GM-CSF Modulates Autoantibody Production and Skin Blistering in Experimental Epidermolysis Bullosa Acquisita

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GM-CSF Modulates Autoantibody Production and Skin Blistering in Experimental Epidermolysis Bullosa Acquisita

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GM-CSF activates hematopoietic cells and recruits neutrophils and macrophages to sites of inflammation. Inhibition of GM-CSF attenuates disease activity in models of chronic inflammatory disease. Effects of GM-CSF blockade were linked to modulation of the effector phase, whereas effects on early pathogenic events, for example, Ab production, have not been identified. To evaluate yet uncharacterized effects of GM-CSF on early pathogenic events in chronic inflammation, we employed immunization-induced epidermolysis bullosa acquisita (EBA), an autoimmune bullous disease caused by autoantibodies to type VII collagen. Compared to wild-type mice, upon immunization, GM-CSF$$^{−/−}$$ mice produced lower serum autoantibody titers, which were associated with reduced neutrophil numbers in draining lymph nodes. The same effect was observed in neutrophil-depleted wild-type mice. Neutrophil depletion in GM-CSF$$^{−/−}$$ mice led to a stronger inhibition, indicating that GM-CSF and neutrophils have additive functions. To characterize the contribution of GM-CSF specifically in the effector phase of EBA, disease was induced by transfer of anti–type VII collagen IgG into mice. We observed an increased GM-CSF expression, and GM-CSF blockade reduced skin blistering. Additionally, GM-CSF enhanced reactive oxygen species release and neutrophil migration in vitro. In immunization-induced murine EBA, treatment with anti–GM-CSF had a beneficial effect on established disease. We demonstrate that GM-CSF modulates both autoantibody production and skin blistering in a prototypical organ-specific autoimmune disease. The Journal of Immunology, 2014, 192: 559–571.

C olony-stimulating factors are a family of cytokines mediating recruitment and activation of hematopoietic cells to sites of inflammation and infection (1–3). There are three major members of the CSF family: CsF1 (G-CSF), CsF2 (GM-CSF), and CsF3 (M-CSF). Among these, GM-CSF is most potent regarding recruitment of neutrophils and macrophages to sites of inflammation. GM-CSF is secreted as a glycosylated protein and consists of a single polypeptide chain. The GM-CSF receptor consists of a specific binding subunit (CSF2R$\alpha$) and a common signal transduction subunit (CSF2R$\beta$), which in humans is shared with other cytokines, including IL-3 and IL-5 (4–6). After receptor binding, GM-CSF stimulates production of proinflammatory cytokines in granulocytes and macrophages. Local GM-CSF production leads to the formation of a density gradient that induces leukocyte migration toward the site of inflammation (7–9).

In line with these proinflammatory functions, increased GM-CSF expression has been noted in several chronic inflammatory and autoimmune diseases, and its blockade prevented induction of inflammation in models of arthritis (10–13), experimental autoimmune encephalitis (14), psoriasis (15), and nephritis (16). In contrast, in experimental contact hypersensitivity, characterized by an increased GM-CSF expression (17), blockade of this cytokine had no effect on disease manifestation (18). Interestingly, in Crohn’s disease, application of GM-CSF had a beneficial effect on established disease. We demonstrate that GM-CSF modulates both autoantibody production and skin blistering in a prototypical organ-specific autoimmune disease.

Previous studies in the above-mentioned animal models of inflammation documented a contribution of GM-CSF to innate immune mechanism, such as neutrophil-dependent tissue injury. In these models, effects of GM-CSF on adaptive immune functions, such as production of autoantibodies, have not been identified (10–15). Recently, GM-CSF (together with IL-4) has been shown to increase anti-influenza Ab formation in immunodeficient mice reconstituted with human blood lineage cells (21). Furthermore, neutrophils, which are among the main target cells of GM-CSF (5), have been reported to modulate the physiological Ab response in humans. In detail, neutropenic patients had fewer and hypomutated B cells and a lower abundance of preimmune Igs, indicating that neutrophils generate an innate layer of antimicrobial Ig defense by interacting with specific B cell subsets (22). However, the contribution of GM-CSF to adaptive immune functions in autoimmune diseases is unknown.

We therefore evaluated the contribution of GM-CSF in a prototypical organ-specific autoimmune disease. We selected epidermolysis bullosa acquisita (EBA) as a model, because the pathogenic relevance of autoantibodies in this disease has been clearly demonstrated (23–25), and induction of EBA has been described in a patient treated with GM-CSF (26). These autoantibodies are directed to type VII collagen (COL7), a major component of the hemidesmosomal adhesion complex of the dermal–epidermal junction (DEJ) (27, 28). After binding to their target Ag in the skin, a proinflammatory milieu is generated,
leading to both neutrophil extravasation and activation. Reactive oxygen species and proteolytic enzymes released from neutrophils then lead to subepidermal blister formation (29–31).

In animal models of EBA, we noted increased levels of GM-CSF in both serum and skin. Based on this observation, we first evaluated the effects of GM-CSF blockade in a model of autoantibody transfer-induced EBA and documented a profound proinflammatory activity of this cytokine mediated by innate immune mechanisms. Subsequently, we investigated the contribution of GM-CSF in modulating adaptive immune functions in immunization-induced EBA and identified a significant contribution of GM-CSF to the formation of autoantibodies.

Materials and Methods
Experiments with human blood samples
Blood for isolation of polymorphonuclear cells (PMNs) was obtained from EDTA anticoagulated blood drawn from healthy blood donors. Sera from EBA patients and controls were obtained by venipuncture. All experiments using human samples were approved by the Ethics Committee of the University of Lübeck and were performed according to the Declaration of Helsinki. Blood donors gave their written informed consent prior to study participation.

Mice
C57BL/6, B6.SJL-H2a, and BALB/c mice were initially obtained from Charles River Laboratories (Sulzfeld, Germany). GM-CSF−/− mice were provided by Prof. Jeff Whitsett (Division of Pulmonary Biology, Cincinnati Children’s Hospital, Cincinnati, OH) (32). Mice included in experiments were from the offspring of all the strains. For experiments, mice aged 6–10 wk were used. Mice were held at specific pathogen-free conditions and fed standard mouse chow and acidified drinking water ad libitum. All clinical examinations, biopsies, and bleedings were performed under anesthesia using i.p. administration of a mixture of ketamine (100 mg/g) and xylazine (15 mg/g). Animal experiments were approved by local authorities of the Animal Care and Use Committee (Kiel, Germany) and performed by certified personnel.

Generation of anti-mouse COL7 IgG
Rabbit anti-mouse COL7 IgG was generated as described elsewhere (24). In brief, recombinant fragments of murine COL7 (termed mCOL7A, mCOL7B, and mCOL7C) tagged with GST were produced using a prokaryotic expression system and purified by glutathione affinity chromatography. IgG from rabbits immunized with these recombinant fragments of mCOL7 and from nonimmunized rabbits was purified by affinity chromatography using protein G-Sepharose affinity chromatography (Amersham Biosciences, Heidelberg, Germany). Reactivity of IgG fractions was analyzed by immunofluorescence (IF) microscopy on murine skin.

Induction of experimental EBA by autoantibody transfer
C57BL/6 and GM-CSF−/− mice aged 6–10 wk were used for induction of experimental EBA by autoantibody transfer. In brief, experimental EBA was induced in mice by transfer of rabbit anti-mouse COL7 IgG as described (24). Clinical disease manifestation, expressed as percentage of body surface area covered by EBA skin lesions, was determined before and after the initial anti-COL7 IgG injection. From these data, the area under the curve (AUC) was used to calculate the overall EBA severity. Injection of normal rabbit IgG (NR-IgG) served as negative control.

Induction of immunization induced EBA
C57BL/6, B6.SJL-H2a, and GM-CSF−/− mice aged 6–10 wk were used for experiments with immunization-induced EBA. All clinical examinations, biopsies, and bleedings were performed under anesthesia with i.p. administration of a mixture of ketamine (100 mg/g) and xylazine (15 mg/g). Mice were immunized as described elsewhere (33). In brief, mice were immunized i.p. with 100 μg anti-GM-CSF Abs (clone MP1-22E9; Biocell, Frankfurt, Germany) and 3 days later with 100 μg anti-COL7 Abs (clone 22E5; Biocell, Frankfurt, Germany) served as control (rat IgG2a K isotype, clone eBR2a; Biocell, Frankfurt, Germany). Both treatments were carried out over a time period of 4 wk. Extent of skin blistering was expressed as the percentage of body surface area affected by skin lesions every week in relationship to the score at the beginning of treatment. Total disease severity during observation period was calculated as AUC of the recorded disease severity.

Analysis of G-CSF and GM-CSF in mouse and human sera
At the indicated time points, sera was obtained from mice and analyzed for serum concentrations of G-CSF and GM-CSF. This was performed by a commercial supplier using the Bio-Plex system (BioPlex Biosciences, Heidelberg, Germany). Reactivity of IgG fractions was analyzed by immunofluorescence (IF) microscopy on murine skin. Expression of GM-CSF was determined by Western blotting. Serum from healthy blood donors (n = 52) served as a reference. Analysis of GM-CSF serum concentrations was performed by a commercial supplier using the Bio-Plex system.

IF microscopy
Biopsies of nonlesional skin were obtained 2 d after last injection of IgG (day 12), and IgG and C3 deposits were detected by direct IF microscopy on frozen sections prepared from tissue biopsies using 100-fold diluted FITC-/Texas Red–labeled Abs specific to rabbit IgG (Dako, Glostrup, Denmark, and Abcam, Cambridge, U.K.), as well as FITC-labeled murine C3 (MP Biomedicals, Solon, OH) following previously published protocols (34).

Histopathology
Skin sections from corresponding anatomical sites were obtained 2 d after the last injection of IgG (day 12) and prepared for examination by histopathology as described. Briefly, skin tissues collected from experimental animals were fixed in 4% buffered formalin and, subsequently, sections from paraffin-embedded tissues were stained with H&E (35). For visualization of GM-CSF, sections from frozen skin tissue samples were air dried at room temperature and fixed in cold acetone for 10 min. Endogeneous H2O2 was blocked using EnzymeBlock (Dako) for 10 min. Sections were incubated in blocking solution (5% BSA in PBS) for 1 h. To evaluate the expression of GM-CSF, tissue sections were incubated with goat anti-mouse GM-CSF Ab (sc-52532; Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:50 dilution for 1 h at 37°C. For controls, isotype (goat total IgG) was used. Sections were rinsed and incubated with HRP-labeled donkey anti-goat secondary Abs (sc-2020; Santa Cruz Biotechnology) at 1:200 dilution for 1 h. Slides were developed using Histoprinte using the protocol provided by the manufacturer.

For staining of draining lymph node lymphocytes, inguinal lymph node lymphocytes were obtained from immunized mice at week 6. Lymph node sections (6 μm) were fixed in acetone at −20°C for 10 min and blocked using 5% BSA. Sections were stained with Ly6G coupled with PE (clone 1A8; BD Pharmingen, Heidelberg, Germany) at 4°C for detection of neutrophils. Simultaneously, recombinant murine wFPA2 protein conjugated with DyLight 488 (according to the manufacturer’s protocol; Thermo Scientific, Rockford, IL) was added to detect COL7-specific B cells (36). After washing slides, they were counterstained with DAPI (Life Technologies, Darmstadt, Germany) and mounted for microscopy. Cell numbers with positive staining in the images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD). Presence of BAFF in the draining lymph node was detected using Abs (orb-13821; Biobyt, Cambridge, U.K.) coupled with FITC and counterstained with DAPI (Life Technologies).

Differential blood counts in mice
EDTA anticoagulated blood was collected from mice on the last day of the experiment and samples were analyzed by a commercial supplier (Laboklin, Bad Kissingen, Germany).

Measurement of reactive oxygen species and GM-CSF release from human leukocyte populations
Blood for isolation of PMNs was obtained from EDTA anticoagulated blood collected from healthy blood donors. Neutrophil activation was assessed by determination of immune complex (IC)–induced reactive oxygen species

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FIGURE 1. Expression patterns of GM-CSF in experimental and human EBA. (A) Increased serum concentrations of GM-CSF in Ab transfer-induced EBA. GM-CSF levels were found to be significantly increased in sera from mice injected with mCOL7-IgG (pIgG) starting at day 4 of the experiment compared with NR-IgG–injected mice sera (*p = 0.01, one-way ANOVA). (B) Increased serum concentrations of GM-CSF in immunization-induced EBA. GM-CSF concentrations were significantly increased starting 2 wk after immunization compared with control sera (*p = 0.01, one-way ANOVA). (C) Correlation of GM-CSF serum concentrations with clinical disease severity assessed in C57BL/6 mice before and 3, 6, and 12 d after the initial anti-COL7 IgG injection. Levels of GM-CSF in serum increases exponentially with affected area (r = 0.9675, p < 0.001, nonlinear regression). (D) Representative staining from skin specimens obtained from a total of 12 mice per group injected with NR-IgG or anti-COL7–injected mouse. (E) Expression of GM-CSF in serum is increased in human EBA patients (n = 26) compared with healthy controls (n = 52; p < 0.001, Mann–Whitney rank sum test). Owing to the nonparametric distribution, data are presented as median (black line), 25th/75th percentiles (boxes), and 5th/95th percentiles (error bars), and green/red dots represent actual results for each sample.
GM-CSF IN EBA

Detection of circulating vWFA2-specific Abs

To detect circulating vWFA2-specific Abs, ELISA was performed. Briefly, 96-well microtiter plates (Maxisorb; Nunc, Roskilde, Denmark) were coated with 250 ng murine vWFA2. Nonspecific binding was reduced by blocking plates with 1% BSA in PBS-T at room temperature (RT) for 1 h. Subsequently, plates were incubated on a 96-well plate (Greiner Bio-One) coated with immune complexes formed by monoclonal anti-COL7 Abs and recombinant fragments located within the human NC1 domain of COL7 and incubated at 37°C on a shaker. At different time points (start and after 1, 2, and 3 h), cell supernatants were collected from different wells. GM-CSF release was analyzed by using a Legend Max human GM-CSF ELISA kit (BioLegend, Fell, Germany) following the protocol provided by the manufacturer. Supernatants from unstimulated cells were used as controls.

GM-CSF neutralization and neutrophil depletion using Abs

Endogenous GM-CSF in mice was neutralized using 50 μg anti-GM-CSF (clone MP1-22E9; eBioscience) injected i.p. on alternate days in the autoantibody transfer model of EBA. In immunization-induced EBA, a similar amount of anti-GM-CSF was administered three times per week. For controls, an appropriate isotype Ab was used (rat IgG2a K isotype, clone eBR2a; eBioscience). Neutrophils were depleted using 1A8 Abs (BioLegend, West Lebanon, NH) as described elsewhere (39) with minor modifications. In immunization-induced EBA, mice were injected with 100 μg Abs three times per week.

Statistical analysis

Unless otherwise noted, data are presented as means ± SE. For comparisons of two groups, a t test or Mann–Whitney rank sum test was used as appropriate. For comparison of more than two groups, ANOVA was used. For equally distributed data, a one-way ANOVA, followed by a Bonferroni t test for multiple comparisons, was used; when data were nonparametric, an ANOVA by ranks (Kruskal–Wallis) was applied, followed by a Bonferroni t test for multiple comparisons. Pearson product moment correlation was used to test for correlations. In all tests, a p < 0.05 was considered significant. All statistical analysis was performed using SigmaPlot 12.0 (Systat Software, Erkrath, Germany).

FIGURE 2. Reduced blister formation in GM-CSF–deficient mice after injection of anti-COL7 IgG. Experimental EBA was induced by repetitive injections of anti-COL7 IgG into either C57BL/6 or GM-CSF–deficient (GM-CSF−/−) mice. (A) Clinical EBA severity assessed by the percentage of body surface area covered by skin lesions 4, 8, and 12 d after the initial anti-COL7 IgG injection. Reduction of affected body surface area in GM-CSF–deficient mice is evident 12 d after the initial anti-COL7 IgG injection. (B) Overall disease severity expressed as the AUC. Data indicate significant reduction in GM-CSF−/− mice (n = 12 mice/group; p < 0.001, Mann–Whitney rank sum test). Owing to the nonparametric distribution, data are presented as median (black line), 25th/75th percentiles (boxes), 5th/95th percentiles (error bars), and values outside the 95 percentile (dots). (C) Representative clinical presentations.
Results

Increased expression of GM-CSF, but not G-CSF, in experimental and human EBA

Repetitive injections of anti-COL7 IgG into either C57BL/6 or BALB/c mice induced experimental EBA as described (24). In both strains, comparable and equally distributed serum concentrations of G-CSF and GM-CSF were noted in control mice. In contrast, induction of experimental EBA lead to a sharp, 8- to 38-fold increase of both CSFs in the tested mouse strains. Additionally, a great variation in the serum levels of both G-CSF and GM-CSF concentrations in mice with experimental EBA was noted. Overall, a significant increase was evident for GM-CSF, but not G-CSF, serum concentrations in C57BL/6 as reported (40), as well as BALB/c (23-fold) mice after EBA induction (Supplemental Fig. 1A). In C57BL/6 mice, serum was also obtained before the initial anti-COL7 IgG injection and 3, 6, and 12 d thereafter. In this experiment, GM-CSF serum concentrations strongly and exponentially correlated with the clinical disease activity, expressed as the body surface area affected by EBA skin lesions (Fig. 1A–C). These GM-CSF serum concentrations continuously increased throughout the observation period (Fig. 1A–C). Expression of GM-CSF in the skin of C57BL/6 mice 12 d after the injection of NR-IgG was not observed. In contrast, cutaneous GM-CSF expression was constantly detected 12 d after the administration of anti-COL7 IgG (Fig. 1D). We also observed an increased GM-CSF serum concentration in immunization-induced EBA, which was first noticed 2 wk after immunization and increased until week 4. Thereafter, a plateau was maintained until the end of the 6-wk observation period (Fig. 1B).

In line with these findings, we also observed increased GM-CSF serum concentrations in EBA patients, compared with healthy controls. In detail, in healthy controls (n = 52), GM-CSF serum levels were 30.3 pg/ml (median; 25th percentile, 27.4–31.33 pg/ml; Fig. 1E), whereas in EBA patients levels were 60.6 pg/ml (median; range, 54.7–67.43 pg/ml; p < 0.001, Mann–Whitney rank sum test). Ex vivo stimulation of isolated human neutrophils and monocytes with EBA-IC induced a time-dependent release of GM-CSF by these two leukocyte populations (Supplemental Fig. 1B).

Induction of skin blistering in experimental EBA is hindered in GM-CSF−/− mice

To test whether the increased serum and cutaneous GM-CSF expression in experimental EBA is of functional relevance, anti-COL7 IgG was injected into mice lacking GM-CSF expression (n = 12) and wild-type control C57BL/6 mice (n = 12). Skin lesions were evident in 11 of 12 C57BL/6 mice with a median of 0.2% (range, 0–0.6%) of affected body surface area as early as 4 d after the initial anti-COL7 IgG injection. Similar disease incidence and severity were observed in GM-CSF−/− mice. In detail, 11 of 12 mice presented with skin lesions with a median of 31.5 ± 2.3% (range, 27.4–64.3%) of affected body surface area.

Table I. EBA-associated neutrophilia is absent in GM-CSF−/− mice

<table>
<thead>
<tr>
<th>Strain/IgG</th>
<th>NR-IgG</th>
<th>Anti-COL7 IgG</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td>16.6 ± 2.2</td>
<td>31.5 ± 2.3</td>
</tr>
<tr>
<td>GM-CSF−/−</td>
<td>13.4 ± 1.4</td>
<td>17.6 ± 4.3</td>
</tr>
</tbody>
</table>

Numbers in the table correspond to the percentage of circulating neutrophils, determined by differential blood count 12 days after the injection of the respective IgG preparation (mean ± SEM). Significant differences are observed comparing NR-IgG- and anti-COL7 IgG-injected C57BL/6 mice, as well as in comparison of anti-COL7 IgG-injected C57BL/6 mice with GM-CSF−/− mice. Data are from 5–6 mice per group.

Anti-COL7 IgG, Rabbit anti-murine type VII collagen IgG; NR-IgG, normal rabbit IgG.

FIGURE 3. Decreased blister formation in anti–GM-CSF–treated mice after injection of anti-COL7 IgG. (A) Experimental EBA was induced by repetitive injections of anti-COL7 IgG into either anti–GM-CSF or isotype control–injected C57BL/6 mice. Clinical EBA severity was assessed by the percentage of body surface area covered by EBA skin lesions 4, 8, and 12 d after the initial anti-COL7 IgG injection. Reduction of affected body surface area in anti–GM-CSF–treated mice is evident 12 d after the initial anti-COL7 IgG injection. (B) Overall clinical disease severity, expressed as the AUC shown graphically, indicates significant reduction after GM-CSF neutralization using Abs (n = 5 mice/group; p = 0.003, Mann–Whitney rank sum test). Owing to the nonparametric distribution, data are presented as median (black line) and 25th/75th percentiles (boxes). (C) Representative clinical presentations 12 d after the initial anti-COL7 IgG injection.
0.4% (range, 0–1.4%) affected body surface area. This difference in median disease severity was not statistically significant. Eight days after the initial anti-COL7 IgG injection, blister formation affected all mice. Although not statistically significant ($p = 0.053$), a trend toward a lower disease scores in GM-CSF$^{-/-}$ mice was observed. Mean affected body surface area in C57BL/6 mice was 2.9% (0.6–10.3%), as opposed to 1.3% (0.3–2.6%) in mice lacking GM-CSF expression. This trend was validated 12 d after the initial anti-COL7 IgG injection. Whereas in C57BL/6 mice 8.4% (1.6–14.1%) of the body surface area was affected by blistering, this was significantly lower ($p < 0.001$) in GM-CSF$^{-/-}$ mice, which had a mean of 3.0% (0.6–6.1%) affected body surface area (Fig. 2A). Calculation of the overall disease activity, taking onset and maximum severity into account and expressed as the AUC in

![Graph A](image1)

![Graph B](image2)

![Graph C](image3)

![Graph D](image4)

**FIGURE 4.** Decreased neutrophil infiltration into blood and skin in GM-CSF$^{-/-}$ mice after injection of anti-COL7 IgG. (A) Injection of anti-COL7 IgG significantly increased neutrophil numbers in peripheral blood of C57BL/6 mice compared with GM-CSF$^{-/-}$ mice starting from 8 d after initial injection ($p = 0.01$, two-way ANOVA). (B) Likewise, injection of anti-COL7 IgG induces a significant increase in dermal leukocyte infiltration in C57BL/6 compared with GM-CSF$^{-/-}$ mice ($p < 0.001$, two-way ANOVA). (C) Correlation of neutrophil number in blood with those in dermal infiltrate in both strains after anti-COL7 IgG injection ($r = 0.895$, $p = 0.001$, Pearson product-moment correlation). (D) Representative H&E-stained sections from both strains injected with anti-COL7 IgG and NR-IgG (original magnification ×200).
C57BL/6 mice, reached a median of 29 (range, 4–97). In GM-CSF−/− mice the AUC was reduced to 26% of wild-type mice (median, 8; range, 3–20; Fig. 2B). These clinical changes were accompanied by unaltered deposition of IgG and C3 at the DEJ (Fig. 2C). Injection of NR-IgG did not lead to the development of skin lesions in both strains (n = 5/strain) throughout the entire observation period.

Decreased skin blistering in experimental EBA after anti–GM-CSF injection

We further tested the impact of GM-CSF in experimental EBA by using neutralizing Abs against GM-CSF. Anti-COL7 IgG was injected into C57BL/6 mice (n = 5) and anti–GM-CSF Abs (50 μg/mouse) were simultaneously administered. Control mice were injected with anti-COL7 IgG and appropriate isotype control Ab (n = 5). Skin lesions were evident in control mice with a median of 0.3% (range, 0–0.6%) of affected body surface area as early as after 4 d of the initial anti-COL7 IgG injection. In anti–GM-CSF–treated mice, no blisters were observed at the same time point (Fig. 3A). Overall clinical disease severity expressed as the AUC reached a median of 36.2 (range, 26.2–45.3) in isotype Ab–injected mice, whereas extent of blister formation was >2-fold reduced in mice treated with anti–GM-CSF, reaching a median AUC of 13.1 (range, 10.2–18.4; p = 0.003, Mann–Whitney rank sum test; Fig. 3B). Representative clinical images are shown in Fig. 3C. Differences in clinical disease activity were independent of IgG or C3 deposition at the DEJ, which was similar between both treatment groups (data not shown).

Absence of EBA-associated neutrophilia in GM-CSF-deficient mice

Given the pivotal role of neutrophils in the development of autoantibody-induced tissue injury in this EBA model (41) and the effects of GM-CSF on neutrophil activation (5, 42), levels of circulating neutrophils were determined in both mouse strains 12 d after the injection of either anti-COL7 or NR-IgG. In NR-IgG–injected C57BL/6 and GM-CSF−/− mice the proportion of circulating neutrophils was comparable, that is, 16.6 ± 2.2 and 13.4 ± 1.4%, respectively. Compared to NR-IgG, injection of anti-COL7 IgG into C57BL/6 mice was associated with a marked and statistically significant (p < 0.001, Mann–Whitney rank sum test) increase in circulating neutrophils (31.5 ± 2.3%). This was not observed in GM-CSF−/− mice, which showed similar neutrophil counts after injection of either NR-IgG or anti-COL7 IgG (Table I). In line with this observation, in the differential blood count, the proportion of circulating neutrophils was significantly decreased in GM-CSF−/− mice after injection of anti-COL7 IgG compared with wild-type controls (p = 0.015, Mann–Whitney rank sum test). Similar observations were made evaluating the absolute neutrophil number in the circulation (Fig. 4A).

Decreased dermal infiltration of neutrophils in GM-CSF–deficient mice after injection of anti-COL7 IgG

Parallel to the effects of GM-CSF deficiency on circulating neutrophil counts after the injection of anti-COL7 IgG, the impact of lacking GM-CSF expression on the neutrophil-rich dermal leukocyte infiltration in experimental EBA (30) was analyzed.

**FIGURE 5.** GM-CSF increases IC-induced neutrophil activation. (A) Normalized ROS release of neutrophils after incubation with the indicated combinations of COL7 Ag, anti-COL7 mAbs, and different doses of GM-CSF. Data are from five independently performed experiments. (B) Representative ROS release expressed as relative fluorescent units (RFU) from one of experiment. fMLF was used as positive control.
duction of experimental EBA in C57BL/6 mice led to a pronounced increase in dermal leukocyte infiltration ($p = 0.01$, two-way ANOVA). This phenomenon was also observed in GM-CSF$^{-/-}$ mice ($p = 0.03$, two-way ANOVA). However, after injection of anti-COL7 IgG, compared with C57BL/6 mice, dermal leukocyte infiltration was significantly lower in GM-CSF$^{-/-}$ mice ($p \leq 0.001$, two-way ANOVA; Fig. 4B). Furthermore, a close correlation between circulating and skin-infiltrating neutrophils was observed ($r = 0.895$, $p \leq 0.001$, Pearson product-moment correlation; Fig. 4C). Representative H&E staining images are shown in Fig. 4D.

**GM-CSF enhances IC-induced neutrophil activation**

To test whether IC-induced neutrophil activation is modulated by GM-CSF, ROS release from IC-activated neutrophils was measured in the absence and presence of GM-CSF. Incubation of neutrophils with GM-CSF in the presence of either monoclonal anti-COL7 Abs, or corresponding proteins located within the NC1 domain, had no effect on neutrophil ROS release. In contrast, ROS was readily released from neutrophils after incubation with ICs of anti-COL7 Ab and COL7 protein. This IC-induced neutrophil activation was further enhanced by addition of GM-CSF in a dose-dependent manner (Fig. 5A). A representative ROS release curve shown in Fig. 5B.

**Anti–GM-CSF treatment delays disease progression in already established immunization-induced EBA**

To test whether modulation of GM-CSF function could be potentially used as a treatment, experimental EBA was induced in B6.SJL-H2s mice ($n = 16$) by immunization with an immunodominant protein located within murine COL7. Thereafter, mice were scored every week and allocated into treatment and isotype groups when 2% or more of the body surface area showed skin lesions in individual mice. Mice were randomly allocated to anti–GM-CSF (50 μg three times per week, $n = 8$) or appropriate isotype control Ab ($n = 8$). Disease progression was monitored weekly and is expressed in relationship to the score at allocation to treatment. No difference in disease severity was detected 1 wk after treatment had been initiated (Fig. 6A). At later time points, clinical disease manifestation in isotype-injected mice increased close to 7-fold (Fig. 6A). In contrast, mice treated with anti–GM-CSF showed a significantly lower disease progression at these time points (Fig. 6A). In line with this observation, overall disease activity expressed as AUC reached a median of 47.6 (range, 38.7–54.4) in isotype-treated mice, whereas disease severity was significantly decreased in mice treated with anti–GM-CSF presented with a median AUC of 33.2 (range, 26.1–37.4; $p \leq 0.001$, Mann–Whitney rank sum test; Fig. 6B). Representative clinical images are shown in Fig. 6C. Differences in clinical disease activity were independent of circulating Ag-specific IgG (subclasses) Abs, IgG, or C3 deposition at the DEJ, which were similar between both treatment groups (data not shown).

**Lower levels of Ag-specific autoantibody concentrations after immunization of GM-CSF–deficient mice with recombinant murine COL7: correlation with numbers of neutrophils in draining lymph nodes**

Owing to the long half-life of autoreactive anti-COL7–producing B cells (43), as well as the long persistence of circulating and tissue-bound COL7 autoantibodies (44), we investigated whether GM-CSF is required for autoantibody production. To address this, C57BL/6 and GM-CSF$^{-/-}$ mice ($n = 8$) were immunized with an immunodominant protein located within murine COL7 and levels of circulating Abs were determined by ELISA until 6 wk after immunization. Autoantibodies against COL7 were detected at 2 wk after immunization at equal titers in both strains.
of mice (Fig. 7A). After this time point, C57BL/6 mice showed significantly higher autoantibody serum levels compared with GM-CSF−/− mice (Fig. 7A). This effect of GM-CSF deficiency on specific autoantibody production affected all Ab subclasses (Fig. 7B).

Recently, GM-CSF has been shown to contribute to formation of anti-influenza IgG (21). Furthermore, Ab generation in humans has been demonstrated to be modulated by B cell helper neutrophils (22). Owing to the long persistence of circulating and tissue-bound anti-COL7 Abs (44), we investigated whether neutrophil homing to draining lymph nodes is changed in GM-CSF−/− mice after immunization. Interestingly, at the end of the observation period, we found significantly lower neutrophil numbers in the draining lymph nodes of GM-CSF−/− mice compared with C57BL/6 mice (Fig. 7C). We also observed a colocalization of neutrophils with BAFF in draining lymph nodes 6 wk after immunization (Supplemental Fig. 2). The ratio of neutrophils versus total number of cells (Ly6G/DAPI) showed significant reduction in GM-CSF−/− mice compared with C57BL/6 mice (n = 5/group; p = 0.001, Student t test; Fig. 7D). Staining of draining lymph node sections with labeled Ag, reflecting the total number of COL7-specific B cells (36), was identical in both groups (Fig. 7E). Furthermore, the number of Ly6G+ cells in lymph nodes significantly correlated with the circulating anti–mCOL7-IgG at the end of the experiment (Fig. 7F; r = 0.917; p ≤ 0.0001, Pearson correlation test).

**FIGURE 7.** Reduced autoantibody serum levels in GM-CSF-deficient mice after immunization: correlation with reduced neutrophil numbers in draining lymph nodes. COL7-specific Ab serum levels in C57BL/6 or GM-CSF−/− mice were analyzed weekly after immunization using ELISA. (A) GM-CSF−/− mice mounted a significantly lower autoantibody response compared with C57BL/6 mice from week 4 until the end of the experiment (*p ≤ 0.001, Student t test). (B) Comparison of autoantibody IgG subclasses in mice showed a significantly decreased production at week 6. (C) Representative sections of draining lymph nodes stained with DAPI (blue), Ly6G (red), and COL7 (green) (original magnification ×200). (D) Percentage of neutrophil numbers in lymph nodes (Ly6G/DAPI) of C57BL/6 and GM-CSF−/− mice at the end of the experiment showed a significant reduction of the Ly6G/DAPI ratio in GM-CSF−/− mice (n = 5; p = 0.001, t test). (E) Percentage of COL7-specific B cells in lymph nodes (COL7/DAPI) of C57BL/6 and GM-CSF−/− mice (n = 5) showed no significant difference. (F) Neutrophil numbers in lymph nodes correlate with anti-mCOL7 IgG serum levels after 6 wk of immunization in C57BL/6 and GM-CSF−/− mice (r = 0.917; p ≤ 0.0001, Pearson correlation test).
Lower serum levels of Ag-specific autoantibodies in neutrophil-depleted mice

To evaluate the impact of neutrophils on production of specific autoantibodies, we depleted neutrophils in C57BL/6 mice and GM-CSF−/− mice (n = 5 mice/group). In these mice, EBA was induced by immunization with COL7. As observed before (Fig. 7A), autoantibodies were detected 2 wk after immunization (Fig. 8A). At this time point, equal serum levels of anti-COL7 Abs were observed in neutrophil-depleted and isotype control mice. After this time point, isotype Ab-injected C57BL/6 mice mounted a significantly higher immune response compared with neutrophil-depleted mice (p ≤ 0.001, one-way ANOVA). Three weeks after immunization, GM-CSF−/− mice (both isotype- and 1A8-injected) showed a similar Ab response, which was significantly lower than that observed in C57BL/6 mice (both isotype- and 1A8-injected; p ≤ 0.001, one-way ANOVA). The difference in mounting a specific immune response was even more pronounced 6 wk after immunization. At this time point, GM-CSF−/− mice injected with 1A8 showed a very low specific autoantibody response (Fig. 8A).

Furthermore, C57BL/6 mice (1A8-injected) and GM-CSF−/− mice (isotype-injected) had serum levels of anti-COL7 IgG significantly lower compared with isotype-injected C57BL/6 mice (mean, 2.5 ± 0.1) than that observed in C57BL/6 mice (both isotype- and 1A8-injected; p ≤ 0.001, one-way ANOVA; Fig. 8A) and significantly higher levels compared with 1A8-injected GM-CSF−/− mice (p = 0.01 and p = 0.02, respectively, one-way ANOVA; Fig. 8A).

Analysis of the total amount of anti-COL7 IgG produced during the 6-wk observation period (expressed as AUC, Fig. 8B) revealed significant inhibition of autoantibody production in neutrophil-depleted C57BL/6 mice (1A8-injected; mean AUC, 3.4 ± 0.2; p = 0.003, two-way ANOVA), isotype-injected C57BL/6 mice (mean, 4.5 ± 0.2; p ≤ 0.001, two-way ANOVA), or compared with isotype-injected GM-CSF−/− mice (mean, 3.2 ± 0.2; p = 0.002, two-way ANOVA; Fig. 8B). The effect of neutrophil depletion in GM-CSF−/− mice on autoantibody production is cumulative but not synergistic, as interaction analysis of genotype (C56BL/6 or GM-CSF−/−) and treatment (isotype or 1A8) showed no significance (p = 0.236, two-way ANOVA; Fig. 8B).

Discussion

The data presented in this study provide strong evidence for proinflammatory functions of GM-CSF in an experimental model of a prototypic organ-specific autoantibody-mediated disease. Both genetic and pharmacological blockade of GM-CSF led to significant decrease in clinical disease severity in two different models of EBA. Interestingly, blockade of GM-CSF not only impaired neutrophil functions, but also significantly contributed to autoantibody production.

More specifically, induction of experimental EBA led to increased levels of GM-CSF both locally (skin) and systemically (serum). These increased GM-CSF levels are of functional relevance, as in GM-CSF−/− mice induction of skin blistering by Ab transfer is significantly impaired. Furthermore, in already established EBA, blockade of GM-CSF significantly attenuated clinical disease progression. Our findings are in line with observations in experimental models of arthritis (10–12), but in contrast to data obtained in murine models of contact hypersensitivity, where an increased GM-CSF expression was found in skin and serum samples (17); however, GM-CSF deficiency alone has no effect on disease manifestation (18). These observations underscore the

**FIGURE 8.** Neutrophil depletion in immunization-induced EBA has additive effects on production of specific Abs in GM-CSF−/− mice. Specific autoantibody serum levels in COL7-immunized C57BL/6 or GM-CSF−/− mice with (1A8 Ab) or without (isotype Ab) neutrophil depletion were analyzed weekly by ELISA. (A) Control mice (C57BL/6 mice injected with isotype Ab) exhibit significantly higher autoantibody response compared with neutrophil-depleted C57BL/6 mice (B). Compared to isotype-injected C57BL/6 mice, autoantibody production is similarly decreased in isotype-injected GM-CSF−/− mice (B). Neutrophil depletion in GM-CSF−/− mice (Δ) has additive effects, that is, a significantly lower autoantibody response compared with the three other experimental groups of the experiment (⁎p = 0.02, **p = 0.01, ***p ≤ 0.001, p ≤ 0.001, Δp ≤ 0.001, n = 5/group, two-way ANOVA). (B) Summarized OD450 readings of autoantibody serum levels (AUC). Neutrophil depletion (p ≤ 0.001) and GM-CSF deficiency (p ≤ 0.001) both cumulatively reduce autoantibody serum levels, but not synergistically (interaction p = 0.236).
importance of validating morphologic findings or expression data in appropriate experimental systems to fully understand their contribution to disease pathogenesis.

For the effector phase of experimental EBA, that is, autoantibody-induced tissue injury, neutrophil depletion has been shown to protect mice from induction of skin blisters (41). In line with this observation, CD18-deficient mice with impaired leukocyte extravasation into the skin (45) are also completely resistant to blister induction (41). Additionally, impaired release of ROS (41) or proteolytic enzymes (46) from neutrophils has similar protective effects. In this context, our findings provide novel insights into EBA pathogenesis: GM-CSF contributes to EBA-associated tissue injury by modulating several neutrophil functions (Fig. 9).

First, GM-CSF is required to induce autoantibody-induced neutrophilia. It is tempting to speculate that the increase in circulating neutrophils allows a higher number of neutrophils to migrate into the skin. In general, both G-CSF and GM-CSF are capable of recruiting neutrophils from the bone marrow into the blood (47, 48). Interestingly, in experimental EBA, this recruitment appears to be mainly mediated by GM-CSF, as, after injection with Ab to COL7, neutrophil numbers were significantly lower in GM-CSF−/− mice. Second, GM-CSF is required for neutrophil extravasation from blood into skin, as after EBA induction, the dermal leukocyte infiltrate was significantly reduced in GM-CSF−/− compared with C57BL/6 mice. Based on the observation of a significant increase in dermal leukocyte infiltration in GM-CSF−/− mice, neutrophil extravasation into the skin, in contrast to recruitment into the blood, is not predominantly mediated by GM-CSF. Furthermore, given that GM-CSF deficiency led to impaired blistering, the effect of GM-CSF on neutrophil recruitment into the skin may be indirect rather than a direct enhancement of neutrophil migration (49). More specifically, we hypothesize that GM-CSF activates neutrophils in the skin, resulting in the release of proinflammatory mediators by neutrophils, which leads to continued and increased neutrophil migration into the skin (50). Third, IC-mediated activation of neutrophils is also enhanced by GM-CSF, similar to that described for many, but not all, known neutrophil agonists (42). This enhancement of IC-induced neutrophil activation contributes to reactive oxygen release by neutrophils, which is required for autoantibody-induced tissue injury in different models of EBA (41). Additionally, GM-CSF may also enhance other effector functions of neutrophils, such as release of proinflammatory mediators or proteolytic enzymes (Fig. 9).

Our findings confirm and extend previous reports on GM-CSF inhibition in the effector phase of arthritis. However, in these models, GM-CSF inhibition had no effect on autoantibody production (11, 12). In contrast, in the context of infection, GM-CSF (together with IL-4) was shown to stimulate an Ab response to avian influenza in immunodeficient mice engrafted with human hematopoietic stem cells by promoting T, B, and dendritic cell maturation (21). Furthermore, neutrophils, which are among the main target cells of GM-CSF (5), have profound effects on B cell functions. More specifically, neutrophils induced Ig class switching, somatic hypermutation, and Ab production by activating B cells through mechanisms involving BAFF, APRIL, and IL-21 (22). The observed colocalization of neutrophils with BAFF in draining lymph nodes after immunization with COL7 in draining lymph nodes suggests that neutrophils are also involved in shaping

**FIGURE 9.** Contribution of GM-CSF to pathogenesis of experimental EBA. Schematic diagram summarizing the observed effects of GM-CSF in experimental models of EBA. (A) Mechanisms leading to the loss of tolerance to COL7 and anti-COL7 autoantibody production (path 1). In experimental EBA, a complex interplay with APCs and CD4+ T cells leads to a COL7-specific B cell response (33). In path 2, this results in production of circulating autoantibodies in peripheral lymph nodes, which are released into the circulation (path 3). As shown in this study (Fig. 8), both GM-CSF and neutrophils contribute to formation of anti-COL7 IgG in immunization-induced EBA, presumably by mediating the influx of (I) APCs and (II) neutrophils (Fig. 7) into the peripheral lymph nodes. (B) Subsequently, autoantibodies locate from the circulation to the DEJ in the skin (path 4). Furthermore, neutrophils are recruited from bone marrow (BM) into the circulation. (III) As shown in Fig. 4, GM-CSF modulates neutrophil recruitment from the bone marrow into the circulation. (IV) After autoantibody binding, a proinflammatory milieu is generated in the skin (62), leading to neutrophil recruitment into the skin (path 6). (V) This process also depends on the presence of GM-CSF (Fig. 4). Thereafter, neutrophils bind to the immune complexes located at the DEJ in an FcγR-dependent fashion, leading to neutrophil activation and ultimately blister formation (path 7). (V) As shown in Fig. 5, IC-induced neutrophil activation is enhanced in the presence of GM-CSF.
T cell–dependent Ab production. However, the precise mechanism through which neutrophils modulate the Ab response is unclear. In this study we report a reduced autoantibody response in GM-CSF−/− mice in induction-immunized EBA, which is associated with lower, but not completely absent, neutrophil numbers and unchanged numbers of COL7-specific B cells in draining lymph nodes compared with C57BL/6 mice. Based on these findings, we hypothesize that GM-CSF is an important factor, but not the only driving factor, leading to neutrophil recruitment into draining lymph nodes after immunization (Fig. 9). In addition to GM-CSF, PGs have been demonstrated to recruit neutrophils into lymph nodes after immunization with protein Ags emulsified in adjuvants (51). However, neutrophils recruited by PGs into lymph nodes inhibited CD4 T cell functions (51). Therefore, the contribution of innate immune cells, such as neutrophils, seems to have a differential contribution on adaptive immune functions, most likely in the context of location and timing.

The identification of the significant contribution of GM-CSF to the effector phase of EBA as well as the autoantibody production has a potential impact on the management of patients with EBA and other subepidermal autoimmune skin blistering diseases with a similar pathogenesis, for example, bullous pemphigoid (53), or other autoimmune diseases, such as experimental autoimmune encephalomyelitis. In experimental autoimmune encephalomyelitis, GM-CSF is required to sustain neuroinflammation via myeloid cells infecting the CNS (14). Given the rising incidence of autoimmune diseases in general and autoimmune bullous dermatoses specifically (53, 54), the far more limited therapeutic options (55, 56), and the high morbidity and mortality of the patients (54), there is a clear need for novel treatment strategies. In several autoimmune diseases, such as rheumatoid arthritis, inhibition of cytokines or growth factors has dramatically improved the management of these conditions (57–61). In contrast, with the exception of a few case reports, such innovative strategies have not yet been implemented in patients with autoimmune bullous dermatoses (62).

In the present study we demonstrate that GM-CSF not only modulates the effector phase of EBA by blocking neutrophil functions, but it is also required for mounting an autotaining-specific B cell response. A similar inhibitory effect on autoantibody production was observed in neutrophil-depleted mice. Combining GM-CSF inhibition and neutrophil depletion showed additive effects. Our findings extend the potential therapeutic spectrum of GM-CSF inhibition to autoantibody-mediated diseases.

Disclosures
The authors have no financial conflicts of interest.

References


**Supplement figure 1:** Expression and sources of GM-CSF in EBA

**A.** G- and GM-CSF serum concentrations (pg/ml) in different mouse strains after repetitive injections of either normal rabbit IgG (NR-IgG) or anti-type VII collagen IgG (anti-COL7 IgG). Data has been obtained from at least 3 mice per group, *indicates statistical significance (student's t-test). §Data on B6 mice has already been published elsewhere (Samavedam UK et al, 2013), but has been included here for completeness.

**B.** Ex vivo stimulation of isolated human neutrophils and monocytes with EBA-IC, induced a time-dependent release of GM-CSF (*/***/*** p=0.01, Mann-Whitney Rank Sum Test)
Supplement figure 2: Reduced BAFF expressing neutrophils in draining lymphnodes

A. Representative sections of draining lymph nodes stained with DAPI (blue), Ly6G (red) and BAFF (green).

B. Average number of Ly6G & BAFF co-localized cells in lymph nodes of C57Bl/6 and GM-CSF−/− mice at the end of the experiment showed a significant reduction Ly6G and BAFF cells in GM-CSF−/− mice (n=5 (10 fields), *p<0.001, t-test).