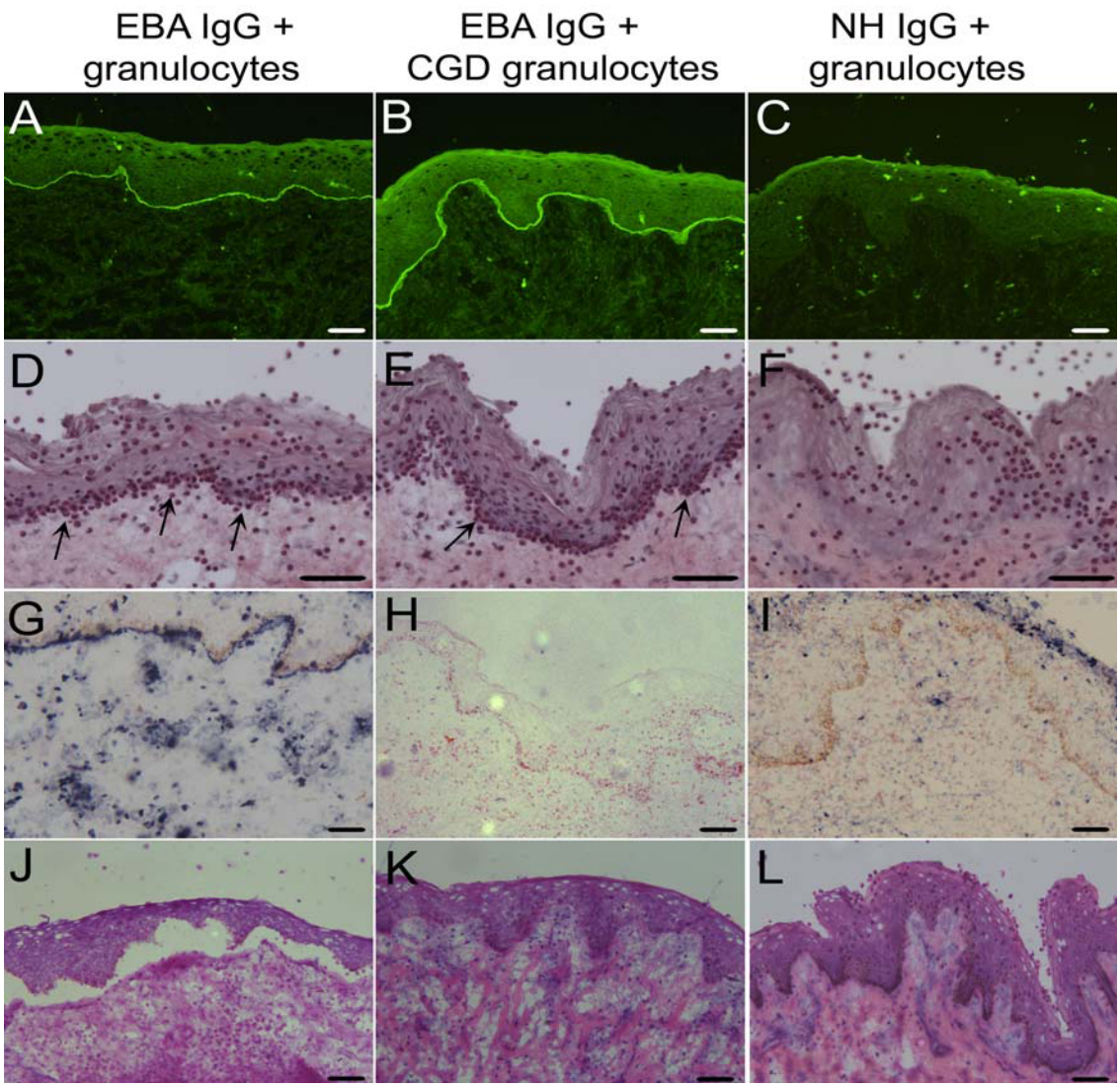

Figure 8 Granulocytes from patients with chronic granulomatous disease are not able to mediate autoantibody-induced tissue damage. Immunofluorescent staining reveals linear IgG deposition at the dermal epidermal junction in cryosections incubated with (**A,B**) 100 µl IgG from an EBA patient (EBA2) but not with (**C**) the same amount of IgG from an healthy control (NH IgG). 1 h incubation of granulocytes from (**D**) a healthy donor or (**E**) a patient with chronic granulomatous disease (CGD #2) results in their attachment to the dermal-epidermal junction (arrows) in cryosections treated with EBA IgG. (**F**) In contrast, no leukocyte recruitment at the dermal-epidermal junction is present in cryosections incubated with control IgG and leukocytes from a healthy donor. In the presence of 0.05% nitroblue tetrazolium, formazan precipitates are found at the dermal-epidermal junction in cryosections treated with (**G**) EBA IgG and granulocytes from a healthy donor, but not with (**H**) EBA IgG and granulocytes from a chronic granulomatous disease patient or with (**I**) normal IgG and granulocytes from a healthy donor. Two h incubation with granulocytes results in dermal-epidermal separation in cryosections treated with (**J**) EBA IgG and granulocytes from a healthy donor. In contrast, no subepidermal splits develop in (**K**) cryosections incubated with EBA IgG and granulocytes from a chronic granulomatous disease patient or (**L**) in cryosections incubated with normal IgG and granulocytes from a healthy donor. Scale bars, all 50 µm.

Granulocytes are required for blistering induced by antibodies against type VII collagen in mice

The chronology of events leading to skin blistering following s.c. injections of antibodies against type VII collagen was documented over a 6-day period in BALB/c mice (n=3 per time point) and are summarized in **Figure 9**. Levels of rabbit antibodies against type VII collagen in serum of mice reached a peak at 6 h after the first injection and did not increase with subsequent injections (**Figure 9A**). Deposition of rabbit IgG and murine C3 at the DEJ was found by direct IF microscopy after the first administration of antibodies against type VII collagen and slightly increased with further injections (**Figure 9B**). Histological examination revealed infiltration of granulocytes and dermal-epidermal separation starting with day 4. Clinically, mice developed extensive blisters and erosions with crusts 5 days after the first injection (**Figure 9B**).

To demonstrate the pathogenic relevance of granulocytes for tissue damage in experimental EBA, we induced and maintained depletion of neutrophils for 6 days by treating mice with the anti-Gr-1 RB6-8C5 antibody. Treatment with the antibody against type VII collagen caused blistering in all BALB/c mice (n=12) treated with the mock rat antibody (**Figure 10A**). By day 6, none of the BALB/c mice injected with the anti-Gr-1 (n=12) and the pathogenic antibody against type VII collagen (**Figure 10B**) or with control rabbit antibody (n=4) (**Figure 10C**) had developed skin alterations. IF microscopy of skin showed deposits of rabbit IgG at the DEJ in all mice (n=6 per group) injected with antibodies against type VII collagen (**Figure 5D,E**), but not in mice treated with control antibody (n=2) (**Figure 10F**). Deposition of C3 was more intense in the skin of mice treated with the mock antibody (**Figure 10G**) compared with deposits in mice that had received anti-Gr-1 antibody (**Figure 10H**), before

treatment with the antibody against type VII collagen. However, this difference in C3 deposition did not reach statistical significance (anti-Gr-1 versus normal rat IgG: 1.83 ± 0.40 versus 1.33 ± 0.33; *p*, 0.36, n = 6 per group). No C3 deposition was found at the DEJ in mice injected with control rabbit antibody (**Figure 10I**). Histological analysis revealed subepidermal cleavage and a neutrophil-rich dermal inflammatory infiltrate in BALB/c mice injected with mock antibody and IgG against type VII collagen (**Figure 10J**), but not in mice receiving anti-Gr-1 antibody and antibodies against type VII collagen (**Figure 10K**) or treated with normal rabbit IgG (**Figure 10L**). Despite repeated administration of anti-Gr-1 antibody, neutropenia could not be maintained for longer than 7 days, after which the neutrophil counts in peripheral blood gradually increased up to 80% of baseline levels by day 13 (not shown). Starting with day 7, similarly to BALB/c mice (n=6) injected with mock antibody and IgG against type VII collagen, BALB/c mice (n=6) treated with anti-Gr-1 and antibodies against type VII collagen also developed skin blistering (**Figure 11A**). Histological analysis at day 12 showed subepidermal cleavage and a neutrophil-rich inflammatory infiltrate in all BALB/c mice injected IgG against type VII collagen irrespective of the administration of the anti-Gr1 or mock antibody (not shown). In serum of all mice injected with IgG against type VII collagen, levels of circulating antibodies specific for this autoantigen were comparable (**Figure 11B**). At day 13, infiltration of granulocytes was detected in the skin of mice treated with the anti-Gr-1 antibody as assessed by measuring the MPO activity (**Figure 11C**).

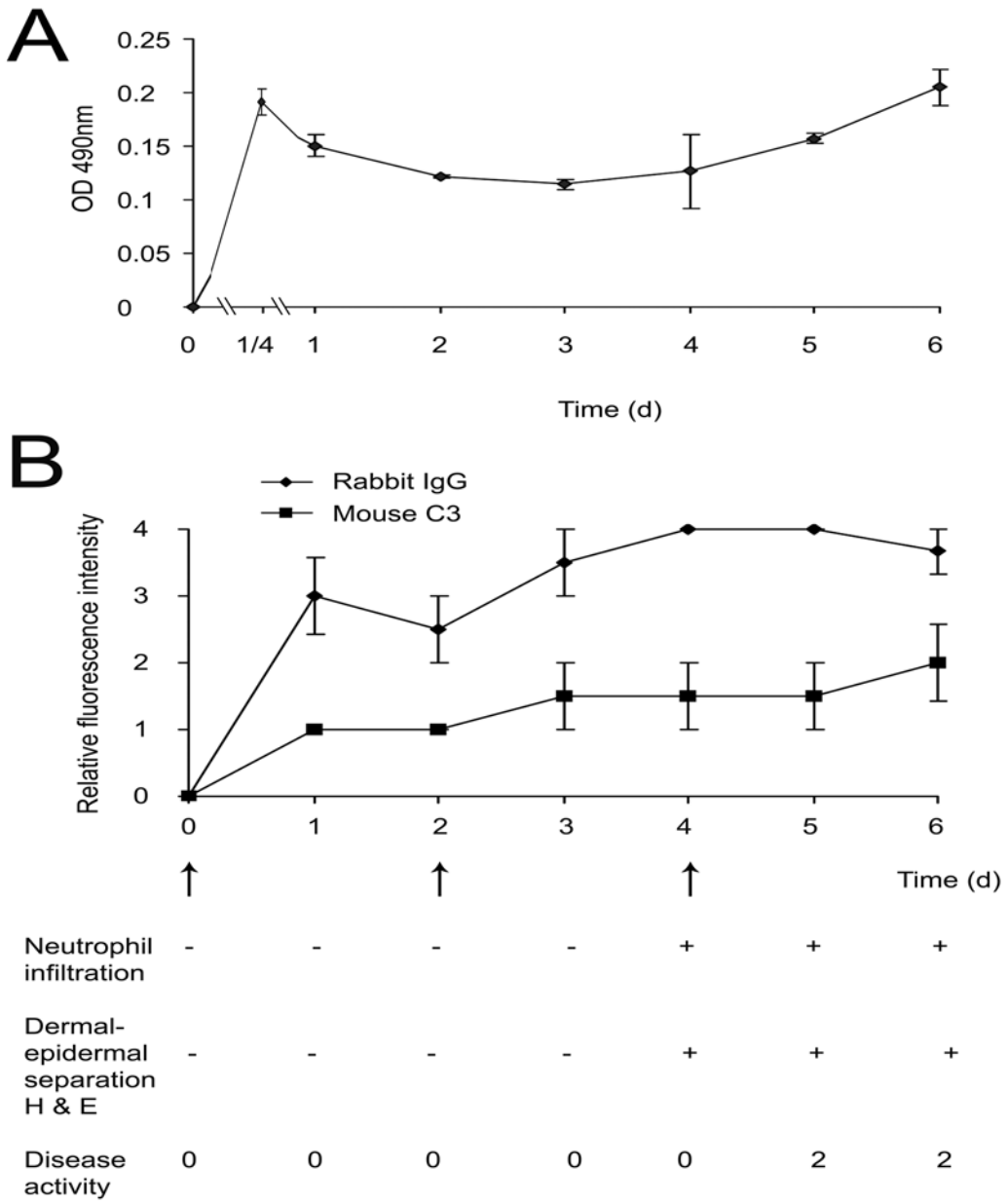

Figure 9 Time course of antibody binding, complement activation, and skin blistering. BALB/c mice were injected with 7.5 µl/g body weight rabbit antibody against type VII collagen every second day and evaluated daily for skin lesions. (**A**) Levels of antibodies to type VII collagen in serum of mice (n=3 per time point) were detected by ELISA using recombinant antigen as described in Methods. (**B**) Biopsies of perilesional or normal appearing skin, obtained each day from 3 mice, were evaluated for IgG and complement C3 deposits at the epidermal basement membrane as described in Methods. Biopsies of the ears from the same mice processed for histopathology were assessed for the presence of neutrophil infiltrates and dermal-epidermal separation. Disease activity was scored as described in Methods. All values are means ± s.e.m.

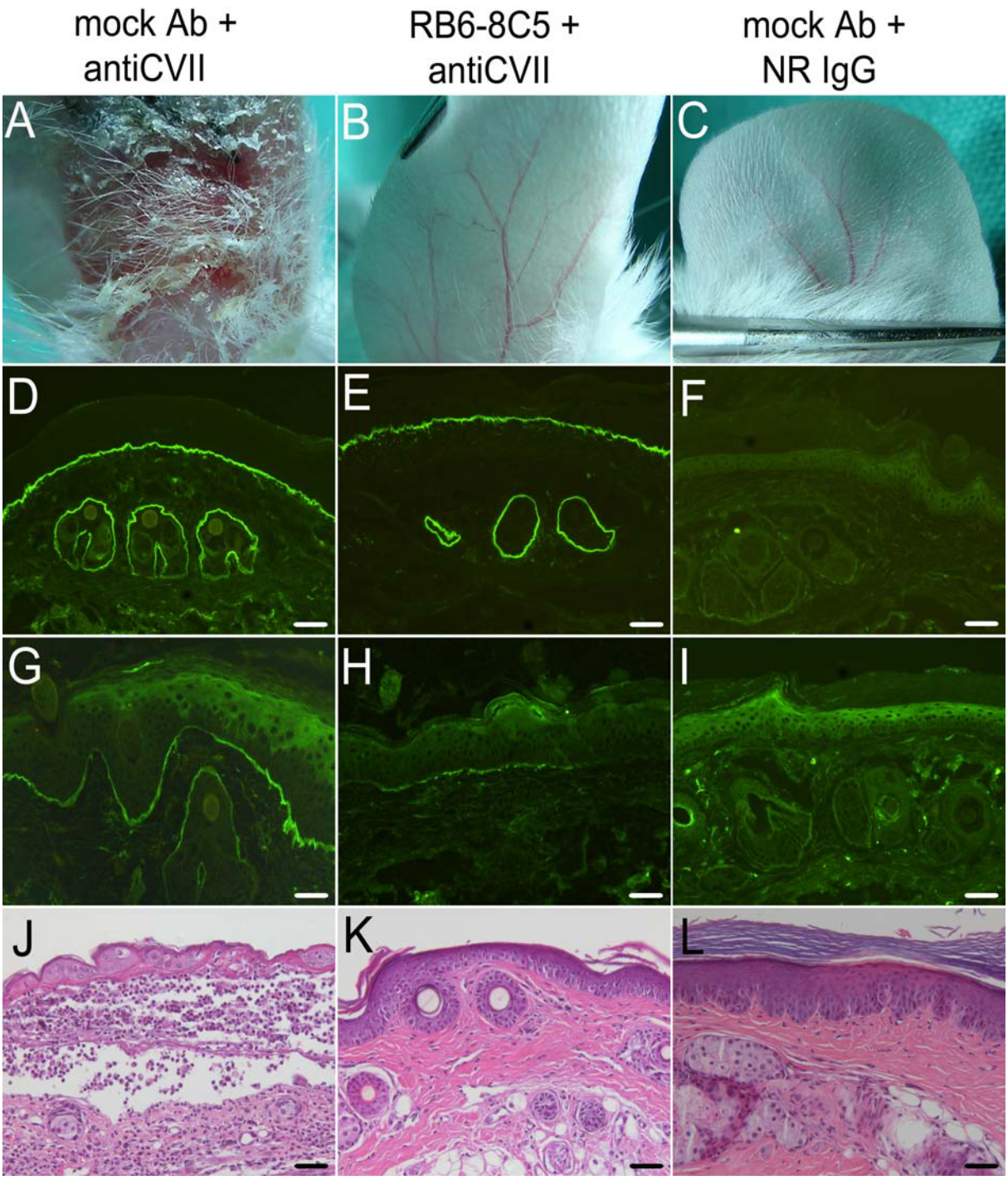

Figure 10 Neutrophil depletion protects mice from skin blistering induced by antibodies to type VII collagen. BALB/c mice were injected with 0.25 mg of the depleting rat anti-Gr-1 RB6-8C5 antibody or control rat IgG 24h before the first injection of rabbit IgG and at 3 days interval thereafter. (**A**) Injection of a total dose of 45 µl/g body weight of IgG against type VII collagen (antiCVII) results in extensive skin lesions, including blisters, erosions, partly covered by crusts, and epidermal detachment on ears in a BALB/c mouse treated with the mock rat antibody. (**B**) A BALB/c mouse injected with pathogenic IgG against type VII collagen and RB6-8C5, like (**C**) a mouse injected with control rabbit IgG, did not develop skin lesions. Immunofluorescence microscopy of murine skin shows linear deposits of rabbit IgG at the dermal-epidermal junction in all mice injected with (**D,E**) antibodies to type VII

collagen, but not with (**F**) control antibody. Complement C3 deposits are found at the dermal-epidermal junction of (**G,H**) mice receiving the antibody against type VII collagen, but not in (**I**) the mouse injected with control rabbit antibody. Histological analysis of murine skin reveals subepidermal cleavage and a neutrophil-rich inflammatory infiltrate in a (**J**) BALB/c injected with IgG against type VII collagen and mock rat antibody, but not in a (**K**) BALB/c mouse, receiving antibodies to type VII collagen and rat anti-Gr1 RB6-8C5. Normal histological appearance in a (**L**) BALB/c mouse treated with control antibody. Scale bars, all 50 μm.

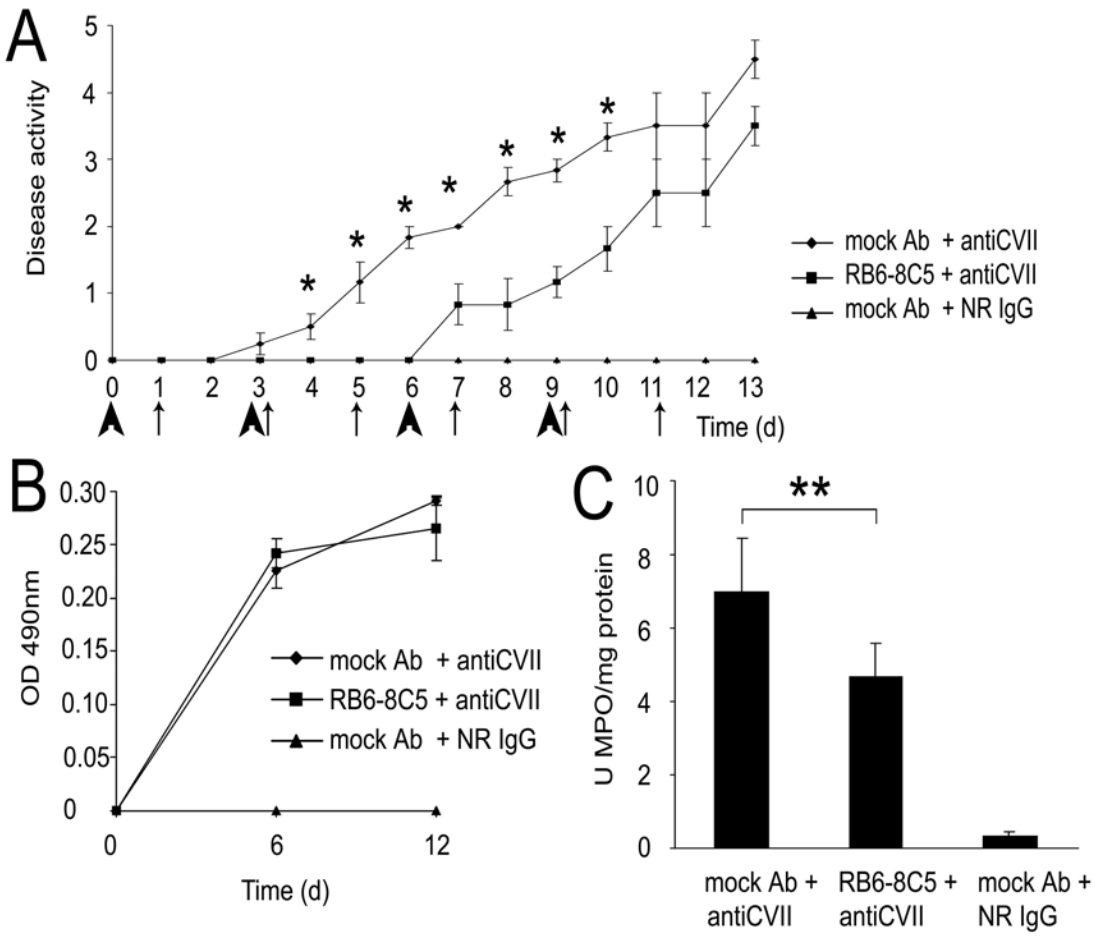

Figure 11 Reappearance of granulocytes in the peripheral blood is associated with recruitment of granulocytes into murine skin and onset of skin blisters. BALB/c mice were injected with the Gr-1 specific RB6-8C5 antibody (n=6) or with a mock antibody (arrow heads) (n=6) and with anti mouse type VII collagen (antiCVII) or normal rabbit IgG (NR IgG) (arrows) (n=4). (**A**) Clinical disease severity was assessed in mice as described in Methods. *, represents significant difference of disease severity between the 2 groups ($p, < 0.05$). (**B**) Levels of circulating antibodies against type VII collagen in serum of injected mice were measured by an ELISA using recombinant type VII collagen as described in Methods. (**C**) Neutrophil infiltrates in the skin of mice treated with the anti-Gr-1 or mock antibody as assessed by measuring myeloperoxidase (MPO) activity in skin biopsies as described in Methods (** significant difference, *p*, 0.0026). All values are means ± s.e.m..

Emigration of granulocytes into the dermis has been shown to depend on CD11/CD18 and $CD18^{-/-}$ granulocytes cannot be recruited into the inflamed dermis(Mizgerd *et al.*, 1997). To provide further evidence for a pathogenetic involvement of granulocytes in experimental EBA, we injected mice deficient in beta2 integrins ($CD18^{-/-}$) with antibodies against type VII collagen. In contrast to wild type mice (n=10) injected with IgG against type VII collagen, $CD18^{-/-}$ mice (n=10) injected with IgG against type VII collagen, like wild type mice (n=5) treated with control IgG did not develop skin blistering. Despite blood neutrophilia and comparable levels of antibodies against type VII collagen, granulocytes were not recruited into the dermis of the $CD18^{-/-}$ mice injected with IgG against type VII collagen as demonstrated by histological analysis and measurements of the MPO activity in skin biopsies (**Figure 12**). Bypassing the emigration of granulocytes into the dermis by injection of $CD18^{+/+}$ granulocytes s.c. into $CD18^{-/-}$ mice (n=5) treated with antibody against type VII collagen did result in subepidermal blistering (**Figure 13**).

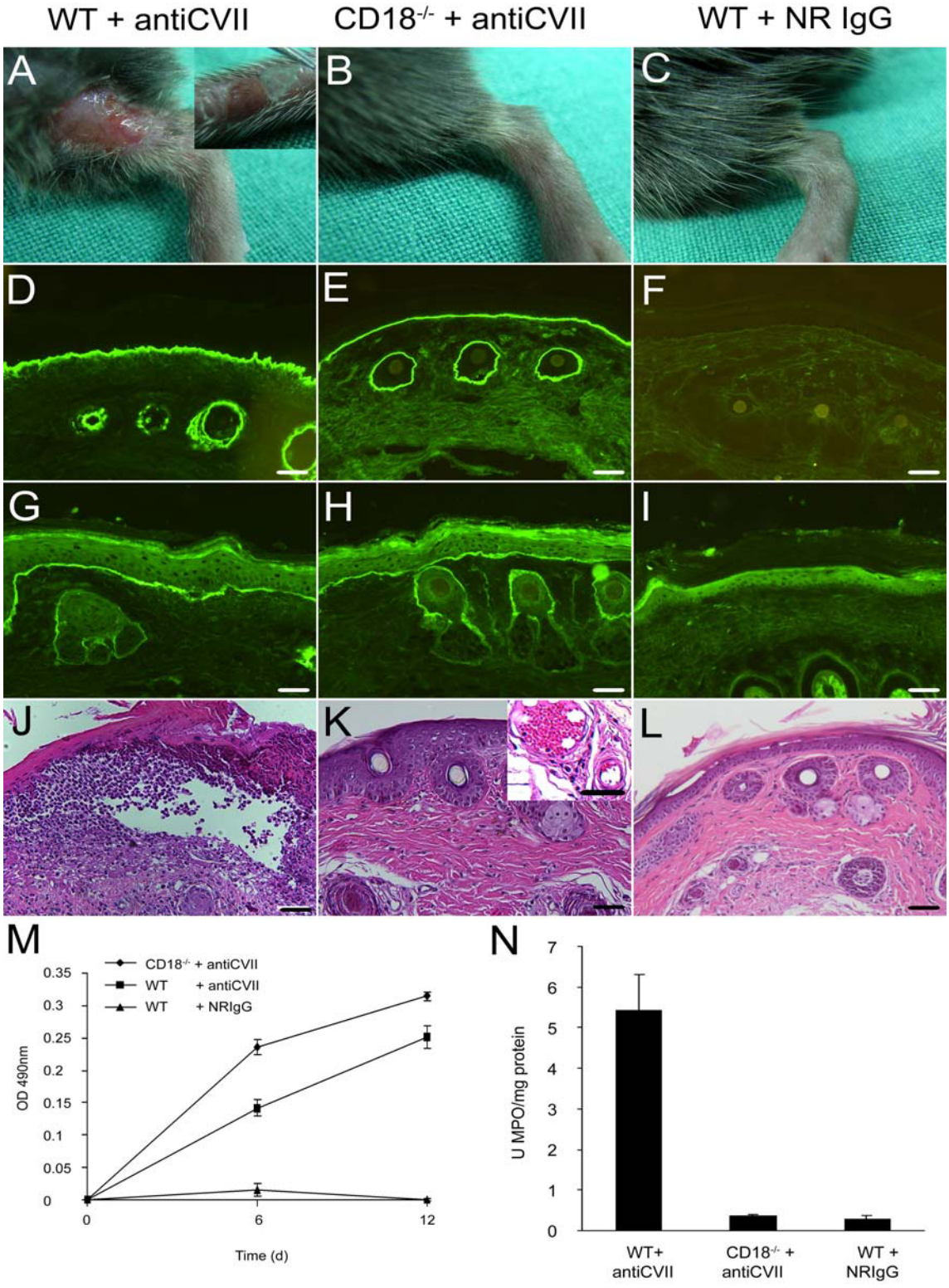

Figure 12 CD18$^{-/-}$ mice are resistant to blister induction by antibodies against type VII collagen. (**A**) Injection of a total dose of 45 µl/g body weight IgG against type VII collagen (antiCVII) results in extensive skin lesions, including blisters, erosions, partly covered by crusts, and epidermal detachment on the hind leg and (**inset**) tail in a C57BL/6J mouse. (**B**) A CD18$^{-/-}$ mouse injected with the same dose of pathogenic IgG against type VII collagen, like (**C**) a C57BL/6J mouse injected with IgG from preimmune rabbit serum, did not develop skin lesions. Immunofluorescence

microscopy of murine skin shows linear deposits of rabbit IgG at the dermal-epidermal junction in mice injected with (**D,E**) antibodies to type VII collagen, but not with (**F**) control antibody. Staining for complement was found at the dermal-epidermal junction in mice injected with (**G,H**) antibodies to type VII collagen, but not with (**I**) control antibody. Histological analysis of lesional murine skin revealed subepidermal cleavage and a neutrophil-rich inflammatory infiltrate in a (**J**) C57BL/6J, but not in a (**K**) CD18$^{-/-}$ mouse receiving antibodies to type VII collagen. (**K, inset**) Note the presence of leukocytes trapped in blood vessels. (**L**) Normal histological appearance in a C57BL/6J mouse treated with control antibody. Scale bars, all 50 µm. (**M**) Circulating antibodies were comparable in wild type (WT) and CD18 -/- mice receiving antibodies against type VII collagen as showed by ELISA using recombinant antigen. (**N**) In contrast to CD18 $^{-/-}$ mice, wild type (WT) animals show high skin infiltration with granulocytes as evaluated by the myeloperoxidase (MPO) assay. All values are means ± s.e.m..

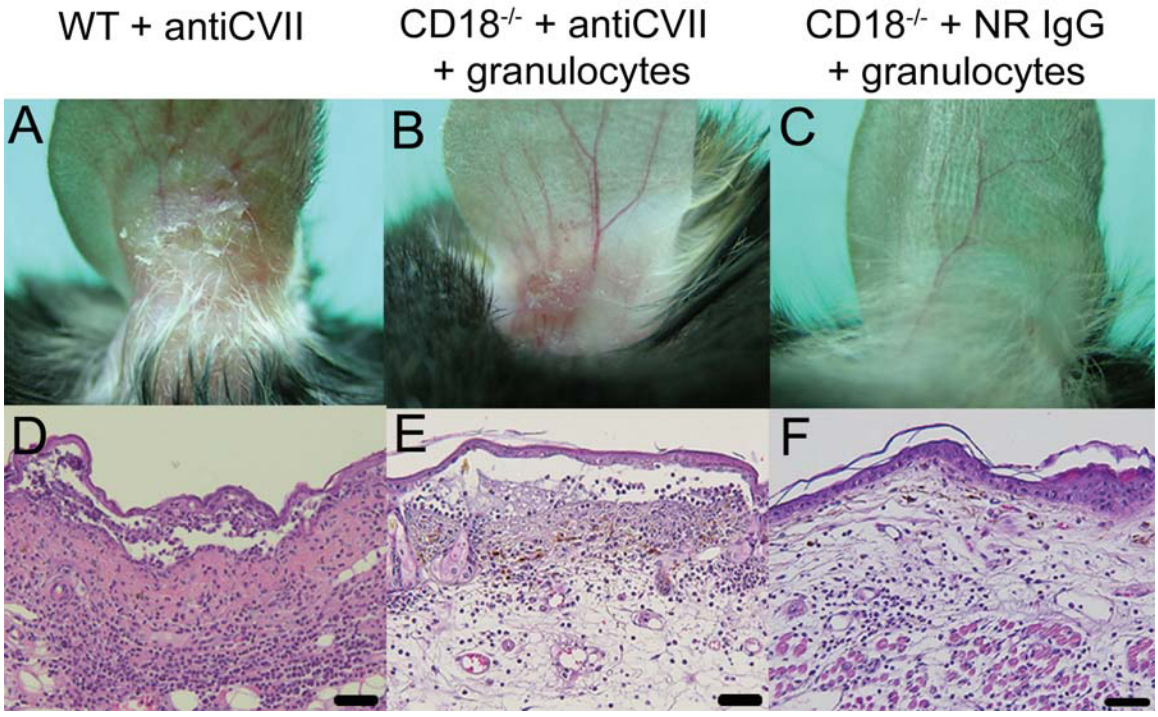

Figure 13 CD18$^{-/-}$ mice reconstituted with CD18$^{+/+}$ granulocytes are susceptible to antibody-induced skin blistering. Mice were injected with 2x10^{6} Ncf1$^{+/+}$ murine granulocytes in 50 µl medium s.c. in the ears. Like a (**A**) wild type mouse injected with antibodies against type VII collagen (antiCVII), a (**B**) CD18$^{-/-}$ mouse injected with antibodies against type VII collagen and locally reconstituted with CD18$^{+/+}$ granulocytes develops blisters and erosions on its ears. (**C**) In contrast, a CD18$^{-/-}$ mouse treated with control rabbit IgG (NR IgG) and reconstituted with CD18$^{+/+}$ granulocytes does not show skin alterations. (**D,E,F**) Histological analysis reveals presence of neutrophils in the dermis of all mice. Dermal-epidermal separation is found in the (**D**) wild type mouse injected with antibodies against type VII collagen and in the (**E**) CD18$^{-/-}$ mouse injected with antibodies against type VII collagen and locally reconstituted with CD18$^{+/+}$ granulocytes, but not in a (**F**) CD18$^{-/-}$ mouse treated with control rabbit IgG and reconstituted with CD18$^{+/+}$ granulocytes. Scale bars, all 50 µm.

Ncf1$^{-/-}$ mice reconstituted with Ncf1$^{+/+}$ granulocytes develop blisters following injection of antibodies to type VII collagen

To demonstrate that neutrophils are the cellular source of Ncf1 required for antibody-induced tissue damage, Ncf1$^{-/-}$ mice were treated with antibodies against type VII collagen and injected s.c. at day 6 with granulocytes purified from Ncf1$^{-/-}$ or Ncf1$^{+/+}$ mice. Ncf1$^{-/-}$ mice injected with antibodies against type VII collagen did not develop blistering when reconstituted with Ncf1$^{-/-}$ granulocytes (n=5) (**Figure 14A**), whereas transfer of Ncf1$^{+/+}$ granulocytes (n=5) resulted in blisters and erosions on their ears (**Figure 14B**). In contrast, Ncf1$^{-/-}$ mice (n=5) treated with control rabbit IgG and reconstituted with Ncf1$^{+/+}$ granulocytes did not show skin alterations (**Figure 14C**). Histological analysis revealed infiltrates of neutrophils in the dermis of all mice (**Figure 14D,E,F**). In Ncf1$^{-/-}$ mice injected with antibodies against type VII collagen, in contrast to transfer of Ncf1$^{-/-}$ granulocytes (**Figure 14D**), administration of Ncf1$^{+/+}$ granulocytes (**Figure 14E**) resulted in dermal-epidermal separation. No subepidermal cleavage was seen in Ncf1$^{-/-}$ mice treated with control IgG and reconstituted with Ncf1$^{+/+}$ granulocytes (**Figure 14F**).

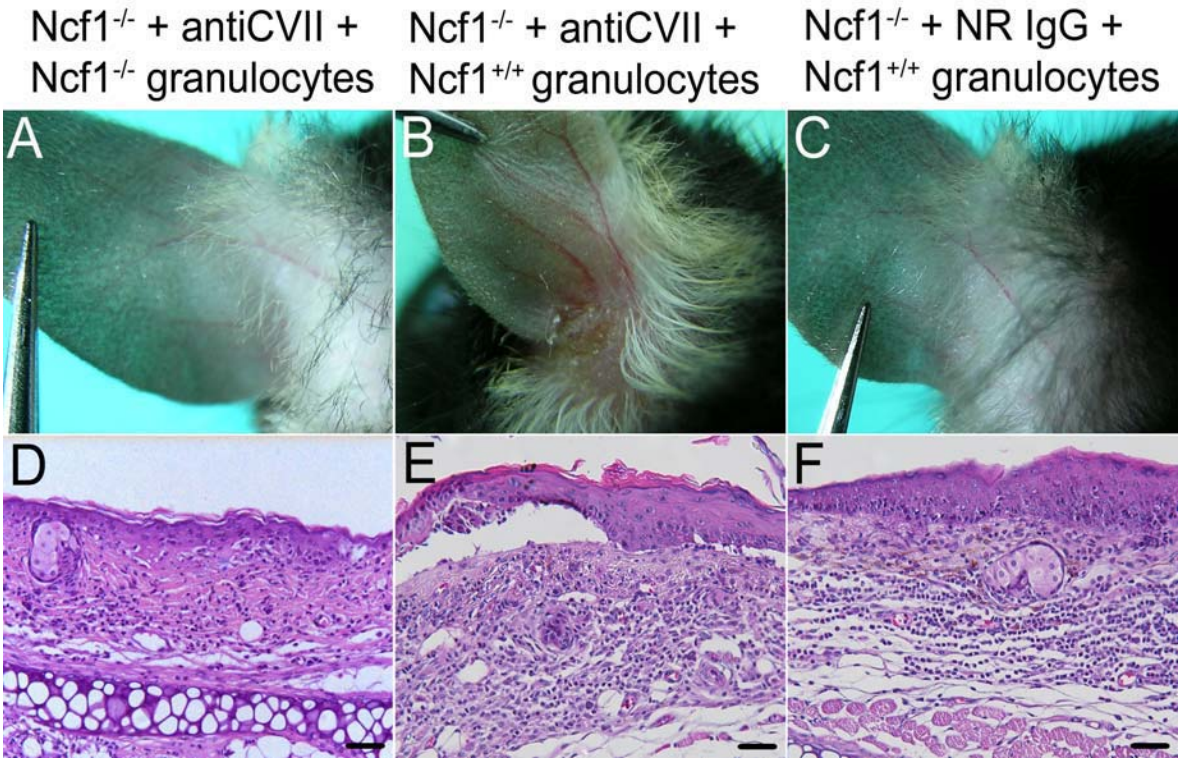

Figure 14 Reconstitution with Ncf1$^{+/+}$ granulocytes renders Ncf1$^{-/-}$ mice susceptible to antibody-induced skin blistering. Mice were treated with the antibody against type VII collagen (antiCVII) or control antibody (NR IgG) and injected at day 6 with 2x10^{6} Ncf1$^{-/-}$ or Ncf1$^{+/+}$ murine granulocytes in 50 µl medium s.c. in the ears. In Ncf1$^{-/-}$ mice injected with antibodies against type VII collagen (**A**) local reconstitution with Ncf1$^{-/-}$ granulocytes does not result in skin lesions, whereas (**B**) reconstitution with Ncf1$^{+/+}$ granulocytes induces blisters and erosions on their ears. (**C**) A Ncf1$^{-/-}$ mouse treated with control rabbit IgG and reconstituted with Ncf1$^{+/+}$ granulocytes does not show skin alterations. (**D,E,F**) Histological analysis reveals an infiltrate of neutrophils in the dermis of all mice. In animals injected with antibodies against type VII collagen, dermal-epidermal separation is not observed in the Ncf1$^{-/-}$ mouse reconstituted with (**D**) Ncf1$^{-/-}$ granulocytes, but occurs in (**E**) the Ncf1$^{-/-}$ reconstituted with Ncf1$^{+/+}$ granulocytes. (**F**) The Ncf1$^{-/-}$ mouse treated with control rabbit IgG and reconstituted with Ncf1$^{+/+}$ granulocytes shows no dermal-epidermal separation. Scale bars, all 50 µm.

Discussion

ROS produced by tissue infiltrating leukocytes are believed to contribute significantly to the pathogenesis of several inflammatory diseases (Weiss, 1989). However, the capacity of NADPH oxidase-derived ROS to mediate tissue damage in autoimmune inflammatory diseases has been controversial as it is unclear whether activation of NADPH oxidase protects from or augments tissue damage. In addition, the relevance of NADPH oxidase for the afferent or the efferent limb of the autoimmune response has not yet been characterized. To address these questions, we here used experimental models of blistering induced by passive transfer of antibodies against type VII collagen, which represent the T cell-independent inflammatory phase in EBA. We demonstrate that gene defects or pharmacological inhibition of NADPH oxidase abolish blistering induced by autoantibodies and mediated by granulocytes both *in vivo* and *ex vivo*.

Some functions of phagocyte NADPH oxidase have been firmly established. The enzyme is clearly essential in immune defense as demonstrated by life threatening infections with certain bacteria and fungi in both human and experimental CGD (Baehner and Karnovsky, 1968; Jackson *et al.*, 1995; Pollock *et al.*, 1995; Huang *et al.*, 2000). However, studies in animal models of autoimmune diseases led to conflicting results with regard to the role of NADPH oxidase in disease pathogenesis. In the T cell-mediated EAE, Ncf1 knock-out B6/129 mice were found resistant to myelin oligodendroglial glycoprotein (MOG) peptide-induced disease (van der Veen *et al.*, 2000), whereas Ncf1 naturally mutated mice immunized with native MOG protein developed an enhanced autoimmune phenotype (Hultqvist *et al.*, 2004).

Irrespective of its stimulatory or inhibitory effects, Ncf1 is thought to modulate the level of T cell autoimmune responses in EAE (Hultqvist *et al.*, 2004; Hultqvist and Holmdahl, 2005).

In animal models of arthritis, different effects of NADPH oxidase on disease expression were reported. Scavenging the NADPH oxidase-derived superoxide using superoxide dismutase or its nonpeptidyl mimetic resulted in amelioration of arthritis induced by streptococcal cell walls, adjuvant or immunization with type II collagen (Schalkwijk *et al.*, 1985; Skaleric *et al.*, 1991; Shingu *et al.*, 1994; Salvemini *et al.*, 1999). In addition, collagen-induced arthritis is enhanced in mice genetically deficient in extracellular superoxide dismutase (Ross *et al.*, 2004). NADPH oxidase-derived ROS induce chondrocyte death and aggravates metalloproteinases-mediated cartilage destruction in interferon gamma-stimulated immune complex arthritis (van Lent *et al.*, 2005). Interestingly, NADPH oxidase deficiency has no effect on the K/BxN serum transfer arthritis (Wipke and Allen, 2001) but causes increased connective tissue destruction in zymosan and immune complex-mediated arthritis in mice (van de Loo *et al.*, 2003). The Ncf1 gene was found to be responsible for the severity of arthritis in rats (Olofsson *et al.*, 2003) and a natural mutation is associated with an enhanced disease phenotype in mice (Hultqvist *et al.*, 2004). These different effects of NADPH oxidase-derived ROS inhibition on the outcome of experimental autoimmune diseases still need to be elucidated. In this context, our present study clearly demonstrates that NADPH oxidase is a key effector of granulocyte-dependent tissue injury triggered by autoantibodies with specificity for the diseased organ.

In a first set of experiments, we delineated the relevance of NADPH oxidase for antibody-induced blistering *in vivo* using Ncf1$^{-/-}$ mice injected with antibodies against type VII collagen. The resistance of these mice to experimental EBA demonstrates that NADPH oxidase is required for the antibody-mediated tissue destruction in this model and strongly suggests that ROS cause tissue damage in antibody-dependent inflammatory diseases. In line with our present results, platelet NADPH oxidase-derived ROS have been shown to be a prerequisite for the thrombocytopenia induced by antibodies against the platelet membrane GPIIIa49-66 (Nardi *et al.*, 2001). In contrast to this condition, in experimental EBA, infiltrating granulocytes, but not cells of target tissue, constitute the main cellular source of NADPH oxidase required for tissue injury.

While the intracellular destruction of phagocytosed particles by CGD granulocytes is clearly impaired (Baehner and Karnovsky, 1968; Katz *et al.*, 1980), the capacity of these cells to mediate antibody-dependent cellular cytotoxicity (ADCC) is controversial. In some studies, CGD granulocytes showed an impaired capacity of ADCC (Borregaard and Kragballe, 1980; Weiss *et al.*, 1981; Miller and Kohl, 1983; Lichtenstein *et al.*, 1989). In other studies, granulocytes from healthy donors and CGD patients were found equally effective in mediating ADCC, especially in the extracellular killing of targets too large to be phagocytosed (Siebens *et al.*, 1979; Katz *et al.*, 1980; van Kessel *et al.*, 1990; Kushner and Cheung, 1991). In our system, granulocytes are recruited by IgG autoantibodies to the dermal-epidermal junction and induce subepidermal splits by releasing proteases that degrade the extracellular matrix (Sitaru *et al.*, 2002a; Shimanovich *et al.*, 2004). Our present observations indicate that destruction of the extracellular matrix by granulocytes

resulting in a disease-specific effect is dependent on functional NADPH oxidase. Exceeding a threshold of ROS production appears to be necessary for blistering because CGD neutrophils with residual NADPH oxidase activity did not cause dermal-epidermal separation. To support these findings, we chose another strategy using the specific flavoprotein inhibitor, diphenylene iodonium which irreversibly binds the enzyme thus blocking the transfer of electrons across the membrane (Cross and Jones, 1986) and the formation of the superoxide anion. Not surprisingly, different concentrations of diphenylene iodonium had a dose-dependent capacity of inhibiting blistering in our model. We found a strong correlation between diphenylene iodonium concentrations and the extent of the dermal-epidermal separation, but concentrations that only partially block superoxide production also cannot completely abolish dermal-epidermal separation.

Neutrophil infiltration at sites of blistering is often seen in patients with EBA (Fine *et al.*, 1989) and in mice immunized against type VII collagen (Sitaru *et al.*, 2006) and is a characteristic feature of diseased mice injected with antibodies against type VII collagen (Sitaru *et al.*, 2005). Neutrophils have been implicated as critical cellular effectors in several other diseases mediated by antibodies (Liu *et al.*, 1997; Wipke and Allen, 2001; Girardi *et al.*, 2003; Nandakumar *et al.*, 2003; Looney *et al.*, 2006). It was suggested that granulocytes may also play an indirect role in tissue destruction seen in EAE, i.e. in orchestrating the recruitment of other mononuclear cells which will subsequently damage the target organ (McColl *et al.*, 1998).

Since granulocytes are the richest source of superoxide anion and were present at sites of blistering, in further experiments, we addressed the role of these cells for

blistering in our *in vivo* experimental model. We hypothesized that in experimental EBA, antibodies bound to type VII collagen at the dermal-epidermal junction activate the complement system and subsequently recruit and activate leukocytes into the upper dermis, thus directing the release of inflammatory mediators, reactive oxygen species and proteases resulting in subepidermal blistering *in vivo*. To provide direct evidence for a *sine qua non* role of granulocytes in experimental EBA, we depleted mice of neutrophils using a monoclonal antibody specific to Gr-1 (Hestdal *et al.*, 1991; Tepper *et al.*, 1992; Conlan and North, 1994). By day 6, none of the mice injected with the depleting Ab. had circulating granulocytes and subsequent injections of IgG against type VII collagen did not lead to skin blistering. In contrast, mice receiving a mock Ab. and subsequently pathogenic IgG against type VII collagen developed typical EBA lesions both clinically and histologically. Interestingly, irrespectively of neutrophil depletion, all mice injected with antibodies against type VII collagen showed almost similar *in situ* deposits of rabbit IgG, murine C3 and circulating specific antibodies as detected by ELISA with recombinant protein. However, starting with day 7, granulocytes reappeared in the peripheral blood, their counts steadily increased, and reached almost baseline levels by the end of the experiment, despite further injections of anti-Gr-1 IgG. These findings are in accordance with previous reports (Jensen *et al.*, 1993; Chen *et al.*, 2000) and might be due to the generation of blocking mouse anti-rat IgG antibodies (Stephens-Romero *et al.*, 2005). But most importantly, reappearance of neutrophils in the peripheral blood was associated with the onset of skin blistering in the mice that were free of lesions until day 7 and disease severity in these mice paralleled the raise in granulocyte counts. In addition, starting with the same day 7, neutrophils were also found in the skin of these mice by histopathology examination and by measuring the

myeloperoxidase activity in skin samples.

Organ specific autoimmune diseases need effector mechanism present it the target tissues. Granulocyte infiltration into these target organs has been demonstrated to be a prerequisite for tissue damage in experimental models of acute lung injury, rheumatoid arthritis and bullous pemphigoid (Liu *et al.*, 1997; Wipke and Allen, 2001; Looney *et al.*, 2006). Granulocyte emigration requires CD11/CD18 complexes. In humans with leukocyte adhesion deficiency type 1, a diseased caused by mutations in the gene for CD18, leukocytes lacking a functional CD11/CD18 integrin cannot migrate to infected gingival, periodontal, peritoneal or dermal sites. Moreover, antibodies against components of this complex inhibit neutrophil emigration during acute infiltration in animals (Arfors *et al.*, 1987; Doerschuk *et al.*, 1990; Liu *et al.*, 2006). Granulocytes from mice with a knock-out for the CD18 subunit are incapable of emigrating in the inflamed skin during induced acute dermatitis (Mizgerd *et al.*, 1997; Scharffetter-Kochanek *et al.*, 1998). To provide additional support for the pathogenic relevance of neutrophils in experimental EBA, we injected mice deficient in CD18 with antibodies against type VII collagen. The antibodies bound to the dermal-epidermal junction activating complement and their serum levels as shown by ELISA were similar to levels in control WT mice receiving IgG against type VII collagen. However, histological examination revealed neither granulocytic infiltration nor dermal-epidermal separation. The lack of granulocytic infiltration in the dermis of CD18 deficient mice was confirmed by the low myeloperoxidase score of extracted skin samples. None of the deficient mice had any clinical lesions of experimental EBA.

Since an impaired recruitment of neutrophils into the dermis abolishes blister formation in CD18-deficient mice, bypassing the defective emigration by local reconstitution with granulocytes should restore the susceptibility of these mice to experimental EBA. We performed this experiment and, indeed, injection of granulocytes into the ears of CD18-deficient mice treated with antibodies against against type VII collagen resulted in blisters and erosions. The DES was accompanied by recruitment of injected granulocytes into the upper dermis. In contrast, $CD18^{-/-}$ mice injected with NR IgG and locally reconstituted with granulocytes do not show any features of EBA despite granulocytes' presence in the dermis.

Even though NADPH oxidase-deficiency was not reported to reduce chemotaxis of granulocytes, inflammatory infiltrates in our present study were significantly lower in $Ncf1^{-/-}$ mice. This reduction of granulocyte recruitment into the dermis may well be an indirect effect related to a reduction of chemoattractant generation. This observation also raised the question whether $Ncf1^{-/-}$ granulocytes were able to produce blistering *in vivo*. However, large numbers of $Ncf1^{-/-}$ granulocytes injected intradermally did not induce blisters. Finally, the transfer of $Ncf1^{+/+}$ granulocytes into $Ncf1^{-/-}$ mice, injected with antibodies against type VII collagen, demonstrated that granulocytes provide the NADPH oxidase required for antibody-induced blistering. Potential non-mutually exclusive mechanisms by which granulocyte-derived NADPH oxidase mediates tissue damage in antibody-mediated inflammatory diseases include: 1) amplification of Fc-mediated neutrophil activation; 2) structural changes of extracellular matrix proteins by ROS resulting in loss of skin adhesive function; 3) activation of latent metalloproteinases; and a 4) direct or indirect inactivation of protease inhibitors.

Granulocyte proteases such as gelatinase B and elastase are required for tissue damage in other inflammatory diseases (Liu *et al.*, 1998; Liu *et al.*, 2000; Opdenakker *et al.*, 2001). It is therefore tempting to speculate at this stage that modulation of their activity by ROS (Weiss *et al.*, 1985; Reddy *et al.*, 1989) is a critical pathogenetic factor in the experimental models used in this study; 5) superoxide generated by the NADPH oxidase can be then converted to other reactive oxygen species including hydrogen peroxide and hypochlorous acid that have been also reported to mediate destruction in inflammatory conditions (Weiss *et al.*, 1981; Weiss *et al.*, 1982). Actually these reactive oxygen species and some of the enzymes implicated in the reaction cascade initiated by the NADPH oxidase are currently under investigation and first results suggest an important role for hypochlorous acid whereas catalase and superoxide-dismutase have been found incapable of protecting against blister formation *ex vivo* (Chiriac *et al.*, 2005). However, further studies *in vivo* should elucidate their precise role.

In conclusion, our study demonstrates that gene defects or pharmacological inhibition of NADPH oxidase abolish blistering induced by (auto)antibodies and granulocytes both *in vivo* and *ex vivo.* Future studies will focus on transferring these mechanistic insights into interventions aiming at arresting the deleterious effects triggered by autoantibodies in inflammatory diseases.

Conclusion

Major advances in our understanding of the pathogenesis of autoimmune blistering diseases have been made over the past 25 years. Many mechanisms have been proposed and some accepted for being implicated in the tissue damage. However, some important members of the pathogenetic chain starting with the production of autoantibodies and ending up with structure's destruction are still being under investigation. Recently, the importance of ROS in antibody-induced tissue damage has been the focus of intense debate and different groups presented contradictory results some sustaining a pro and some a contra effect of ROS. Using both *in vivo* and *ex vivo* models of EBA, we were able to show that NADPH and its major product, i.e. the superoxide anion play a critical role in tissues damage induced by autoantibodies from EBA and BP patients. Our study should facilitate the development of innovative better tolerable therapies for patients suffering from autoantibody-induced autoimmune diseases.

Summary

The contribution of phagocyte-derived reactive oxygen species to tissue injury in autoimmune inflammatory diseases is unclear. Here we report that granulocyte nicotinamide adenine dinucleotide phosphate (NADPH) oxidase crucially contributes to tissue injury in experimental models of the antibody-mediated autoimmune disease epidermolysis bullosa acquisita. Neutrophil cytosolic factor 1-deficient mice lacking functional NADPH oxidase were resistant to skin blistering by the passive transfer of antibodies against type VII collagen. Pharmacological inhibition or deficiency of human NADPH oxidase abolished dermal-epidermal separation caused by autoantibodies and granulocytes *ex vivo*. In addition, recruitment of granulocytes into the skin was required for tissue injury as demonstrated by the resistance to experimental blistering of wild type mice depleted of neutrophils and of CD18-deficient mice. Transfer of neutrophil cytosolic factor 1-sufficient granulocytes into neutrophil cytosolic factor 1-deficient mice demonstrated that granulocytes provide the NADPH oxidase required for tissue damage. Our findings identify granulocyte-derived NADPH oxidase as key molecular effector engaged by pathogenic autoantibodies and provide relevant targets for prevention of tissue damage in granulocyte-mediated autoimmune diseases.

Zusammenfassung

Die Bedeutung der von neutrophilen Granulozyten produzierten Sauerstoffradikale für die Gewebeschädigung ist noch unklar. Die Ergebnisse der vorliegenden Arbeit deuten darauf hin, dass die Aktivität der Nicotinamid-Adenin-Dinucleotid-Phosphat (NADPH)-Oxidase eine zwingende Voraussetzung für die Blasenbildung der experimentellen Epidermolysis bullosa acquisita darstellt. Mäuse, die defizient für den *Neutrophil cytosolic factor 1* ($Ncf1^{-/-}$) sind und keine NADPH-Oxidase Funktion aufweisen, waren resistent gegenüber Blasenbildung durch Antikörper gegen Typ VII Kollagen. Sowohl die pharmakologische Inhibition der NADPH-Oxidase als auch die Verwendung der Granulozyten von Patienten mit septischer Granulomatose führten zur Aufhebung der Blasenbildung in einem Kryoschnitt *ex vivo*-Modell. Darüber hinaus wurde die patogenetische Bedeutung der Rekrutierung von Granulozyten in die Dermis dadurch unterstrichen, dass CD18-defiziente Mäuse, deren Granulozyten in die Dermis nicht einwandern können, nach Injektion von Antikörpern gegen Typ VII Kollagen keine Blasen entwickelten. Der Transfer normaler Granulozyten in $Ncf1^{-/-}$ Mäuse rekonstituierte die Blasenbildung, was darauf hindeutet, dass Granulozyten die Quelle der Sauerstoffradikalen sind. Die vorliegende Arbeit identifiziert die NADPH-Oxidase als einen Schlüsselfaktor für die durch Autoantikörper-induzierte Blasenbildung und soll die Basis für neue Therapieansätze für die entzündliche Gewebeschädigung durch Autoantikörper darstellen.

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List of own original articles generated during the thesis work

1. **Chiriac MT**, Roesler J, Sindrilaru A, Scharffetter-Kochanek K, Zillikens D, Sitaru C. Neutrophil-derived NADPH oxidase is required for autoantibody-induced tissue damage in experimental epidermolysis bullosa acquisita. ***J Pathol 2007***, DOI: 10.1002/path.2157 (IF 6.21)

This work constitutes the main body of the doctoral thesis.

2. Sitaru C, **Chiriac MT**, Mihai S, Büning J, Gebert A, Ishiko A, Zillikens D. Induction of complement fixing IgG4 autoantibodies against type VII collagen results in subepidermal blistering in mice. ***J Immunol 2006*** 177: 3461-8. (IF 6.38)

Some methods and protocols published in this paper, including ELISA with recombinant protein and evaluation of the disease activity in vivo, were adapted and used in the doctoral thesis.

3. Mihai S, **Chiriac MT**, Takahashi K, Thurman JM, Holers VM, Zillikens D, Botto M, Sitaru C. The alternative pathway of complement activation is critical for blister induction in experimental epidermolysis bullosa acquisita. ***J Immunol 2007,*** *in press* (IF 6.38)

The role of complement for blistering in experimental epidermolysis bullosa acquisita was characterized in depth in this paper. Some of the techniques performed here, including the detection of granulocyte infiltration using the myeloperoxidase assay in extracted skin samples and the local in vivo reconstitution with granulocytes, had been established and were previously described in the doctoral thesis.

4. Sitaru C, Mihai S, Otto C, **Chiriac MT**, Haußer I, Dotterweich B, Saito H, Rose C, Ishiko A, Zillikens D. Induction of dermal-epidermal separation in mice by passive transfer of antibodies to type VII collagen. ***J Clin Invest*** 2005, 115: 870-878. (IF 15.05)

The work presented here describes the first passive transfer model for the autoimmune blistering skin disease epidermolysis bullosa acquisita. With some modifications, most of the protocols published in this study, including the purification of antibodies, the injection protocols and the scoring for the immunoreactants deposited in situ were successfully applied throughout the in vivo experiments performed in the doctoral thesis.

5. Mihai S, **Chiriac MT,** Herrero-Gonzales JE, Goodall M, Jefferis R, Savage CO, Zillikens, D, Sitaru C. Non-complement-fixing IgG4 autoantibodies activate leukocytes and induce dermal-epidermal separation. Submitted

The ex vivo model used in this paper for characterizing the pathogenicity of different IgG subclasses in bullous pemphigoid was modified to focus on the role of reactive oxygen species and thereafter successfully utilized throughout the experiments with epidermolysis bullosa acquisita and bullous pemphigoid patients performed in the doctoral thesis.

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List of abstracts published during the thesis work

1. Sitaru C, Mihai S, Otto C, **Chiriac MT**, Hausser I, Dotterweich B, Saito H, Rose C, Ishiko A, Zillikens D. Induction of dermal-epidermal separation in mice by passive transfer of antibodies to type VII collagen. Joint Annual Meeting of the German and Dutch Societies for Immunology (JAMI), 20-23 October, Maastricht, The Netherlands. Immunobiology 2004; 209: 302.

2. Sitaru C, **Chiriac MT**, Mihai S, Ishiko A, Zillikens D. Development of an active disease model for epidermolysis bullosa acquisita in mice. 32nd Annual Meeting of the Arbeitsgemeinschaft Dermatologische Forschung (ADF), 03.03-05.03.2005, Innsbruck. Austria. Arch Dermatol Res 2005; 296: 389.

3. **Chiriac MT**, Zillikens D, Sitaru C. Inhibition of NADPH-oxidase abolishes leukocyte-dependent dermal-epidermal separation induced by autoantibodies from patients with bullous pemphigoid. 32nd Annual Meeting of the Arbeitsgemeinschaft Dermatologische Forschung (ADF), 03.03-05.03.2005, Innsbruck, Austria. Arch Dermatol Res 2005; 296: 410.

4. Sitaru C, **Chiriac MT**, Mihai S, Ishiko A, Zillikens D. Development of an active disease model for epidermolysis bullosa acquisita in mice. 10th European Meeting on Complement in Human Disease, 9-13 September 2005, Heidelberg, Germany. Mol Immunol 2006; 43: 158.

5. Sitaru C, **Chiriac MT**, Mihai S, Ishiko A, Zillikens D. Immunization against type VII collagen induces an active subepidermal autoimmune blistering disease in mice. 35th Annual Meeting of the European Society for Dermatological Research (ESDR), 22-24 September 2005, Tübingen, Germany. J Invest Dermatol 2005; 125: S1.

6. **Chiriac MT**, Zillikens, D, Sitaru, C. Neutrophils are required for the antibody-induced blister formation in a mouse model of epidermolysis bullosa acquisita. 33rd Annual Meeting of the Arbeitsgemeinschaft Dermatologische Forschung (ADF), 23.03–25.03 2006, Aachen, Germany. Exp Dermatol 2006; 15: 209.

7. Sitaru C, **Chiriac MT**, Mihai S, Buning J, Gebert A, Ishiko A, Zillikens D. Induction of complement-fixing autoantibodies against type VII collagen results in

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8. **Chiriac MT**, Zillikens D, Sitaru C. Neutrophils are key cellular effectors in experimental epidermolysis bullosa acquisita. 36th Annual Meeting of the European Society for Dermatological Research (ESDR), 07-09 September 2006, Paris, France. J Invest Dermatol 2006; 126: S3.

9. Mihai S, **Chiriac MT**, Takahashi K, Thurman JM, Holers VM, Botto M, Zillikens D, Sitaru C. The alternative pathway of complement activation is critical for the induction of experimental epidermolysis bullosa acquisita. 36th Annual Meeting of the European Society for Dermatological Research (ESDR), 07-09 September 2006, Paris, France. J Invest Dermatol 2006; 126: S3.

10. Sesarman AV, Mihai S, **Chiriac MT**, Olaru F, Thurman JM, Zillikens D, Sitaru C. Blocking the interaction with complement and Fc receptors abolishes antibody-induced blistering in experimental epidermolysis bullosa acquisita. 34th Annual Meeting of the Arbeitsgemeinschaft Dermatologische Forschung (ADF), 08.03–10.03 2006, Freiburg, Germany. Exp Dermatol 2007; 16: 223.

11. **Chiriac MT**, Roesler J, Sindrilaru A, Scharffetter-Kochanek K, Zillikens D, Sitaru D. NADPH oxidase is required for neutrophil-dependent tissue damage induced by autoantibodies against type VII collagen. 34th Annual Meeting of the Working Group for Dermatological Research (ADF), 08.03–10.03 2006, Freiburg, Germany. Exp Dermatol 2007; 16: 223.

12. **Chiriac MT**, Roesler J, Sindrilaru A, Scharffetter-Kochanek K, Zillikens D, Sitaru D. Neutrophil-derived NADPH oxidase is required for autoantibody-induced tissue damage in experimental epidermolysis bullosa acquisita. 68th Annual Meeting of the Society for Investigative Dermatology (SID), May 9–12, 2007 Los Angeles, USA. J Invest Dermatol 2007; *in press*.

13. Sesarman AV, Mihai S, **Chiriac MT**, Olaru F, Thurman JM, Zillikens D, Sitaru C. Complement- and leukocyte-activating capacity of antibodies against type VII collagen determines their blister-inducing potential. 68th Annual Meeting of the Society for Investigative Dermatology (SID), May 9–12, 2007 Los Angeles, USA. J Invest Dermatol 2007; *in press*.