Journal of Pathology
J Pathol 2007; **212**: 56–65
Published online 23 March 2007 in Wiley InterScience
(www.interscience.wiley.com) **DOI:** 10.1002/path.2157

Original Paper

NADPH oxidase is required for neutrophil-dependent autoantibody-induced tissue damage

MT Chiriac,[1] J Roesler,[2] A Sindrilaru,[3] K Scharffetter-Kochanek,[3] D Zillikens[1] and C Sitaru[1]*

[1] *Department of Dermatology, University of Lübeck, Lübeck, Germany*
[2] *Department of Paediatrics, University Clinic Carl Gustav Carus, Dresden, Germany*
[3] *Department of Dermatology, University of Ulm, Ulm, Germany*

**Correspondence to:*
C Sitaru, Department of Dermatology, University of Lübeck, Ratzeburger Allee 160, 23538 Lübeck, Germany.
E-mail: csitaru@fastmail.fm

No conflicts of interest were declared.

Received: 19 December 2006
Revised: 31 January 2007
Accepted: 4 February 2007

Abstract

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 a key molecular effector engaged by pathogenic autoantibodies and provide relevant targets for prevention of tissue damage in granulocyte-mediated autoimmune diseases.

Keywords: autoimmunity; inflammation; skin

Introduction

In many inflammatory conditions, the ability of phagocytes to produce reactive oxygen species (ROS) is thought to be crucially involved in host defence and tissue damage [1]. NADPH oxidase is responsible for the generation of the superoxide anion (O_2^-) and other ROS [2]. The biological relevance of NADPH oxidase is evident from inherited or targeted gene disruptions resulting in chronic granulomatous disease (CGD) [3–6].

The contribution of NADPH oxidase to tissue injury in autoimmune diseases is still unclear. Depending on the experimental setting, inhibition of ROS production by pharmacological agents or gene defects resulted in diminution [7,8] or augmented severity of experimental autoimmune conditions [9]. Therefore, the capacity of NADPH oxidase to mediate tissue injury still remains one of the outstanding questions of their involvement in autoimmune inflammatory diseases.

In a group of autoimmune diseases, autoantibodies mediate activation of leukocytes and tissue damage. Epidermolysis bullosa acquisita (EBA) is such an autoimmune disease characterized by sub-epidermal blisters and autoantibodies against type VII collagen of the dermal–epidermal junction (DEJ) [10]. The pathogenic relevance of (auto)antibodies against type VII collagen has been conclusively demonstrated *ex vivo* and in experimental animals [11–15]. In experimental EBA, tissue damage is independent of T cells, since blistering can be induced in nude mice and *ex vivo* with purified granulocytes [11,13].

In the present study, to evaluate the relevance of NADPH oxidase for tissue damage induced by antibodies, we used experimental models of blister induction by the passive transfer of antibodies against type VII collagen. We demonstrate that gene defects or pharmacological inhibition of NADPH oxidase abolishes tissue injury induced by (auto)antibodies and granulocytes both in mice and in an *ex vivo* human model.

Materials and methods

Patients

Sera were collected from patients with EBA ($n = 5$) and bullous pemphigoid (BP) ($n = 5$), before initiation

of treatment, as well as from healthy donors ($n = 10$) [11,13,16]. Leukocytes were obtained from the peripheral blood of healthy donors ($n = 10$) or from patients with chronic granulomatous disease (CGD) ($n = 8$). Characteristics of the CGD patients are summarized in Supplementary Table 1 available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2157.html. We obtained institutional approval issued by the Ethics Committee at the Medical Faculty of the University of Lübeck. In adherence to the Helsinki principles, we obtained informed consent from all patients whose material was used in this study.

Antibody-induced dermal–epidermal separation

IgG from serum of patients and rabbits was purified by using Protein G and analysed as described previously [11,13]. Preparations of rabbit IgG against type VII collagen were adjusted to an end-point titre of 1 : 128 000 by immunofluorescence (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. The blister-inducing potential of patient IgG was assessed *ex vivo* using a model of autoantibody-induced leukocyte-dependent dermal–epidermal separation in cryosections of normal human skin [11,12] (see also Supplementary text available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2157.html).

Passive transfer studies followed published protocols with minor modifications [13]. Neutrophil infiltration of murine skin was assayed as described previously [17], with minor modifications (see Supplementary text available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2157.html). All *in vivo* experiments were approved by the local authorities of the Animal Care and Use Committee (Kiel, Germany) and performed by certified personnel.

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 [15], with minor modifications (see also Supplementary text available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2157.html).

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 previously described [18] with minor modifications (see also Supplementary text available online at http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2157.html).

.

Results

Ncf1 (neutrophil cytosolic factor 1)$^{-/-}$ mice are resistant to blistering induced by antibodies against type VII collagen

In initial experiments, we injected Ncf1$^{-/-}$ ($n = 18$) and Ncf1$^{+/+}$ ($n = 14$) mice with IgG against 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 1A). There was no evidence of clinical lesions in 17 of 18 Ncf1$^{-/-}$ mice (Figure 1B) treated with antibody against type VII collagen or in either Ncf1$^{-/-}$ ($n = 2$) or Ncf1$^{+/+}$ mice ($n = 2$) (Figure 1C) that received control antibody at any time during the observation period (Supplementary Figure 1A available online as mentioned before). Immunofluorescence (IF) microscopy revealed linear deposits of rabbit IgG (Figures 1D and 1E) and murine complement C3 (Figures 1G and 1H) at the DEJ in mice that received IgG specific to type VII collagen, but not in mice injected with control IgG (Figures 1F and 1I). Histological examination of lesional skin biopsies from diseased Ncf1$^{+/+}$ mice injected with IgG against type VII collagen revealed dermal–epidermal separation and dense neutrophil infiltrates (Figure 1J). 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 1K). In Ncf1$^{+/+}$ mice injected with control IgG, we found no subepidermal cleavage and no inflammatory infiltrate (Figure 1L). Neutrophil skin infiltration was assessed using a myeloperoxidase (MPO) assay. In mice injected with IgG against type VII collagen, a significantly lower MPO activity was found in Ncf1$^{-/-}$ mice compared with Ncf1$^{+/+}$ mice (Supplementary Figure 1C online).

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

Skin cryosections were incubated with IgG from patients with EBA ($n = 5$) and BP ($n = 5$), and with leukocytes from healthy donors. IgG from patients' sera (Figures 2A and 2B), but not from healthy controls (Figure 2C), bound to the DEJ, as revealed by indirect IF microscopy. Addition of leukocytes resulted in their recruitment at the DEJ in cryosections incubated with EBA and BP autoantibodies both in the absence (Figure 2D) and in the presence (Figure 2E) of 75 μM diphenylene iodonium, but not in those

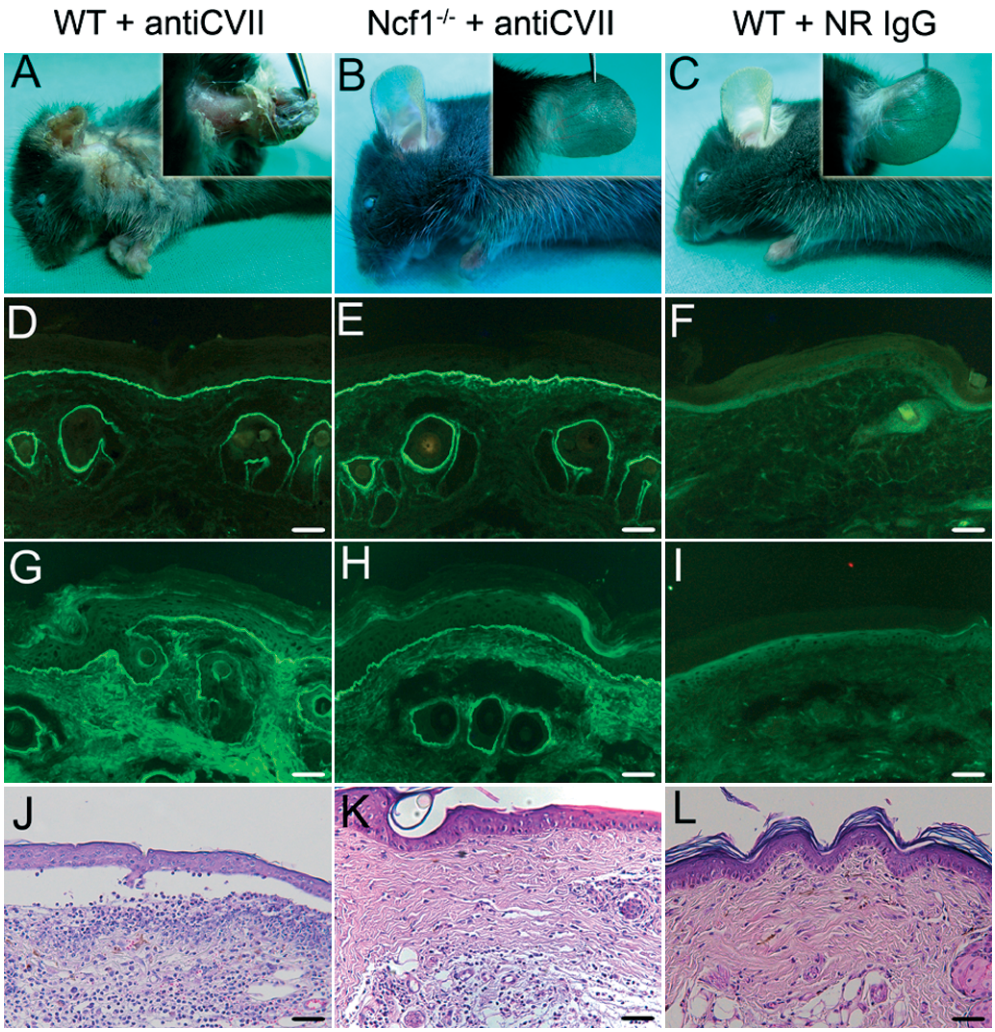

Figure 1. Ncf1$^{-/-}$ mice are resistant to blisters induced by antibodies against type VII collagen. (A) Injection of a total dose of 45 μl/g body weight of IgG against type VII collagen (antiCVII) results in extensive skin lesions, including blisters, erosions, partly covered by crusts, and epidermal 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 sub-epidermal cleavage and a neutrophil-rich inflammatory infiltrate in (J) a C57BL/6J mouse, but only a moderate infiltrate of neutrophils in (K) an Ncf1$^{-/-}$ mouse, receiving antibodies to type VII collagen. Normal histological appearance in (L) a C57BL/6J mouse treated with control antibody. Scale bars = 50 μm

incubated with control IgG (Figure 2F). When leukocyte suspensions were supplemented with nitroblue tetrazolium, deposits of formazan were observed at the DEJ in cryosections incubated with EBA and BP IgG and leukocytes (Figure 2G), but not in those incubated with EBA and BP IgG, leukocytes and diphenylene iodonium (Figure 2H), or with control IgG and leukocytes (Figure 2I). Dermal–epidermal separation was seen in cryosections incubated with EBA and BP IgG and leukocytes (Figure 2J), but not in those incubated with EBA and BP IgG, leukocytes and diphenylene iodonium (Figure 2K), or with control IgG and leukocytes (Figure 2L). Diphenylene iodonium caused a dose-dependent inhibition of both superoxide production from phorbol ester-stimulated human granulocytes and autoantibody-induced dermal–epidermal separation (Supplementary Figure 2 online). In the presence of diphenylene iodonium, superoxide production from granulocytes correlates highly with the extent of dermal–epidermal separation ($r = 0.99$; $p < 0.001$).

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

Skin cryosections were incubated with IgG purified from patients with EBA ($n = 2$) and BP ($n = 2$), and subsequently with granulocytes from patients with CGD ($n = 8$) or from healthy donors ($n = 5$) (Supplementary Figure 3 online). EBA and BP IgG (Figures 3A and 3B), but not IgG from healthy controls (Figure 3C), bound to the DEJ, as revealed

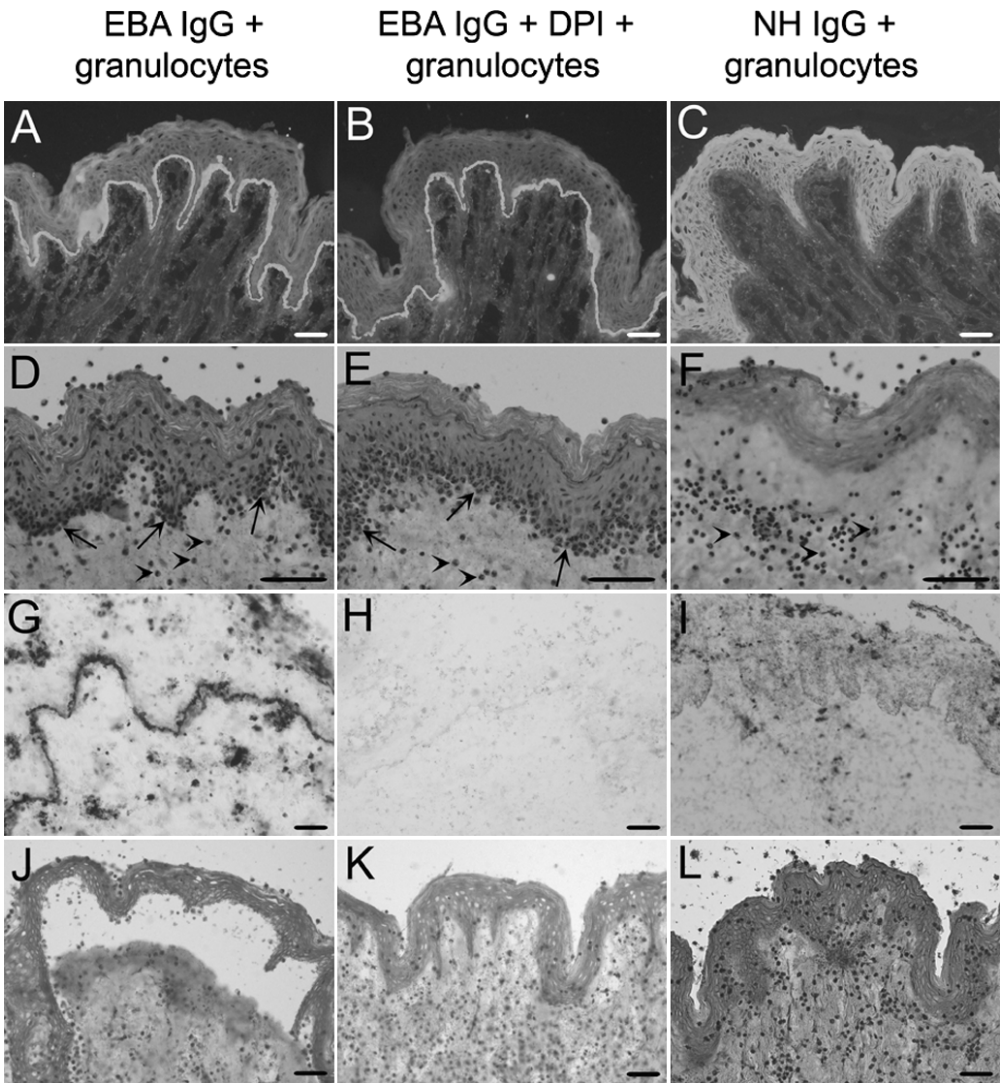

Figure 2. Inhibition of NADPH oxidase abolishes dermal–epidermal separation induced by autoantibodies in cryosections of human skin. Frozen sections of normal human skin were incubated with 100 μl of IgG from a patient with epidermolysis bullosa acquisita (EBA1) or a healthy control. (A, B) EBA IgG autoantibodies, in contrast to (C) normal human (NH) IgG, bind to the dermal–epidermal junction, as revealed by immunofluorescence microscopy. One-hour incubation with 1.5×10^7 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 absence (D) and in the presence (E) of the NADPH oxidase inhibitor diphenylene iodonium (DPI). (F) In cryosections incubated with IgG from the healthy control, no recruitment of leukocytes at the dermal–epidermal junction is observed. In all sections, leukocytes, randomly scattered over the entire epidermis and dermis (arrowheads), are seen. 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-hour incubation with granulocytes results in dermal–epidermal separation in cryosections treated with IgG from our EBA patient in the absence (J) of diphenylene iodonium. In contrast, no sub-epidermal splits develop in (K) cryosections incubated with autoantibodies and leukocytes when diphenylene iodonium was added or in (L) cryosections incubated with IgG from the healthy control and leukocytes. Scale bars = 50 μm

by IF microscopy. Addition of granulocytes from both healthy donors (Figure 3D) and CGD patients (Figure 3E) resulted in their recruitment at the DEJ in cryosections that had been incubated with EBA and BP autoantibodies. No attachment of leukocytes at the DEJ was found in cryosections incubated with control IgG and granulocytes from healthy donors (Figure 3F). When leukocyte suspensions were supplemented with nitroblue tetrazolium, deposits of formazan were observed in cryosections incubated with EBA and BP IgG and granulocytes from healthy donors (Figure 3G), but not in those incubated with EBA and BP IgG and CGD granulocytes (Figure 3H), or with control IgG and leukocytes from healthy donors (Figure 3I). Dermal–epidermal separation was seen only in cryosections incubated with EBA and BP IgG and granulocytes from healthy donors (Figure 3J). In contrast, no sub-epidermal splits were observed in cryosections treated with EBA and BP IgG and CGD granulocytes (Figure 3K), or with control IgG and leukocytes from healthy donors (Figure 3L).

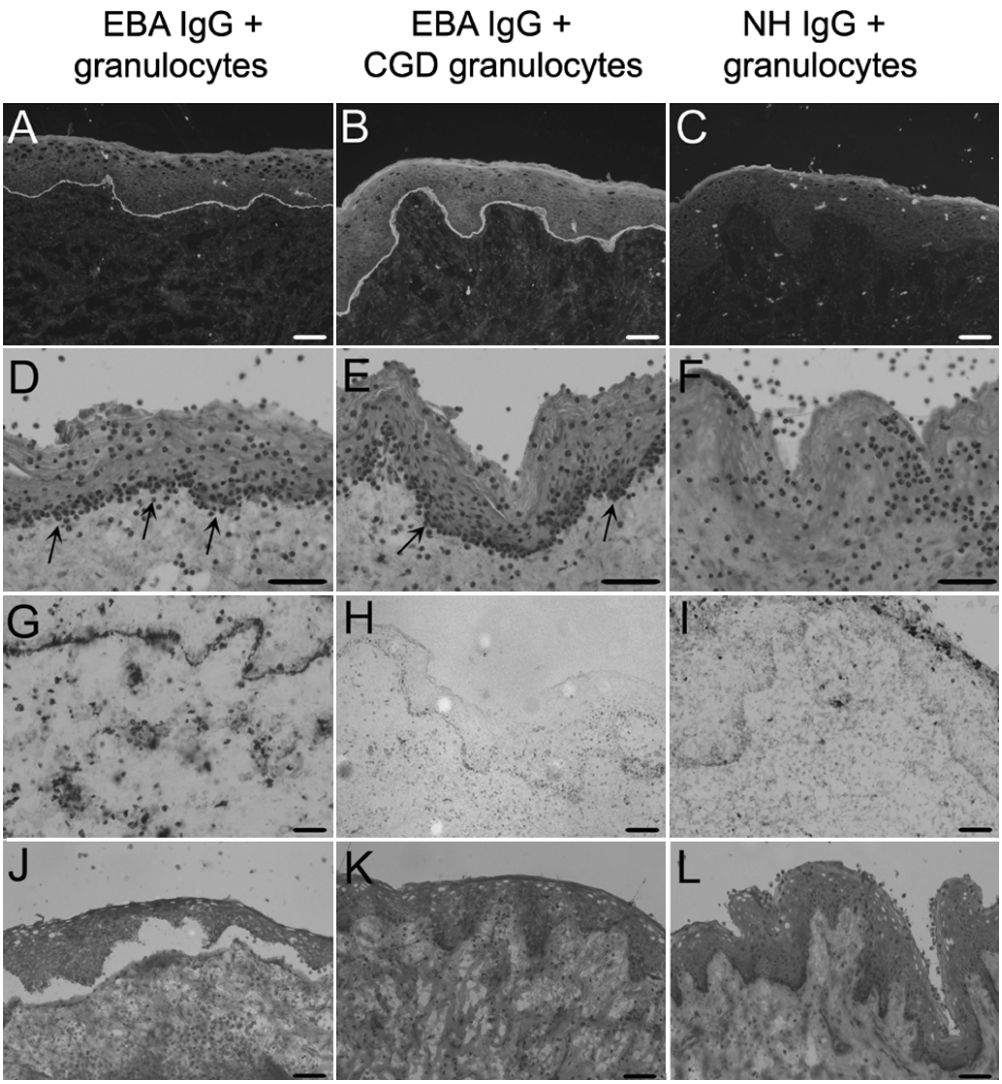

Figure 3. Granulocytes from patients with chronic granulomatous disease are not able to mediate autoantibody-induced tissue damage. Immunofluorescent staining reveals linear IgG deposition at the dermal–epidermal junction in cryosections incubated with (A, B) 100 μl of IgG from an EBA patient (EBA2) but not with (C) the same amount of IgG from a healthy control (NH IgG). One-hour incubation of granulocytes from (D) a healthy donor or (E) a patient with chronic granulomatous disease (CGD2) 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-hour incubation with granulocytes results in dermal–epidermal separation in cryosections treated with (J) EBA IgG and granulocytes from a healthy donor. In contrast, no sub-epidermal splits develop in (K) cryosections incubated with EBA IgG and granulocytes from a chronic granulomatous disease patient or in (L) cryosections incubated with normal IgG and granulocytes from a healthy donor. Scale bars = 50 μm

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

Infiltration of granulocytes into the dermis precedes the development of clinical blisters following subcutaneous injections of antibodies against type VII collagen in BALB/c mice (= 3 per time point) (Supplementary Figure 4 online). To demonstrate the pathogenic relevance of granulocytes for tissue damage in experimental EBA, we depleted neutrophils for 6 days using 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 4A). By day 6, none of the BALB/c mice injected with the anti-Gr-1 ($n = 12$) and the antibody against type VII collagen (Figure 4B) or with control rabbit antibody ($n = 4$) (Figure 4C) 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 (Figures 4D and 4E), but not in mice treated with control antibody ($n = 2$) (Figure 4F). Deposition of C3 was more intense in the skin of mice treated with the mock antibody (Figure 4G) than in mice that had received anti-Gr-1 antibody (Figure 4H), before treatment with the antibody against type VII

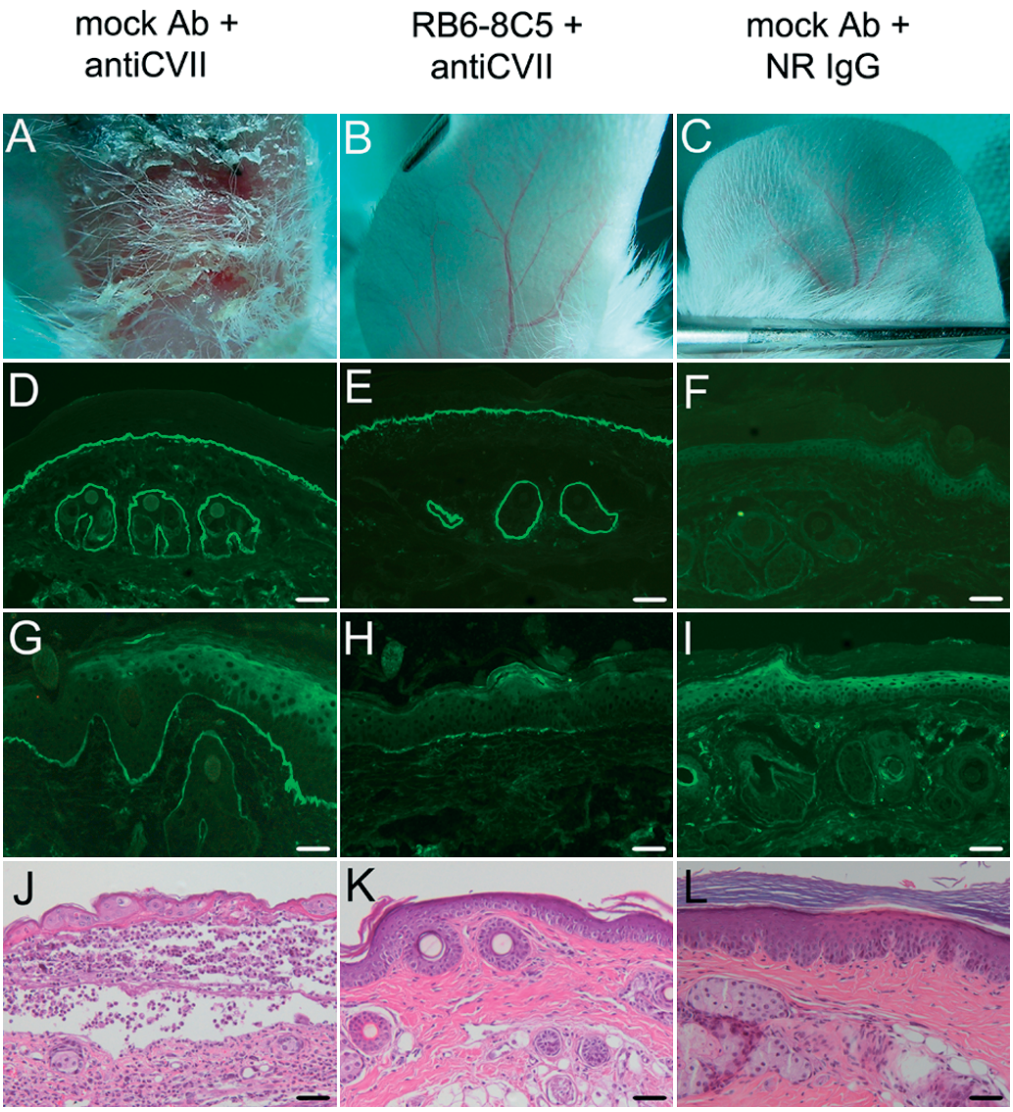

Figure 4. Neutrophil depletion protects mice from skin blistering induced by antibodies to type VII collagen. BALB/c mice were injected with 0.25 mg of the depleting rat anti-Gr-1 RB6-8C5 antibody or control rat IgG 24 h before the first injection of rabbit IgG and at 3-day intervals thereafter. (A) 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 sub-epidermal cleavage and a neutrophil-rich inflammatory infiltrate in (J) a BALB/c mouse injected with IgG against type VII collagen and mock rat antibody, but not in (K) a BALB/c mouse receiving antibodies to type VII collagen and rat anti-Gr1 RB6-8C5. Normal histological appearance in (L) a BALB/c mouse treated with control antibody. Scale bars = 50 μm

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 4I). Histological analysis revealed sub-epidermal cleavage and a neutrophil-rich dermal inflammatory infiltrate in BALB/c mice injected with the mock antibody and IgG against type VII collagen (Figure 4J), but not in mice receiving anti-Gr-1 antibody and antibodies against type VII collagen (Figure 4K) or treated with normal rabbit IgG (Figure 4L). 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 to 80% of baseline levels by day 13 (not shown). Starting with day 7, similarly to BALB/c mice ($n = 6$) injected with the 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 (Supplementary Figure 5A online). Histological analysis at day 12 showed sub-epidermal 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 the serum of all mice injected with IgG against type VII collagen, serum levels of specific antibodies were comparable (Supplementary Figure 5B online). 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 (Supplementary Figure 5C online).

Emigration of granulocytes into the dermis has been shown to depend on CD11/CD18, and $CD18^{-/-}$ granulocytes cannot be recruited into the inflamed dermis [19]. To provide further evidence for 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 (Supplementary Figure 6 online). Bypassing the emigration of granulocytes into the dermis by injection of $CD18^{+/+}$ granulocytes subcutaneously into $CD18^{-/-}$ mice ($n = 5$) treated with antibody against type VII collagen did result in sub-epidermal blistering (Supplementary Figure 7 online).

$Ncf1^{-/-}$ 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 subcutaneously 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 5A), whereas transfer of $Ncf1^{+/+}$ granulocytes ($n = 5$) resulted in blisters and erosions on their ears (Figure 5B). In contrast, $Ncf1^{-/-}$ mice (n=5) treated with control rabbit IgG and reconstituted with $Ncf1^{+/+}$ granulocytes did not show skin alterations (Figure 5C). Histological analysis revealed infiltrates of neutrophils in the dermis of all mice (Figures 5D–5F). In $Ncf1^{-/-}$ mice injected with antibodies against type VII collagen, in contrast to the transfer of $Ncf1^{-/-}$ granulocytes (Figure 5D), administration of $Ncf1^{+/+}$ granulocytes (Figure 5E) resulted in sub-epidermal splits. No sub-epidermal cleavage

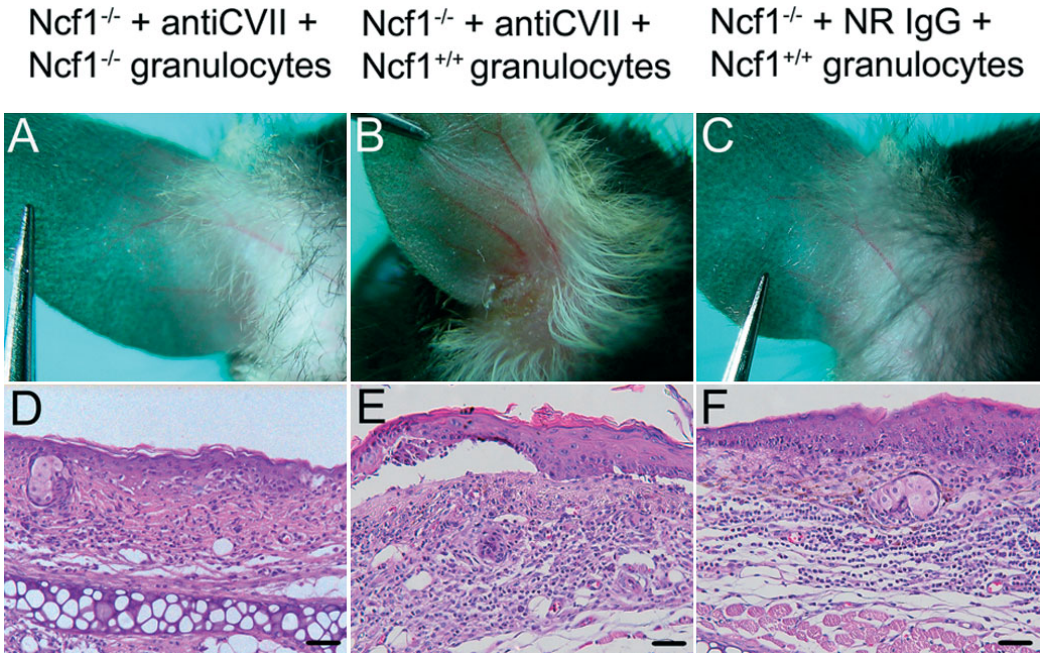

Figure 5. Reconstitution with $Ncf1^{+/+}$ granulocytes renders $Ncf1^{-/-}$ mice susceptible to antibody-induced skin blistering. Mice were treated with the antibody against type VII collagen (antiCVII) or control antibody (NR IgG) and injected at day 6 with 2×10^6 $Ncf1^{-/-}$ or $Ncf1^{+/+}$ murine granulocytes in 50 μl of medium subcutaneously 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) An $Ncf1^{-/-}$ mouse treated with control rabbit IgG and reconstituted with $Ncf1^{+/+}$ granulocytes does not show skin alterations. (D–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 (D) the $Ncf1^{-/-}$ mouse reconstituted with $Ncf1^{-/-}$ granulocytes, but occurs in (E) the $Ncf1^{-/-}$ mouse 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 = 50 μm

J Pathol 2007; **212**: 56–65 DOI: 10.1002/path

was seen in Ncf1$^{-/-}$ mice treated with control IgG and reconstituted with Ncf1$^{+/+}$ granulocytes (Figure 5F).

Discussion

ROS produced by tissue-infiltrating leukocytes are believed to contribute significantly to the pathogenesis of several inflammatory diseases [1]. 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 used here 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 mediated by autoantibodies and granulocytes both *in vivo* and *ex vivo*.

NADPH oxidase is clearly essential in immune defence, as demonstrated by life-threatening infections with certain bacteria and fungi in both human and experimental CGD [3–6]. 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 to be resistant to myelin oligodendroglial glycoprotein (MOG) peptide-induced disease [7], whereas Ncf1 naturally mutated mice immunized with native MOG protein developed an enhanced autoimmune phenotype [9]. Irrespective of its stimulatory or inhibitory effects, Ncf1 is thought to modulate the level of T-cell autoimmune responses in EAE [9,20].

In animal models of arthritis, different effects of NADPH oxidase on disease expression have been reported. Scavenging the NADPH oxidase-derived superoxide using superoxide dismutase or its non-peptidyl mimetic resulted in amelioration of arthritis induced by streptococcal cell walls, adjuvant or immunization with type II collagen [21–24]. In addition, collagen-induced arthritis is enhanced in mice genetically deficient in extracellular superoxide dismutase [8]. NADPH oxidase-derived ROS induce chondrocyte death and aggravate metalloproteinase-mediated cartilage destruction in interferon gamma-stimulated immune complex arthritis [25]. Interestingly, NADPH oxidase deficiency has no effect on the K/BxN serum transfer arthritis [26] and causes increased connective tissue destruction in zymosan and immune complex-mediated arthritis in mice [27]. The Ncf1 gene was found to be responsible for the severity of arthritis in rats [28] and a natural mutation is associated with an enhanced disease phenotype in mice [9]. 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.

While the intracellular destruction of phagocytosed particles by CGD granulocytes is clearly impaired [3,29], the capacity of these cells to mediate antibody-dependent cellular cytotoxicity (ADCC) is controversial [29–36]. In our system, granulocytes are recruited by IgG autoantibodies to the DEJ and induce subepidermal splits by releasing proteases that degrade the extracellular matrix [11,12]. Our present observations indicate that destruction of the extracellular matrix by granulocytes resulting in a disease-specific effect is dependent on functional NADPH oxidase.

Neutrophil infiltration at sites of blistering is often seen in patients with EBA [37] and in mice immunized against type VII collagen [15], and is a characteristic feature of diseased mice injected with antibodies against type VII collagen [13]. Neutrophils have been implicated as critical cellular effectors in several other diseases mediated by antibodies [26,38–41]. We therefore hypothesized that antibodies bound to type VII collagen recruit and activate leukocytes *in situ* and thus direct tissue destruction at the DEJ *in vivo*. Indeed, depletion of neutrophils abolished the blistering phenotype and CD18$^{-/-}$ mice, in which granulocytes cannot be recruited into the skin, are resistant to experimental EBA.

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 observation raised the question of whether Ncf1$^{-/-}$ granulocytes were able to produce blistering *in vivo*. However, 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 amplification of Fc-mediated neutrophil activation; structural changes of extracellular matrix proteins by ROS, resulting in loss of skin adhesive function; activation of leukocyte proteases; and direct or indirect inactivation of protease inhibitors. Bacterial killing by neutrophils essentially

depends on proteases [42]. In addition, granulocyte proteases are required for blistering induced by autoantibodies [12]. It is therefore tempting to speculate that in experimental EBA, proteases are instrumental for tissue damage and their activity is modulated by NADPH oxidase.

In conclusion, our study demonstrates that gene defects or pharmacological inhibition of NADPH oxidase abolishes 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.

Acknowledgements

We are grateful to Dr F Sendo for providing the hybridoma cell line RB6-8C5, D Paul for assistance with the preparation of CGD granulocytes, and Drs L Pricop, SJ Weiss, and R Holmdahl for advice and critically reading the manuscript. This work was supported by grants SI 1281/1-1 and Zi 439/6-2 (to CS and DZ) and SFB 497/C7 (to AS and KS-K) from the Deutsche Forschungsgemeinschaft and from the Doktor Robert Pfleger-Stiftung.

Supplementary material

Supplementary material may be found at the web address http://www.interscience.wiley.com/jpages/0022-3417/suppmat/path.2157.html.

References

1. Weiss SJ. Tissue destruction by neutrophils. *N Engl J Med* 1989;**320:**365–376.
2. Babior BM. NADPH oxidase. *Curr Opin Immunol* 2004;**16:**42–47.
3. Baehner RL, Karnovsky ML. Deficiency of reduced nicotinamide-adenine dinucleotide oxidase in chronic granulomatous disease. *Science* 1968;**162:**1277–1279.
4. Pollock JD, Williams DA, Gifford MA, Li LL, Du X, Fisherman J, *et al*. Mouse model of X-linked chronic granulomatous disease, an inherited defect in phagocyte superoxide production. *Nature Genet* 1995;**9:**202–209.
5. Jackson SH, Gallin JI, Holland SM. The p47phox mouse knockout model of chronic granulomatous disease. *J Exp Med* 1995;**182:**751–758.
6. Huang CK, Zhan L, Hannigan MO, Ai Y, Leto TL. P47(phox)-deficient NADPH oxidase defect in neutrophils of diabetic mouse strains, C57BL/6J-m db/db and db/+. *J Leukoc Biol* 2000;**67:**210–215.
7. van der Veen RC, Dietlin TA, Hofman FM, Pen L, Segal BH, Holland SM. Superoxide prevents nitric oxide-mediated suppression of helper T lymphocytes: decreased autoimmune encephalomyelitis in nicotinamide adenine dinucleotide phosphate oxidase knockout mice. *J Immunol* 2000;**164:**5177–5183.
8. Ross AD, Banda NK, Muggli M, Arend WP. Enhancement of collagen-induced arthritis in mice genetically deficient in extracellular superoxide dismutase. *Arthritis Rheum* 2004;**50:**3702–3711.
9. Hultqvist M, Olofsson P, Holmberg J, Backstrom BT, Tordsson J, Holmdahl R. Enhanced autoimmunity, arthritis, and encephalomyelitis in mice with a reduced oxidative burst due to a mutation in the Ncf1 gene. *Proc Natl Acad Sci U S A* 2004;**101:**12646–12651.
10. Sitaru C, Zillikens D. Mechanisms of blister induction by autoantibodies. *Exp Dermatol* 2005;**14:**861–875.
11. Sitaru C, Kromminga A, Hashimoto T, Bröcker EB, Zillikens D. Autoantibodies to type VII collagen mediate Fcgamma-dependent granulocyte activation and induce dermal–epidermal separation in cryosections of human skin. *Am J Pathol* 2002;**161:**301–311.
12. Shimanovich I, Mihai S, Oostingh GJ, Ilenchuk TT, Brocker EB, Opdenakker G, *et al*. Granulocyte-derived elastase and gelatinase B are required for dermal–epidermal separation induced by autoantibodies from patients with epidermolysis bullosa acquisita and bullous pemphigoid. *J Pathol* 2004;**204:**519–527.
13. Sitaru C, Mihai S, Otto C, Chiriac MT, Haußer I, Dotterweich B, *et al*. Induction of dermal–epidermal separation in mice by passive transfer of antibodies to type VII collagen. *J Clin Invest* 2005;**115:**870–878.
14. Woodley DT, Ram R, Doostan A, Bandyopadhyay P, Huang Y, Remington J, *et al*. Induction of epidermolysis bullosa acquisita in mice by passive transfer of autoantibodies from patients. *J Invest Dermatol* 2006;**126:**1323–1330.
15. Sitaru C, Chiriac MT, Mihai S, Büning J, Gebert A, Ishiko A, *et al*. Induction of complement-fixing autoantibodies against type VII collagen results in subepidermal blistering in mice. *J Immunol* 2006;**177:**3461–3468.
16. Sitaru C, Schmidt E, Petermann S, Munteanu SL, Bröcker EB, Zillikens D. Autoantibodies to bullous pemphigoid antigen 180 induce dermal–epidermal separation in cryosections of human skin. *J Invest Dermatol* 2002;**118:**664–671.
17. Bradley PP, Priebat DA, Christensen RD, Rothstein G. Measurement of cutaneous inflammation: estimation of neutrophil content with an enzyme marker. *J Invest Dermatol* 1982;**78:**206–209.
18. Tan AS, Ahmed N, Berridge MV. Acute regulation of glucose transport after activation of human peripheral blood neutrophils by phorbol myristate acetate, fMLP, and granulocyte–macrophage colony-stimulating factor. *Blood* 1998;**91:**649–655.
19. Mizgerd JP, Kubo H, Kutkoski GJ, Bhagwan SD, Scharffetter-Kochanek K, Beaudet AL, *et al*. Neutrophil emigration in the skin, lungs, and peritoneum: different requirements for CD11/CD18 revealed by CD18-deficient mice. *J Exp Med* 1997;**186:**1357–1364.
20. Hultqvist M, Holmdahl R. Ncf1 (p47phox) polymorphism determines oxidative burst and the severity of arthritis in rats and mice. *Cell Immunol* 2005;**233:**97–101.
21. Schalkwijk J, van den Berg WB, van de Putte LB, Joosten LA, van den Bersselaar L. Cationization of catalase, peroxidase, and superoxide dismutase. Effect of improved intraarticular retention on experimental arthritis in mice. *J Clin Invest* 1985;**76:**198–205.
22. Shingu M, Takahashi S, Ito M, Hamamatu N, Suenaga Y, Ichibangase Y, *et al*. Anti-inflammatory effects of recombinant human manganese superoxide dismutase on adjuvant arthritis in rats. *Rheumatol Int* 1994;**14:**77–81.
23. Skaleric U, Allen JB, Smith PD, Mergenhagen SE, Wahl SM. Inhibitors of reactive oxygen intermediates suppress bacterial cell wall-induced arthritis. *J Immunol* 1991;**147:**2559–2564.
24. Salvemini D, Wang ZQ, Zweier JL, Samouilov A, Macarthur H, Misko TP, *et al*. A nonpeptidyl mimic of superoxide dismutase with therapeutic activity in rats. *Science* 1999;**286:**304–306.
25. van Lent PL, Nabbe KC, Blom AB, Sloetjes A, Holthuysen AE, Kolls J, *et al*. NADPH-oxidase-driven oxygen radical production determines chondrocyte death and partly regulates metalloproteinase-mediated cartilage matrix degradation during interferon-gamma-stimulated immune complex arthritis. *Arthritis Res Ther* 2005;**7:**R885–R895.
26. Wipke BT, Allen PM. Essential role of neutrophils in the initiation and progression of a murine model of rheumatoid arthritis. *J Immunol* 2001;**167:**1601–1608.
27. van de Loo FA, Bennink MB, Arntz OJ, Smeets RL, Lubberts E, Joosten LA, *et al*. Deficiency of NADPH oxidase components p47phox and gp91phox caused granulomatous synovitis and increased connective tissue destruction in experimental arthritis models. *Am J Pathol* 2003;**163:**1525–1537.

28. Olofsson P, Holmberg J, Pettersson U, Holmdahl R. Identification and isolation of dominant susceptibility loci for pristane-induced arthritis. *J Immunol* 2003;**171:**407–416.
29. Katz P, Simone CB, Henkart PA, Fauci AS. Mechanisms of antibody-dependent cellular cytotoxicity: the use of effector cells from chronic granulomatous disease patients as investigative probes. *J Clin Invest* 1980;**65:**55–63.
30. Borregaard N, Kragballe K. Role of oxygen in antibody-dependent cytotoxicity mediated by monocytes and neutrophils. *J Clin Invest* 1980;**66:**676–683.
31. Weiss SJ, Young J, LoBuglio AF, Slivka A, Nimeh NF. Role of hydrogen peroxide in neutrophil-mediated destruction of cultured endothelial cells. *J Clin Invest* 1981;**68:**714–721.
32. Miller GP, Kohl S. Antibody-dependent leukocyte killing of *Cryptococcus neoformans*. *J Immunol* 1983;**131:**1455–1459.
33. Lichtenstein A, Seelig M, Berek J, Zighelboim J. Human neutrophil-mediated lysis of ovarian cancer cells. *Blood* 1989;**74:**805–809.
34. Siebens H, Tevethia SS, Babior BM. Neutrophil-mediated antibody-dependent killing of herpes-simplex-virus-infected cells. *Blood* 1979;**54:**88–94.
35. Kushner BH, Cheung NK. Clinically effective monoclonal antibody 3F8 mediates nonoxidative lysis of human neuroectodermal tumor cells by polymorphonuclear leukocytes. *Cancer Res* 1991;**51:**4865–4870.
36. van Kessel KP, van Strijp JA, van Kats-Renaud HJ, Miltenburg LA, Fluit AC, Verhoef J. Uncoupling of oxidative and non-oxidative mechanisms in human granulocyte-mediated cytotoxicity: use of cytoplasts and cells from chronic granulomatous disease patient. *J Leukoc Biol* 1990;**48:**359–366.
37. Fine JD, Tyring S, Gammon WR. The presence of intralamina lucida blister formation in epidermolysis bullosa acquisita: possible role of leukocytes. *J Invest Dermatol* 1989;**92:**27–32.
38. Liu Z, Giudice GJ, Zhou X, Swartz SJ, Troy JL, Fairley JA, *et al*. A major role for neutrophils in experimental bullous pemphigoid. *J Clin Invest* 1997;**100:**1256–1263.
39. Girardi G, Berman J, Redecha P, Spruce L, Thurman JM, Kraus D, *et al*. Complement C5a receptors and neutrophils mediate fetal injury in the antiphospholipid syndrome. *J Clin Invest* 2003;**112:**1644–1654.
40. Looney MR, Su X, Van Ziffle JA, Lowell CA, Matthay MA. Neutrophils and their Fcgamma receptors are essential in a mouse model of transfusion-related acute lung injury. *J Clin Invest* 2006;**116:**1615–1623.
41. Nandakumar KS, Svensson L, Holmdahl R. Collagen type II-specific monoclonal antibody-induced arthritis in mice: description of the disease and the influence of age, sex, and genes. *Am J Pathol* 2003;**163:**1827–1837.
42. Reeves EP, Lu H, Jacobs HL, Messina CG, Bolsover S, Gabella G, *et al*. Killing activity of neutrophils is mediated through activation of proteases by K^+ flux. *Nature* 2002;**416:**291–297.

Note added in press

This article was published online on 23 March 2007. An omission was subsequently identified in the acknowledgements section, and corrected by an erratum notice that was published online only. This printed version incorporates the amendment identified by the erratum notice.