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Monocytes enhance neutrophil-induced blister formation in an *ex vivo* model of bullous pemphigoid

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Abbreviations:

BFA, brefeldin A; BP, bullous pemphigoid; BPS, bullous pemphigoid serum; DEJ, dermal-epidermal junction; DES, dermal-epidermal separation; DHR, dihydro-rhodamine; DPI, diphenyleneiodonium chloride; FcR, Fc receptor; ICAM, intercellular adhesion molecule; ROS, reactive oxygen species; NE, neutrophil elastase; NOS, nitric oxide synthase; MMP-9, matrix metalloproteinase-9; MPO, myeloperoxidase; PBMC, peripheral blood mononuclear cells;

Abstract

Background: Lesions of bullous pemphigoid (BP), an autoimmune subepidermal blistering disease characterized by the presence of tissue-bound and circulating autoantibodies to hemidesmosomal antigens, harbour a mixed inflammatory cellular infiltrate. In various models, neutrophils, eosinophils, mast cells, monocytes as well as B and T cells have been shown to be involved in the pathogenesis of BP. However, their interactions with and effective role in blister formation remains uncertain. This study was aimed at investigating the effect of monocyte/neutrophil interaction on blister formation in an *ex vivo* BP model.

Methods: Skin cryosections were incubated with purified human neutrophils and monocytes, in the presence or absence of BP autoantibodies. Production of reactive oxygen species (ROS), degranulation, mediator release (neutrophil elastase (NE), myeloperoxidase (MPO), matrix metalloproteinase-9 (MMP-9)), binding of Fc γ receptor (CD16, CD32, CD64), and cell adhesion (CD18, ICAM-1) were investigated using appropriate inhibitors. Dermal-epidermal separation (DES) was assessed by light microscopy and quantified by Fiji software.

Results: Monocytes and neutrophils synergistically interact resulting in a significantly higher DES compared to either monocytes or neutrophils separately ($P < 0.0001$). Monocyte/neutrophil-induced DES was associated with increased ROS production and was dependent on adhesion and Fc γ RIII binding. Upon stimulation by the granule-poor fraction of monocyte supernatants, neutrophils increased their release of MMP-9, thereby also DES at the dermal-epidermal junction of skin cryosections.

Conclusion: Our observations suggest that the interaction of cells, as shown here for monocytes and neutrophils, enhances mediator release resulting in an increased subepidermal blister formation. Thus, blocking intercellular cross-talk promises a new therapeutic approach for blocking tissue damage in BP.

INTRODUCTION

Bullous pemphigoid (BP), the most common autoimmune subepidermal blistering disorder in Western Europe and North America, is characterized by circulating and tissue-bound antibodies targeting two hemidesmosomal antigens: BP180 (BPAG2, type XVII collagen) and BP230 (BPAG1) (1-3). BP180 is considered to be the major autoantigen and its extracellular non-collagenous 16A (NC16A) domain is the immunodominant region in BP (2, 4). Histology demonstrates subepidermal blisters with an infiltration of large numbers of eosinophils and the presence of neutrophils, but also lymphocytes, monocyte-macrophages (MOMA) and basophils in early lesions (3-5).

There is evidence that both eosinophils and neutrophils contribute to the splitting at the dermal-epidermal junction (6-10). Upon stimulation with BP auto-antibodies, *in vitro* cultured normal human epidermal keratinocytes secreted interleukin (IL)-8, a chemoattractant cytokine for neutrophils (11). Myeloperoxidase (MPO) (12), neutrophil elastase (NE) (13, 14) and matrix metalloproteinase-9 (MMP-9, also known as 92-kDa type IV collagenase, gelatinase B) (9) have all been detected in BP lesions and blister fluids. NE and MMP-9, that both may degrade the extracellular matrix, were required for blister formation in experimental BP (13, 14). Furthermore, the production of reactive oxygen species (ROS) by neutrophils has been shown to be essential for autoantibody-induced tissue damage in a cryosection model of BP (9).

In addition to neutrophils and eosinophils, autoreactive T cells recognizing BP180 epitopes (15, 16), B cells, e.g. by expressing B-cell activating factor (BAFF) (17), and monocytes (18) have been reported to contribute to the pathogenesis of BP. Furthermore, significantly elevated levels of interferon (IFN)- γ -inducible protein-10 (IP-10, CXCL10) and monocyte chemo-attractant protein (MCP)-1, which is known to chemo-attract and activate monocytes, basophils and lymphocytes, have been detected in BP patients (19).

The presence of different inflammatory cells as well as corresponding chemokines and mediators suggests an interplay in the induction and progression of BP. Monocytes and macrophages, as well as neutrophils have been shown to interact in the initiation and termination of inflammation as they cooperatively promote their mutual recruitment. Furthermore, macrophages take up apoptotic neutrophils (20). By amplifying the infiltration of neutrophils in a mast cell-dependent manner, macrophages were found to be crucial for the blister formation in an animal BP model (21). So far, it is not clear whether inflammatory cells directly interact with neutrophils in BP lesions and whether they enhance or limit blister formation. Therefore, we have aimed to investigate the effects of peripheral blood mononuclear cells (PBMC), in particular monocytes, on neutrophil-induced dermal-epidermal separation (DES) in an *ex vivo* skin model of BP.

METHODS

Patient materials

Serum samples were obtained from healthy donors, for normal human serum (NHS), and from BP patients prior to therapy (BP serum; BPS). BD Biosciences (Erembodegem, Belgium) Vacutainer blood collection tubes with no anti-coagulant added (# 368861) were used. In BPS, the mean titres for BP180 and BP230 antibodies were 102.90 units/ml and 39.47 units/ml, respectively, as assessed by ELISA. Leukocytes were obtained from peripheral blood samples from healthy donors without any history of allergy or autoimmunity, collected in BD Biosciences Vacutainer blood collection tubes containing lithium heparin (# 367526). Healthy human foreskin was obtained from routine circumcision. The study had been approved by the Cantonal Ethics Committee (KEK) Bern. Written informed consent was obtained from all patients prior to enrolment in the study.

Induction and evaluation of DES

The cryosection model was used as previously described with minor modifications (6, 22). Briefly, human foreskin was washed, prepared and embedded at an optimum cutting temperature in Tissue-Tek® O.C.T.™ compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, The Netherlands) and stored at -20°C. Six foreskin cryosections (transverse, 6-µm-thick) were placed on adhesive microscope slides

(Starfrost®, Knittel Glass, Medite Service AG, Dietikon, Switzerland). BPS and NHS were diluted 1:2 with phosphate-buffered saline and applied to the skin sections for 2 h at 37°C, followed by washing the slides with phosphate buffered saline. Leukocytes resuspended in DMEM (DMEM plus GlutaMAX™, Gibco®, Life Technologies Europe B.V., Zug, Switzerland) were subsequently added in the prepared chambers for a further incubation of 4 h at 37°C. Tissue sections were then fixed with 3.7% formalin and stained with hematoxylin and eosin (H&E). DES was analysed by light microscopy. The total lengths of DES and DEJ, respectively, were calculated using Fiji (23). DES was given as a percentage of separation at the dermal-epidermal junction (DEJ). To assess tissue damage semi-quantitatively in the indicated experiments, we scored the extent of skin damage and presence of DES as previously reported (6). Each experiment was repeated at least three times to confirm reproducibility.

Isolation of human neutrophils and monocytes

The whole leukocyte fraction was obtained after sedimentation through 3% DextranT500 (Amersham Pharmacia Biotech., GE Healthcare Europe GmbH, Glattbrugg, Switzerland) in 0.9% NaCl solution as described previously (22). Peripheral blood neutrophils were isolated by density-gradient centrifugation using Pancoll Human from PAN™ BioTech (PAN-Biotech GmbH, Aidenbach, Germany). The lower layer, containing mainly neutrophils and erythrocytes was treated with erythrocyte lysis solution (155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA, pH 7.3). The resulting cell populations contained > 95% neutrophils as assessed with the Hemacolor® Rapid staining (Merck Millipore, Darmstadt, Germany) followed by light microscopic analysis (24).

Monocytes were isolated from the upper fraction containing PBMC using EasySep™ Human Monocyte Enrichment Kit, immunomagnetic negative selection procedure (STEMCELL Technologies, Grenoble, France). Unwanted cells were removed with tetrameric monoclonal antibody complexes recognizing CD2, CD3, CD16, CD19, CD20, CD56, CD66b, CD123, glycophorin A on dextran-coated magnetic particles. The cocktail also contains an antibody to human FcR to prevent nonspecific binding of monocytes. Labeled cells were separated using an EasySep™ magnet without the use of columns. For the positive selection procedure, monoclonal antibodies directed against CD14 were used (EasySep™ Human CD14

Positive Selection Kit, STEMCELL Technologies). The collected cells were analysed by Hemacolor® Rapid staining and light microscopy. Fractions containing $\geq 95\%$ monocytes were employed for further experiments.

Hoxb8 neutrophils

Mouse neutrophils were generated from stem cell factor (SCF)-dependent, conditional Hoxb8-immortalized myeloid progenitors derived from wild-type (C57BL/6) and NADPH oxidase NOX2-knockout mice, as previously described (25, 26). 3×10^5 cells/ml were passaged in RPMI 1640/GlutaMAX with 10% FCS, 100 U/ml penicillin/100 $\mu\text{g/ml}$ streptomycin, 50 μM 2-ME, mouse SCF (5% of CHO/SCF cell conditioned medium), and 100 nM 4-hydroxytamoxifen for 3–4 d. To initiate the differentiation into neutrophils, 2.5×10^4 cells/ml were washed and then cultured in the same medium in the absence of 4-hydroxytamoxifen. Upon 3 days of differentiation, 5 $\mu\text{g/ml}$ granulocyte colony-stimulating factor (G-CSF) (PeproTech EC Ltd., London, UK) was added to cell cultures and after further incubation for two days, cells were washed twice with phosphate buffered saline, and once with X-VIVO™ 15 medium, and resuspended with DMEM before being used for subsequent experiments. Immunophenotypic characterization of Hoxb8 mouse neutrophils was performed by determining the cell surface expression of Ly6G using flow cytometry.

Pharmacological inhibition of reactive oxygen species, reactive nitrogen species, and degranulation

To block ROS production, monocytes and neutrophils were incubated with diphenyleneiodonium chloride (DPI, Calbiochem, Merck KGaA, Darmstadt, Germany), which is widely used as a general inhibitor of ROS production and blocks NADPH oxidase activity as well as the enzymatic activity of several flavoproteins that are part of the mitochondrial oxidation pathway (27), or L-NAME (L-NAME hydrochloride, Enzo Life Sciences AG, Lausen, Switzerland), a nitric oxide synthase (NOS) inhibitor. Both inhibitors were employed in a concentration-dependent manner (DPI at 400 μM , 800 μM and 1000 μM , and L-NAME at 1, 5, 25 and 50 μM) for 30 min at 37°C prior to their incubation with skin sections. To exclude a potential toxic effect of DPI on cell viability, monocytes and neutrophils were recovered from the chambers after a standard 4-h incubation and stained with annexin V (FITC, BD Pharmingen™, Allschwil, Switzerland) and propidium iodide (PI, Sigma-Aldrich,

Buchs, Switzerland). Apoptosis was quantified by flow cytometry (BD FACSVerse™, BD Biosciences, Allschwil, Switzerland) and FlowJo software (Ashland, USA) as described previously (28, 29).

To quantify ROS production, 1 μ M dihydrorhodamine-123 (DHR, Sigma-Aldrich) was added to monocytes and neutrophils prior to their addition to human skin sections which had been incubated with BPS, NHS or phosphate-buffered saline in a black, glass-bottom 96-well plate (Greiner Bio-One GmbH, Frickenhausen, Germany) (29). Fluorescence activity of the DHR was measured at excitation 485 nm and the fluorescence emission at 538 nm, using a SpectraMaxM2 plate reader (Bucher Biotech, Basel, Switzerland) over a period of 2 h. Negative and positive controls with 50 μ M DPI and 25 nM phorbol-myristate-acetate (PMA, Calbiochem), respectively, were performed. In separate experiments, 5% nitroblue tetrazolium (NBT, Sigma-Aldrich, Buchs, Switzerland) was added to the cells and incubated for 1.5 h at 37°C on BPS or NHS co-incubated cryosections as described by others (9) in order to determine intracellular and extracellular ROS production (30). Slides were stained with hematoxylin and formazan precipitations reflecting ROS production, were analysed by light microscopy and Fiji software (25).

To block degranulation, monocytes and neutrophils were incubated for 30 min at 37°C with 10 μ g/ml brefeldin A (BFA, Sigma-Aldrich co, Buchs, Switzerland) which inhibits vesicular transport and granule emptying (28). Furthermore, monocytes and neutrophils were incubated with inhibitors of serine proteases (10 μ M GW311616A, Sigma-Aldrich), myeloperoxidase (500 μ M ABAH, Calbiochem) or MMP-2/MMP-9 (100 μ M SB-3CT, Enzo Life Sciences AG) for 30 min at 37°C prior to their application to the chambers.

Blocking adhesion molecules and Fc γ receptors (Fc γ R)

To block adhesion, monocytes and neutrophils were incubated with 20 μ g/ml mouse F(ab')₂ fragments directed against human CD18 (Ansell Corporation, Bayport, USA) and/or mouse IgG1 against the intercellular adhesion molecule (ICAM)-1 (CD54, R&D Systems, Minneapolis, USA) for 45 min on ice. To block Fc γ R-mediated effects, cells were incubated with 10 μ g/ml mouse F(ab')₂ fragments against CD16, CD32 and CD64 (all from Ansell Corporation, Bayport, USA) for 45 min on ice. Isotype-matched mouse anti-human F(ab')₂ fragments (Jackson ImmunoResearch

Europe Ltd. Suffolk, UK) and the monoclonal mouse IgG1 whole molecule (Dako Schweiz GmbH, Baar, Switzerland) served as controls.

Supernatant assays

After incubating monocytes and neutrophils on BPS treated cryosections for 4 h, the supernatant was recovered from the chambers, centrifuged (10 min, 500 x g) to remove any left-over cells, and subsequently added, alone or in combination with freshly isolated monocytes and/or neutrophils, to freshly prepared cryosections for incubation for another 4 h. In subsequent experiments monocyte-derived supernatant was ultra-centrifuged (20 min, 27'000 x g) at 4°C in order to obtain a granule-poor supernatant and a granule-rich pellet (31). Together with freshly isolated neutrophils, each was added to freshly prepared cryosections and incubated for 4 h.

Determination of MMP-9 release

The release of MMP-9 in supernatants of monocytes and neutrophils after 4-h co-incubation with cryosections was determined by immunoblot analysis. Briefly, samples were denatured in reducing Laemmle buffer prior to separation on 12 % sodium dodecyl sulfate (SDS) polyacrylamide gel (Expedeon, Lucerna-Chem AG, Luzern, Switzerland). Proteins were blotted on PVDF transfer membrane (Immobilon-FL®, Millipore, Merck&Cie, Schaffhausen, Switzerland) and subsequently probed with primary, rabbit anti-MMP-9 antibody (1:1000; G657; Cell Signalling Technology Europe B.V., Leiden, The Netherlands). Subsequently, membranes were incubated with a secondary, goat anti-rabbit IgG-HRP antibody (P0449, Dako Schweiz GmbH), and signals were detected using enhanced chemiluminescence (ECL, ECL Western Blotting Analysis System, GE Healthcare Europe GmbH, Glattbrugg, Switzerland). Imaging was performed by LI-COR technology using the Odyssey® Fc Imaging System (LI-COR Biosciences GmbH, Bad Homburg, Germany). To further quantify MMP-9 in supernatants, an ELISA was performed using a kit pre-coated with anti-human MMP-9 (LEGEND MAX™, Biolegend Inc., Fell, Germany). Protein quantification was based on the absorbance read at 450 – 570 nm with a SpectraMaxM2 plate reader using an protein concentration standard curve.

Statistical analysis

Data were analysed by Graph Pad Prism 5 software (La Jolla, CA, USA) and are presented as means $\pm$ SEM. *P* values were obtained with one-way ANOVA, followed by Tukey's multiple comparisons test or unpaired two-tailed *t*-test to compare between values of different conditions. *P* values of <0.05 were considered significant.

RESULTS

Synergistic effect of neutrophils and monocytes on DES

To determine the ability of different inflammatory cells to induce DES, cryosections were incubated with either the whole leukocyte population, PBMC fraction, containing B and T lymphocytes, monocytes, basophils and natural killer (NK) cells, or neutrophils. Compared to the extensive DES affecting 85.2 ± 2.9 % of the DEJ which was induced by the whole leukocyte population in the presence of BPS, both neutrophils and PBMC induced less DES, 44.7 ± 6.6 % and 27.8 ± 4.9 %, respectively. Negligible DES was observed when NHS was used ($< 5.8\%$), or eosinophils (1.5 ± 0.4 %) were added (Fig. 1A). As both PBMC and neutrophils could induce DES, we proceeded by co-incubating different ratios of PMBC and neutrophils. The DES induced by 20×10^6 /ml PBMC together with 10×10^6 /ml neutrophils was 76 ± 13.2 % and thus comparable with that induced by to the whole leukocyte fraction. Co-incubation of PBMC with eosinophils at the same ratio did not exhibit any synergistic effect (Fig. 1B and 1E).

In order to identify the cells within the PBMC fraction mediating an increased DES, monocytes isolated by negative selection or PBMC without monocytes (equivalent to positively selected B cells, T cells, basophils and NK cells) were co-incubated with neutrophils. Assuming a mean monocyte portion of 25% in the total PBMC fraction (normal range 2-40% (32), we used 5×10^6 /ml monocytes and 15×10^6 /ml monocyte-deprived PBMC in further experiments. Co-incubation of 10×10^6 /ml neutrophils with 5×10^6 /ml monocytes resulted in a DES of $80.0 \pm 9.1\%$ that was as high as DES induced by neutrophils together with 20×10^6 /ml PBMC (Fig. 1C and 1F). Monocyte-deprived PBMC, even at high cell numbers or when isolated with a negative selection procedure to exclude inhibitory effects of the magnetic particles in the isolation kit, did not enhance neutrophil induced DES (Fig. 1D).

Taken together, monocytes could be demonstrated to synergistically interact with neutrophils, resulting in an enhanced subepidermal blister formation in the cryosection BP model.

Monocytes/neutrophil-induced DES is ROS-dependent

To evaluate the role of ROS in monocyte/neutrophil-induced DES, we tested the effect of DPI, a NADPH oxidase inhibitor, in our *ex vivo* skin model. DPI inhibited DES in a concentration-dependent manner (Fig. 2A and Fig. 2C-E). To exclude cell toxicity caused by high concentrations of DPI, we checked cell viability (28, 29). More than 85% of the monocytes and neutrophils, which were recovered from the cryosection chambers after 4 h incubation, were shown to be ethidium bromide negative (results not shown) and annexin V-/PI negative as assessed by FACS analysis (Supplemental Fig. 1) demonstrating that DPI was not toxic. L-NAME, an inhibitor of NOS, at increasing concentrations did not have any effect on DES, indicating that NOS were not involved in blister formation under the experimental conditions used (Fig. 2B and 2F).

By measuring intracellular ROS using the cell-entering dye DHR, we observed slightly increased ROS production when monocytes and neutrophils were co-incubated as compared to neutrophils ($P=0.5$) or monocytes ($P=0.0225$) alone in the presence of BPS. This could be blocked by DPI (Fig. 2G). The nitroblue tetrazolium (NBT) assay revealed marked ROS production by neutrophils in the presence of BPS that was slightly increased upon co-incubation with monocytes (Fig. 2H-O). These observations suggested that neutrophils were the relevant source of ROS produced at the DEJ in the presence of BPS in the *ex vivo* skin model. To confirm the importance of NADPH oxidase in neutrophil-dependent DES as described previously (9), we generated mature neutrophils from conditional Hoxb8-immortalized myeloid progenitors derived from NOX2-knockout and wild-type mice (24, 25). Mouse Fc γ R can efficiently bind to human IgG subclasses (33). NOX2-knockout mouse neutrophils could not induce DES in any of the experimental conditions, whereas wild-type mouse neutrophils induced DES upon BPS co-incubation (Supplemental Fig. 2).

Monocyte/neutrophil-induced DES depends on adhesion and FcγR

Since adhesion has been shown to play a crucial role for IgG-mediated neutrophil activation (34), we were interested in determining the role of cell adhesion in monocyte/neutrophil-induced DES. Therefore, we blocked CD18, the common β chain shared by leukocyte integrins, and ICAM-1, an important ligand expressed by epidermal keratinocytes in BP lesions, as well as on activated monocytes (35-37). Upon blocking of CD18 or ICAM-1, we observed a decrease of DES induced by monocytes plus neutrophils in the presence of BP autoantibodies, which was highly significant especially upon blocking of both CD18 and ICAM-1 ($P < 0.0001$) (Fig. 3A-C).

In an animal model of BP, FcγR activation was shown to play a critical role (39). In order to investigate the relevance of neutrophil and monocyte FcγRs in the recognition of IgG autoantibodies in BP serum in our *ex vivo* skin model, we used blocking F(ab')₂ fragments directed against CD16 (FcγRIII), CD32 (FcγRII) and CD64 (FcγRI). A significant decrease of DES was noticed when CD16 was blocked, whereas F(ab')₂ fragments directed against CD32 or CD64 or control F(ab')₂ fragments had no effect on DES (Fig. 3D). Our results underline the importance of BP autoantibody through FcγR, in particular FcγRIII binding, in monocyte/neutrophil-induced subepidermal blister formation.

Monocytes enhance neutrophil-induced DES by releasing a granule-poor fraction

As neutrophil granule proteins have been shown to activate macrophages at the site of inflammation (38), we investigated whether neutrophil cell supernatant would be sufficient to activate monocytes, and *vice versa*, resulting in an increased subepidermal blister formation in our model. For this purpose, we collected monocyte and neutrophil supernatants and added them to freshly isolated neutrophils or monocytes, respectively. We could observe a significant increase in neutrophil-induced DES upon addition of monocyte supernatants ($12.6 \pm 3.9\%$ *versus* $60.2 \pm 19.8\%$, $P=0.0211$) (Fig. 4A and 4C). In contrast, no effect on DES was seen when neutrophil supernatants were added to monocytes, or when neutrophil or monocyte supernatants were applied on cryosections (Fig. 4A and 4D).

In order to get insight into relevant mediator(s) within the monocyte supernatant, we applied ultracentrifugation to obtain a granule-rich pellet and a granule-poor fraction (31). When these fractions were added to freshly isolated neutrophils in our BP *ex vivo* model, we observed that the granule-poor fraction significantly increased neutrophil-induced DES, whereas the granule-rich fraction did not (Fig. 4B, 4E and 4F). According to recently published work, cell organelles in the granule-poor fraction include microvesicles, such as exosomes and shedding vesicles, that are involved in multiple biological processes including intercellular communication (39). Taken together, we conclude that monocytes, by releasing mediators as part of the granule-poor fraction, stimulate neutrophils, resulting in an enhanced neutrophil-induced blister formation.

Monocyte/neutrophil-induced DES depends on MMP-2/MMP-9 release

Granule proteins seem to be important in the pathogenesis of BP as NE, MPO and MMP-9 have been detected in blister fluids and skin lesions and BP180 could be cleaved by NE and MMP-9 ((12-14). In order to investigate the role of monocyte and neutrophil granule proteins in our model, we pharmacologically inhibited NE (GW311616A), MPO (ABAH) and MMP-2/MMP-9 (SB-3CT) (Fig. 5A). The inhibition of NE or MPO did not have any effect on DES (Fig. 5A, 5C and 5D), while inhibition of MMP-2/MMP-9 led to a significant decrease in DES ($74.7 \pm 11.0\%$ *versus* $4.0 \pm 0.8\%$, $P=0.0028$) (Fig. 5A and 5E). The decrease of DES upon blocking with SB-3CT was not caused by an effect on cell viability, since more than 90% of the cells were ethidium bromide negative in a viability assay (40).

Monocytes increased neutrophil MMP-9 release independent of autoantibodies, degranulation or adhesion

MMP-9 has been ascribed an important role in the pathogenesis of BP (14, 41, 42). Western blot analysis and protein quantification by ELISA revealed a significantly increased MMP-9 release upon co-incubation of monocytes and neutrophils as compared to the release by either monocytes or neutrophils alone (Fig. 6A and 6B). MMP-9 release by neutrophils co-incubated with monocytes was not dependent on autoantibodies (Fig. 6A and 6B). Since DES was only partially blocked by BFA, but significantly reduced upon inhibition of MMP-2/MMP-9 with SB-3CT, we asked whether BFA affected MMP-9 release. BFA had no effect on MMP-9 release by

neutrophils co-incubated with monocytes (Fig. 6C). Next, we investigated the importance of adhesion for MMP-9 release; however, the inhibition of CD18/ICAM-1 did not decrease MMP-9 protein release by neutrophils co-incubated with monocytes (Fig. 6D).

DISCUSSION

In this report, using a human *ex vivo* model of BP, we demonstrate that the interaction of monocytes and neutrophils results in a synergistic effect on blister formation at the DES. The synergism of monocytes and neutrophils leading to an extensive DES was found: 1. to be associated with increased ROS production and to be dependent on adhesion and Fc γ RIII binding, 2. to be mediated by a granule-poor fraction in the supernatants of monocytes stimulating neutrophils and, 3. to result in an increased MMP-9 release by neutrophils. Recently, mice deficient in neutrophils, mast cells and macrophages were shown to be resistant to experimental BP induced by injection with pathogenic anti-mBP180 IgG (21). In this experimental model, macrophages amplified neutrophil infiltration in a mast cell-dependent manner (21).

As neutrophil-induced DES has been reported to be NADPH oxidase-dependent (9, 43), we investigated the role of ROS and monocyte/neutrophil-induced tissue damage. ROS and reactive nitrogen species, the principle redox molecules in immunity, can be produced by both activated neutrophils and monocytes by means of NADPH oxidases and NOS (44, 45). While the output production of ROS is higher in neutrophils, that of reactive nitrogen species is higher in monocytes (45). In the *ex vivo* BP model used here, DPI significantly inhibited DES induced by neutrophils plus monocytes. As DPI also inhibits other flavoprotein oxidoreductases, such as NOS, in addition to NADPH oxidase (46), we investigated the role of NOS in blister formation. However, inhibiting NOS had no effect, indicating that reactive nitrogen species production was not required for DES induction. In conclusion, our results show that the synergistic effect of neutrophils plus monocytes on DES is linked to an increased ROS production. This finding is in line with the recent observation that monocytes promote neutrophil recruitment and ROS production leading to neutrophil-dependent tissue injury in an experimental model of glomerulonephritis (47).

In agreement with recent observations on the essential role of adhesion in IgG-mediated neutrophil activation (34), we demonstrate a significant reduction in subepidermal blister formation when adhesion was blocked by antibodies directed against CD18 and ICAM-1. CD18 is expressed by both neutrophils and monocytes (35), while ICAM-1, a ligand for CD18 integrins, can be expressed by epidermal keratinocytes in BP lesions and activated monocytes (36, 37). Despite the fact that neutrophils and monocytes could attach to each other through these adhesion molecules, our data suggest that the decrease in DES upon blocking CD18 and ICAM is more likely owing to reduced binding of these cells to the epidermal keratinocytes.

Autoantibodies directed against BP180 present in the serum and tissues of patients with BP have been demonstrated to be essential for subepidermal blister formation (48-51). BPS and purified IgG from the same sera were shown to have identical effects on DES in the *ex vivo* model, as BPS depleted of IgG reactive to NC16A or F(ab')₂ fragments directed against NC16A of BP180 failed to induce DES (22). Therefore, we refrained from purification and detailed characterization of the BPS in the present study. The function of anti-mBP180 IgG depends entirely on its Fc domain (52). In agreement with the recent observation that FcγRIII plays a critical role in the activation of neutrophils in experimental BP (52), we observed a significant decrease in DES in the presence of F(ab')₂ fragments directed against CD16 (FcγRIII), whereas F(ab')₂ fragments directed against CD32 (FcγRII) or CD64 (FcγRI) were without effect. Neutrophils express FcγRIIIB and FcγRIIA in a quiescent state, and FcγRI can be induced upon stimulation, e.g. with granulocyte colony-stimulating factor, while on monocytes, FcγRIIIA, FcγRIIA, and FcγRI have all been detected (33).

Monocytes and neutrophils may interact at sites of inflammation (46). So far, neutrophil granule proteins have been reported to activate macrophages to produce and release cytokines (20). Our study demonstrates that monocytes may further activate neutrophils in as much as the granule-poor fraction of monocyte supernatants stimulates neutrophils, resulting in a significant increase in DES. The granule-poor fraction consists of cell organelles including microvesicles, such as exosomes and shedding vesicles, which have been described to be involved in multiple biological processes (39). Exosomes contain various cytokines, growth factors, proteins and nucleic acids such as messenger RNA and microRNAs (53).

The exact mechanism by which exosomes activate neutrophils needs to be investigated in further studies.

Granule protein release has been shown to play an essential role in the pathogenesis of BP (12-14). In our experimental setting, co-incubation of monocytes and neutrophils resulted in a significant increase of MMP-9 release, and the inhibition of MMP-2/MMP-9 significantly reduced DES. Inhibition of MPO and serine proteases had no effect in our model. This is in contrast to previous studies reporting high levels of MPO and NE in blister fluids (7, 12, 54) and that MPO and NE cleave BP180 (8, 13, 55). NE had also been suggested to be required for blister formation (14), possibly owing to the use of non-specific inhibitors. There is increasing evidence that gelatinases, especially MMP-9, are involved in the pathogenesis of BP (14, 41, 42). BP relapses have been linked to increased concentrations of CXCL10, a chemokine that stimulates MMP-9 release by neutrophils and monocytes (18). Interestingly, MMP-9 release upon monocyte/neutrophil incubation was independent of blocking degranulation with BFA or adhesion. Recently, an ATP-induced MMP-9 release by monocytes that failed to be blocked with BFA has been reported (18).

In summary, our study provides further evidence that there is a close interaction of inflammatory cells which result in synergistic effects on subepidermal blister formation in BP. In our *ex vivo* BP model, a monocyte-dependent augmentation of neutrophil-induced DES was demonstrated. So far, it remains largely unclear whether cells other than monocytes and neutrophils, e.g. eosinophils, mast cells, basophils, might interact in human BP. Understanding these mechanisms will help to identify novel therapeutic targets.

CONFLICT OF INTEREST

The authors report no conflicts of interest.

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Figure legends

Figure 1. Synergistic effects of monocytes and neutrophils on dermal-epidermal separation (DES) in an *ex vivo* skin model. (A-D) Graphs show means $\pm$ SEM of quantitative measures of DES at the DEJ ($n = 3-13$). (A) Quantification of DES in cryosections upon incubation with the whole leukocyte fraction, neutrophils, eosinophils, or PBMC ($30 \times 10^6/\text{ml}$ each) in the presence of autoantibodies (BPS) or normal serum (NHS). (B) DES induced by the whole leukocyte population or PBMC plus neutrophils at different ratios. (C) Quantification of DES induced by monocytes ($5 \times 10^6/\text{ml}$), PBMC ($20 \times 10^6/\text{ml}$), or PBMC deprived of monocytes ($15 \times 10^6/\text{ml}$) with/without co-incubation of neutrophils ($10 \times 10^6/\text{ml}$) in the presence of BPS or NHS. (D) Higher numbers of monocyte-deprived PBMC also fail to induce DES. (E-H) Representative images (H&E) showing DES (arrows) on cryosections in the presence of BPS incubated with PBMC and neutrophils (2:1) (E) or with neutrophils and monocytes (F). No DES was observed when NHS was used (G) or PBMC-deprived of monocytes were co-incubated with neutrophils in the presence of BPS (H). Magnification $\times 200$.

Figure 2. Synergistic effect of monocytes and neutrophils on dermal-epidermal separation (DES) is associated with an increased ROS production. (A, B) Graphs show means $\pm$ SEM of quantitative measures of DES at the DEJ ($n = 4-5$). DES induced by monocytes ($5 \times 10^6/\text{ml}$) and neutrophils ($10 \times 10^6/\text{ml}$) co-incubated with BPS is inhibited by $50 \mu\text{M}$ DPI (A), but not L-NAME (B). (C-D) Representative images show DES (arrows) in human foreskin cryosections, incubated with BPS and monocytes ($5 \times 10^6/\text{ml}$) and neutrophils ($10 \times 10^6/\text{ml}$) in the absence (C) or presence of $1 \mu\text{M}$ DPI (D), $50 \mu\text{M}$ DPI (E) or 1 mM L-NAME (F). Magnification $\times 200$. (G) DHR measurement of intracellular ROS production reveals an increased ROS production upon monocyte/neutrophil co-incubation compared to monocytes or neutrophils alone in the presence of BPS. $50 \mu\text{M}$ DPI and 25 nM PMA were used as controls. Bars show means $\pm$ SEM of fluorescence ($n = 4$). (H) The graph shows mean numbers $\pm$ SEM ($n = 5$) of formazan deposits indicating that ROS release at the DEJ of human skin cryosections is highest when neutrophils were incubated with monocytes in the presence of BPS. (I-K) Representative images of

skin cryosections show a lack of formazan deposits when monocytes ($5 \times 10^6/\text{ml}$) were applied with either (BPS (I) or (K) NHS (K). In contrast, deposits are seen at the DEJ upon incubation with neutrophils ($10 \times 10^6/\text{ml}$) in the presence of BP autoantibodies (L), but not NHS (M). Formazan deposits are also seen following co-incubation of monocytes $5 \times 10^6/\text{ml}$ and neutrophils $10 \times 10^6/\text{ml}$ in the presence of BPS (N), but not NHS (O). Formazan deposits at the stratum corneum (asterisks) were considered unspecific. Magnification $\times 100$.

Figure 3. Synergistic monocyte/neutrophil induced dermal-epidermal separation (DES) is dependent on adhesion and Fc γ RIII binding. (A, D) Graphs show means $\pm$ SEM of quantitative measures of DES at the DEJ ($n = 3-5$). (A) DES of skin sections co-incubated with monocytes ($5 \times 10^6/\text{ml}$), neutrophils ($10 \times 10^6/\text{ml}$) and BPS was blocked by antibodies to the adhesion molecules CD18 and ICAM-1. (B, C) Representative images of human skin cryosections show significantly decreased DES upon blocking with $5 \mu\text{g}/\text{ml}$ anti-CD18 and $10 \mu\text{g}/\text{ml}$ anti-ICAM-1 antibodies (B), while isotype control F(ab')₂ fragments and IgG1 had no effect on DES (C). Arrows indicate DES. Magnification $\times 200$. (D) Monocyte/neutrophil-induced DES is dependent on Fc γ RIII since blocking F(ab')₂ fragments against CD16 decreased DES whereas F(ab')₂ fragments blocking CD32 (Fc γ RII) and CD64 (Fc γ RI) or control isotype F(ab')₂ fragments were without effect.

Figure 4. Monocytes, by releasing a granule-poor fraction, enhance neutrophil-induced dermal-epidermal separation (DES). (A, B) Graphs show means $\pm$ SEM of quantitative measures of DES at the DEJ ($n = 3$). Monocyte and neutrophil supernatants were collected after 4-h incubation with BPS on human skin cryosections. (A) DES of skin cryosections developing after stimulating freshly isolated neutrophils with monocyte supernatant was as extensive as DES induced upon co-incubation of monocytes and neutrophils in the presence of BPS, and significantly higher than neutrophil-induced DES. Supernatants alone or neutrophil supernatants added to freshly isolated monocytes did not increase DES. (B) DES induced by neutrophils co-incubated with the granule-poor fraction of monocyte supernatants was almost as extensive as DES induced by neutrophils stimulated with monocyte supernatants or with monocyte/neutrophil co-incubation in the presence of BPS. (C-F) Representative images show significant DES of skin

cryosections upon co-incubation of neutrophils and monocyte supernatants (C), but lack of DES upon co-incubation of monocytes with neutrophil supernatants (D). In contrast to the granule-rich fraction (E), neutrophils co-incubated with the granule-poor fraction of monocyte supernatants induced extensive DES (F). Arrows indicate separation, asterisks the loss of epidermis as a consequence of complete DES. Magnification x200.

Figure 5. Monocyte/neutrophil-induced dermal-epidermal separation (DES) is mediated by MMP-2/MMP-9 release. (A) Graph shows means $\pm$ SEM of quantitative measures of DES at the DEJ ($n = 3$). (A) DES was inhibited by blocking of MMP-2/MMP-9 (100 μ M SB-3CT), but not the serine proteases (10 μ M GW311616A) or MPO (500 μ M ABAH), and was minimally reduced by an inhibitor of degranulation (10 μ g/ml BFA). (B-E) Representative images of skin cryosections show DES upon co-incubation of monocytes (5×10^6 /ml) and neutrophils (10×10^6 /ml) in the presence of BPS and GW311616A (B), ABAH (C), or BFA (E), but a lack of DES upon blocking with SB-3CT (D). Arrows indicate separation, asterisks the complete loss of the epidermis as a consequence of DES. Magnification x200.

Figure 6. MMP-9 release is independent of autoantibodies, degranulation and adhesion. Release of MMP-9 was evaluated by Western blot (A) and ELISA in supernatants collected from monocytes (5×10^6 /ml) and/or neutrophils (10×10^6 /ml) after 4-h incubation with BPS or NHS on skin cryosections (B-D). (A) MMP-9 amounts in supernatants are increased upon incubation with monocytes plus neutrophils as compared to either monocytes or neutrophils alone. (B-D) Quantification of MMP-9 release. Graphs show mean $\pm$ SEM of MMP-9 levels adjusted for the amount of cells ($n = 3$). The absence of BP-autoantibodies (B), inhibition of degranulation with 10 μ g/ml BFA (C), or blocking adhesion with 5 μ g/ml anti-CD18 and 10 μ g/ml anti-ICAM-1 antibodies (D) did not affect MMP-9 release by neutrophils plus monocytes.

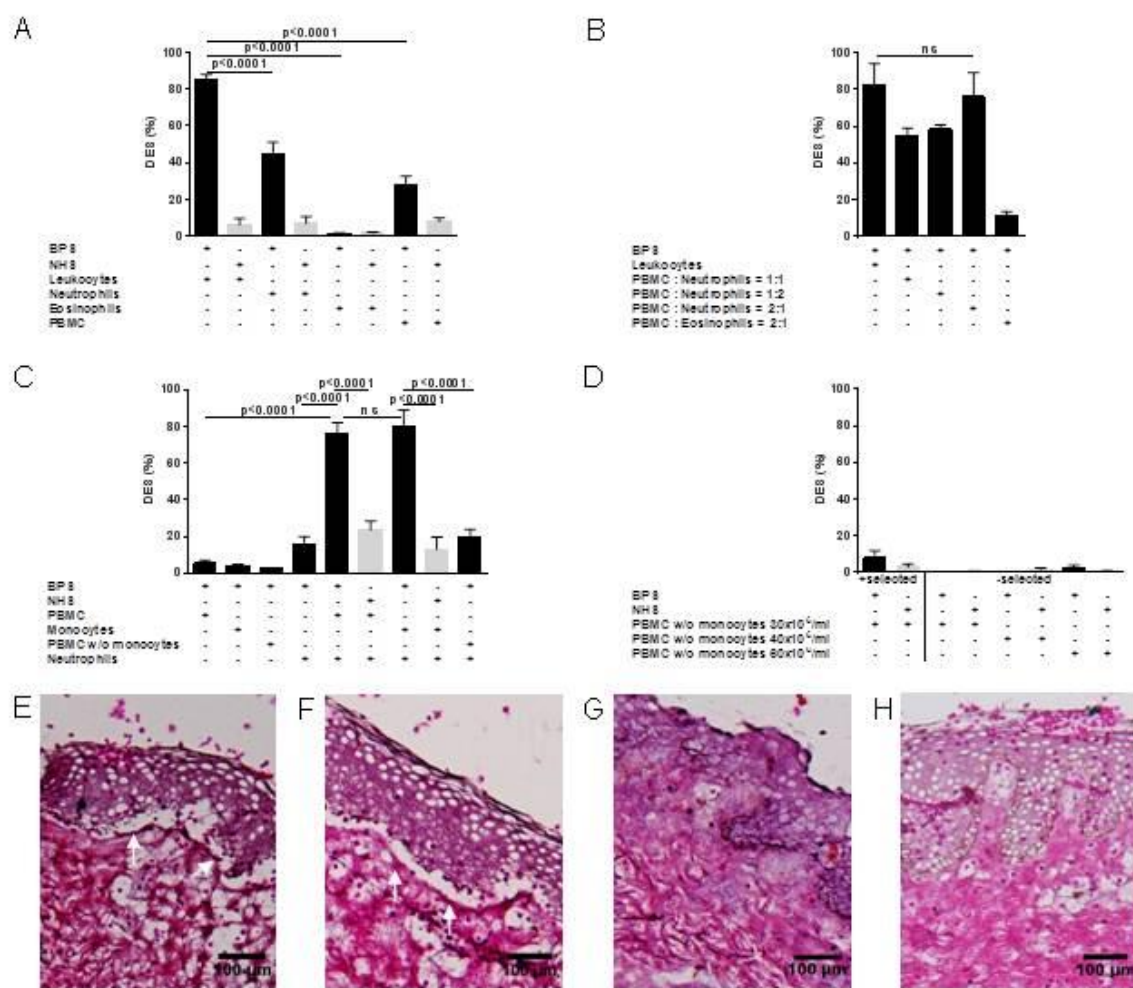


Figure 1

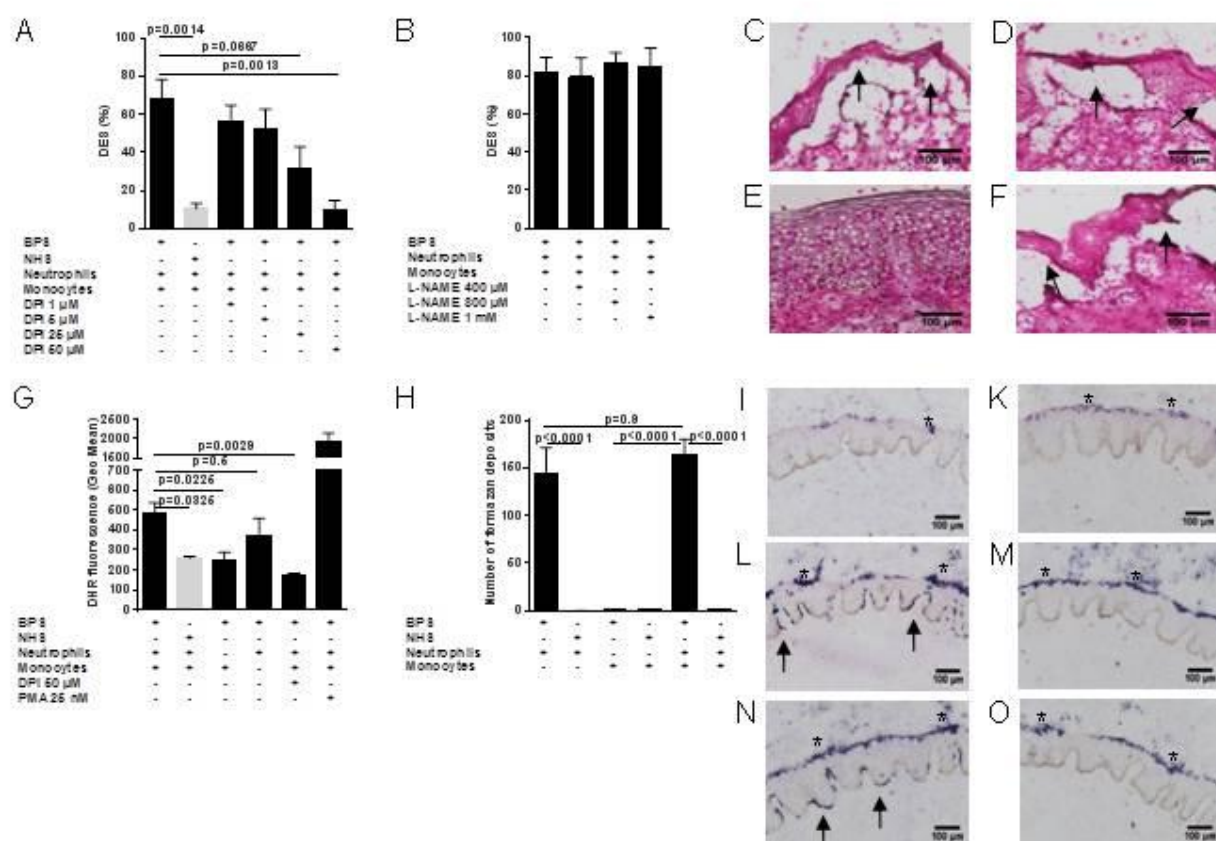


Figure 2

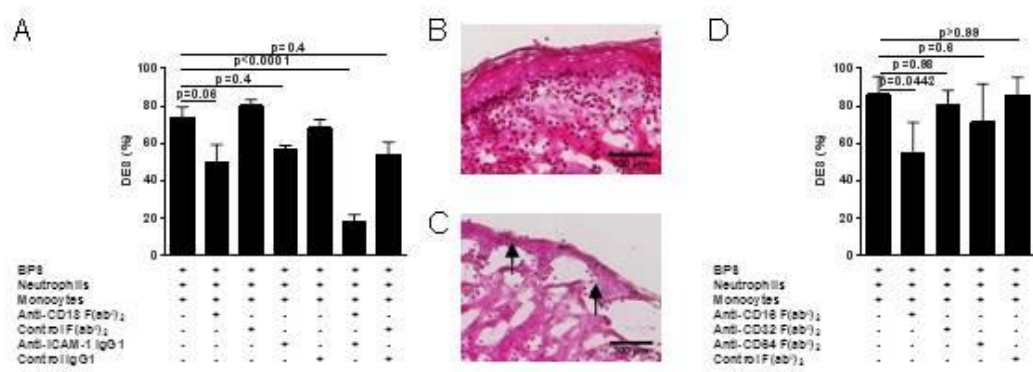


Figure 3

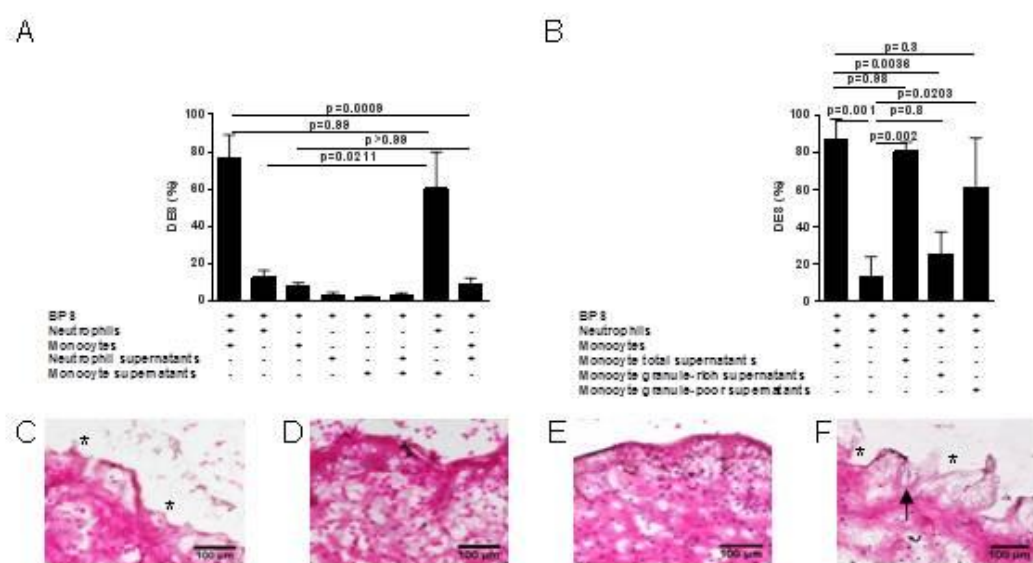


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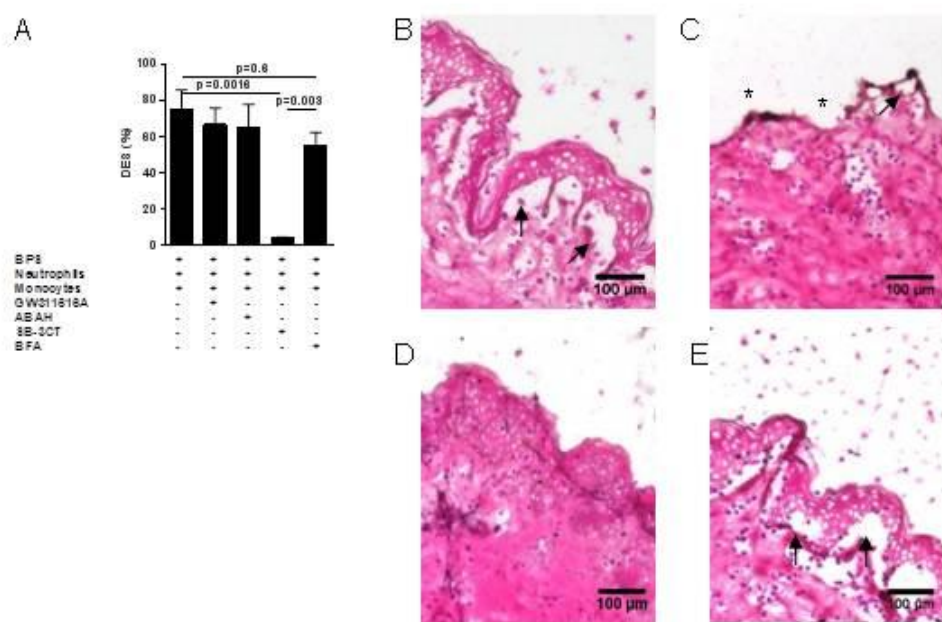


Figure 5

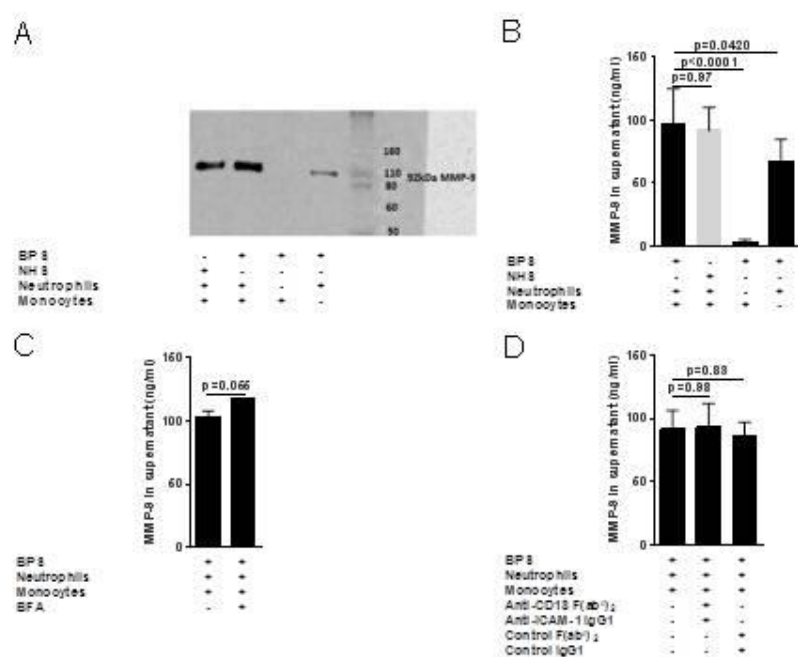
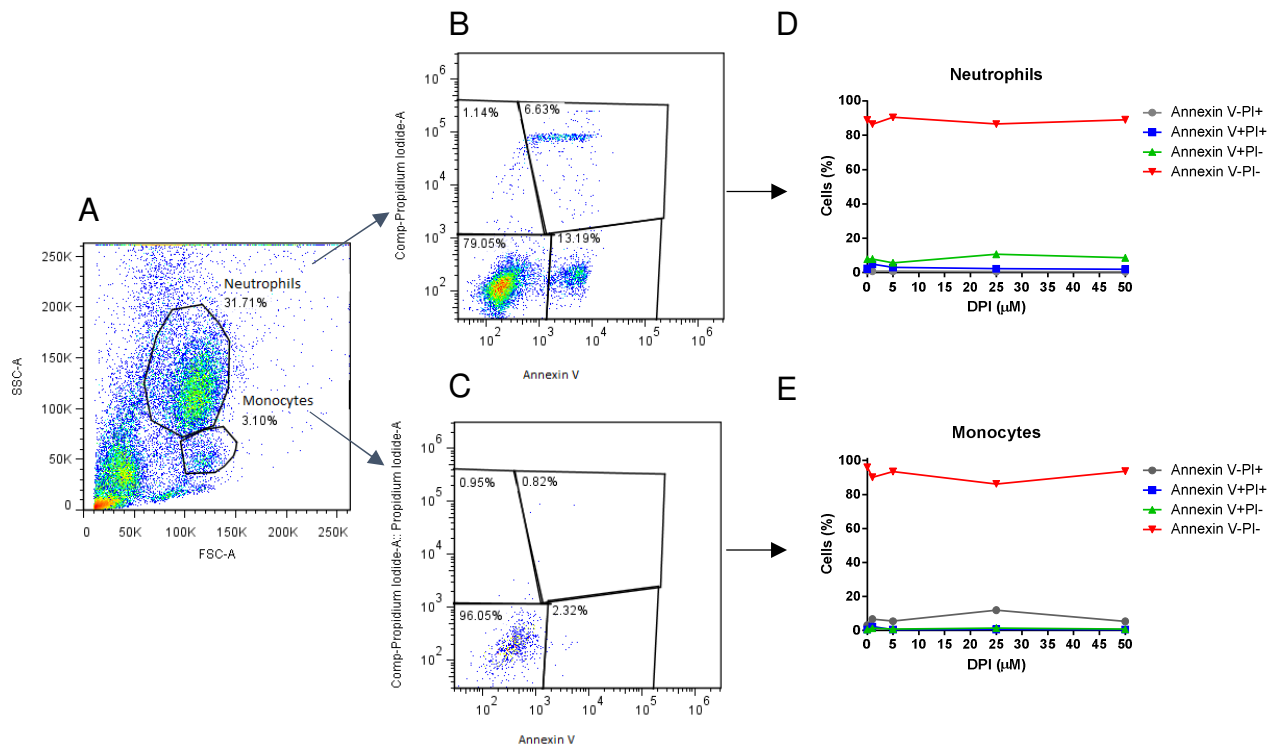


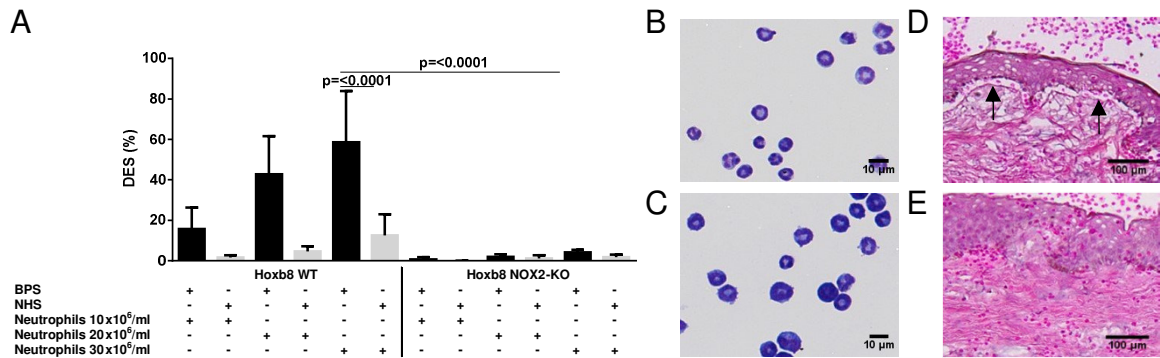
Figure 6

Supplemental Figure 1



Supplemental Figure 1. DPI does not affect the viability of monocytes and neutrophils. To evaluate the viability of monocytes and neutrophils after 4-h incubation in the presence of increasing concentrations of DPI (0 – 50 mM), annexin V/ propidium iodide (PI) staining (24, 25) was performed and analyzed by flow cytometry. After separately gating on neutrophil and monocyte populations (A, representative image), annexin V and PI positive and negative gates were created for neutrophils (B, representative image) and monocytes (C, representative image). In both monocytes and neutrophils more than 80% of the cells are annexin V and PI negative. Data are representative of 3 independent experiments.

Supplemental Figure 2



Supplemental Figure 2. Nox2-knockout neutrophils fail to induce autoantibody-dependent dermal-epidermal separation (DES). (A) Graphs show mean $\pm$ SEM of quantitative measures of DES at the DEJ of skin cryosections ($n = 4$). Wild-type mouse neutrophils ($30 \times 10^6/\text{ml}$) induce autoantibody-dependent DES, whereas Nox2-knockout mouse neutrophils ($30 \times 10^6/\text{ml}$) show no effect. (B-E) Representative images show the morphology of wild-type mouse neutrophils (B) and Nox2-knockout mouse neutrophils (C). DES is induced in skin cryosections incubated with wild-type neutrophils in the presence of BPS (D), but DES is absent when Nox2-knockout neutrophils were used (E). Arrows indicate the separation. Magnification x630 (B, C), x200 (D, E).