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Macrophages, But Not T and B Lymphocytes, Are Critical for Subepidermal Blister Formation in Experimental Bullous Pemphigoid: Macrophage-Mediated Neutrophil Infiltration Depends on Mast Cell Activation

Ruoyan Chen,* Janet A. Fairley,† Ming-Lang Zhao,* George J. Giudice,† Detlef Zillikens,‡ Luis A. Diaz,* and Zhi Liu‡*

Bullous pemphigoid (BP) is a subepidermal blistering disease associated with autoantibodies against two hemidesmosomal proteins, BP180 and BP230. Numerous inflammatory cells infiltrate the upper dermis in BP. We have previously shown by passive transfer studies that Abs to the ectodomain of murine BP180 are capable of triggering blisters in mice that closely mimic human BP. Experimental BP depends on complement activation and neutrophil infiltration. In the present study, we investigated the relative contribution of neutrophils, mast cells (MCs), macrophages (MØ), and lymphocytes and their functional relationship in the immunopathogenesis of this disease model by using mice deficient in these cells. Wild-type, T cell-deficient, and T and B cell-deficient mice injected intradermally with pathogenic anti-murine BP180 IgG exhibited extensive subepidermal blisters. In contrast, mice deficient in neutrophils, MCs, and MØ were resistant to experimental BP. MCs play a major role in neutrophil recruitment into the dermis. Furthermore, MØ-mediated neutrophil infiltration depends on MC activation/degranulation.


Bullous pemphigoid (BP) is an acquired autoimmune skin disease characterized by subepidermal blisters and autoantibodies against two hemidesmosomal Ags, BP230 (BPAG1) and BP180 (BPAG2) (1). These anti-hemidesmosomal autoantibodies are found in the circulation of patients, and can be detected, along with complement components, bound to the dermal-epidermal junction (DEJ) of perilesional skin. The skin blisters of these patients show detachment of basal keratinocytes from the underlying dermis and a dermal inflammatory infiltrate (2). A variety of cellular lineages have been identified in these inflammatory infiltrates, including eosinophils, neutrophils, lymphocytes, mast cells (MCs), and monocyte/macrophages (MØ) (1, 3–8). MCs found in BP lesions exhibit morphological changes suggesting degranulation (7, 9). Lesional skin in BP patients exhibits several granular proteins derived from leukocytes, such as eosinophil cationic protein, eosinophil major basic protein, and neutrophil-derived myeloperoxidase (MPO) (10–12). Various inflammatory mediators that can activate MCs or leukocytes have been identified in lesional skin and/or blister fluids of BP patients, including C5a, eosinophilic/neutrophilic chemoattractants, histamine, leukotrienes, and various cytokines (e.g., IL-1, -2, -5, -6, -8, TNFs, and IFN-γ) (13–20). Several proteases are also found in BP blister fluid, including plasmin, collagenase, elastase, and 92-kDa gelatinase (21–24).

An experimental model of BP that involves the passive transfer of anti-murine BP180 (mBP180) Abs into neonatal BALB/c mice reproduces the key immunopathological features of this human autoimmune disease, i.e., IgG and complement deposition at the DEJ, inflammatory infiltration of the upper dermis, and subepidermal blistering (25). We further showed that the pathogenicity of anti-mBP180 Abs is dependent on complement activation (26), MC degranulation (27), and neutrophil recruitment (28). In the present study, we investigated the role of MØ, T and B lymphocytes, and compared the relative contribution of these inflammatory cells in subepidermal blistering in experimental BP.

Materials and Methods

Laboratory animals

Breeding pairs of C57BL/6J, MC-deficient WCB6F1-MgfSl/MgfSl−d (referred to as MC−/−) mice (29), T cell-deficient nude (referred to as T−/−) mice, T and B cell-deficient C57BL-6J-Rag1tm1Mom mice (referred to as T−/− and B−/−) mice (30) were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained at the Medical College of Wisconsin Animal Resource Center (Milwaukee, WI). Neonatal mice (24–36 h old with body weights between 1.4 and 1.6 g) were used for passive transfer experiments.

Preparation of pathogenic rabbit anti-murine IgG

The preparation of recombinant mBP180 and the immunization of rabbits were performed as previously described (25). The titers of rabbit anti-mBP180 Abs in the rabbit sera and in the purified IgG fractions were assayed by indirect immunofluorescence (IF) using mouse skin cryosections as substrate (25). The pathogenicity of these IgG preparations was tested by passive transfer experiments as described below. One pathogenic anti-mBP180 IgG (referred to as R530) and one control IgG (referred to as R50) were used (26).
A 50-μl dose of sterile IgG in PBS was administered to neonatal mice by intradermal (i.d.; 2.64 mg IgG/g of body weight). The injection techniques have been described elsewhere (25, 28). The skin of neonatal mice from the test and control groups was examined 12 h after the IgG injection. The extent of cutaneous disease was scored as follows: −, no detectable skin disease; 0, mild erythema with evidence of epidermal detachment; +, intense erythema with epidermal detachment sign (this sign was elicited by gentle friction of the mouse skin which, when positive, produced fine, persistent wrinkling of the epidermis); 2+, intense erythema and epidermal detachment sign involving >50% of the epidermis in localized areas; and 3+, intense erythema with frank epidermal detachment sign involving >50% of the epidermis. The animals were then sacrificed and the following specimens were obtained.

The pooled cells were washed twice in HBSS without phenol red.

The pooled cells were done by following published protocols (31, 32) with some minor modifications. Briefly, skin sections were obtained and rinsed in 70% ethanol. The skin sections were cut into 4 × 4-mm sheets and submerged in culture medium into a 6-well plate. Each well contained 4 ml of RPMI 1640 with 25 mM HEPES, 10% heat-inactivated FBS and penicillin/streptomycin. The plate was incubated at 37°C for 6 h in a 5% CO2 incubator. The cells spontaneously emigrating out of the skin sections were pooled and incubated for additional 20 min at 37°C in PBS with 2 mM glucose and without calcium and magnesium. The pooled cells were washed twice in HBSS without phenol red.

The dermal inflammatory cells were identified by characteristic size (forward scatter) and granularity (side scatter) combined with two-color flow cytometric analysis (31, 32). The neutrophils were identified as small cells, Ly-6G bright (RB6-8C5; BD PharMingen, San Diego, CA), and negative for F4/80 (A3-1; Caltag Laboratories, Burlingame, CA) or MHC class II (25-9-17; BD PharMingen). The Mψ were identified as F4/80 positive or Mac-3 positive (M5/84; BD PharMingen) and MHC class II low or negative. The T lymphocytes were identified by their small size and by CD3 (145-2C11; BD PharMingen) expression; B lymphocytes were identified by CD19 (1D3; BD PharMingen); the eosinophils were identified by their (145-2C11; BD PharMingen) expression; B lymphocytes were identified by their CD19 (1D3; BD PharMingen). The viability of the neutrophils was 96% as determined by cell-cytospin and LeukoStat staining (Fisher Diagnostic, Orangeburg, NY). The viability of the neutrophils was >96% as determined by trypan blue exclusion.

In vivo inhibition of MC degranulation

Neutrophil isolation

Mouse neutrophils were isolated from heparinized blood by dextran sedimentation followed by separation on a density gradient as described (41). RBCs were removed from the cell preparation by hypotonic lysis in 0.2% NaCl. Neutrophils were washed and resuspended in cold PBS/10 mM glucose, counted in a hemocytometer, and adjusted to a concentration of 1 × 10^7 cells/ml. Neutrophil purity of the isolated neutrophils was consistently >96% as determined by cell-lysospin and LeukoStat staining (Fisher Diagnostics, Orangeburg, NY). The viability of the neutrophils was >96% as determined by trypan blue exclusion.

i.d. injection of neutrophils

Mψ-deicient mice were injected i.d. with pathogenic anti-mBP180 IgG (2.64 mg/g body weight/50 μl of PBS). Two hours later, these mice received 5 × 10^7 neutrophils i.d. (in 50 μl of PBS/10 mM glucose) at the same site (42). The animals were analyzed 12 h after the IgG injections as described above.

IL-8 pretreatment of Mψ-deicient mice

Recombinant human IL-8, purchased from R&D Systems (Minneapolis, MN), was stored at 1 mg/ml in sterile PBS. Single i.d. injection of IL-8 (50 ng in 50 μl of PBS) or an equivalent amount of BSA, was given into neonatal Mψ-deicient mice 60 min before i.d. injection of rabbit anti-mBP180 IgG (2.64 mg/g body weight in 50 μl) (28). Control animals received an equivalent amount of normal rabbit IgG in place of the anti-mBP180 IgG. Twelve hours after the IgG injections, the animals were analyzed as described above.

Statistical analysis

The data were expressed as mean ± SEM and were analyzed using the Student’s paired t test. A value of p < 0.05 was considered significant.
emigrating from the IgG-injected dermis were recovered and analyzed by flow cytometry. As shown in Fig. 1, the number of neutrophils, Mϕ, T lymphocytes, and B lymphocytes were significantly increased in mice injected with pathogenic IgG than mice injected with control Ab. The influx of eosinophils remains the same between the pathogenic IgG-treated and control groups. These results were similar to findings obtained by routine histology staining of the mouse skin sections.

Neutrophils, MCs, Mϕ, but not B and T lymphocytes, are required for subepidermal blistering in experimental BP

To compare relative contributions of different inflammatory cells in experimental BP, neonatal control, and mice deficient in neutrophils, MCs, Mϕ, T cells, and B and T cells were injected i.d. with pathogenic anti-mBP180 IgG R530 (n = 10 for each group). Twelve hours after IgG injection, wild-type control, T cell-deficient, and B and T cell-deficient mice developed blisters with similar disease scores (Fig. 2, A and G; see also Table I). Direct IF showed deposition of rabbit IgG and murine C3 at the cutaneous BMZ. Histological examination of the skin of mice revealed DEJ separation with neutrophil infiltration in the dermis (Fig. 2, B and H). In contrast, the injected animals deficient in neutrophils, MCs, and Mϕ were resistant to experimental BP (Fig. 2, C, E, and I; see also Table I). There was no blister formation (Fig. 2, D, F, and J), despite the presence of IgG and mouse C3 at the BMZ when examined by direct IF (Table I). The positive direct IF staining ruled out the possibility that deficiency of neutrophils, MCs, or Mϕ impairs the binding of IgG to its target or complement activation.

To quantify the relative contributions of different inflammatory cells to the tissue damage triggered by pathogenic anti-mBP180 IgG, skin neutrophil infiltration, which is directly correlated with the skin disease activity (28), was assessed by MPO assay. The lesional skin of wild-type mice and mice deficient in T cells or T and B cells showed significantly elevated levels of tissue extractable MPO activities as compared with the skin of mice deficient in neutrophils, MCs, and Mϕ (Fig. 3). The relative MPO activities (OD_{438} reading unit per milligram protein) were 1.08 ± 0.16 for wild-type, 1.29 ± 0.19 for T cell-deficient, 1.14 ± 0.12 for T and B cell-deficient, 0.13 ± 0.02 for neutrophil-deficient (p < 0.001), 0.41 ± 0.05 for MC-deficient (p < 0.01), and 0.73 ± 0.08 for Mϕ-deficient mice (p < 0.05), respectively. Taken together, these data demonstrated that neutrophils, MCs, and Mϕ, but not T and B lymphocytes, are involved in subepidermal blistering in experimental BP.

Mϕ are not involved in MC activation, but play an accessory role in recruiting neutrophils in experimental BP

To determine whether Mϕ play a role in MC activation/degranulation, Mϕ-sufficient and Mϕ-deficient mice were injected i.d. with pathogenic IgG. The total number of MCs and the percentage of MC degranulation in the IgG-injected skin were quantified at different time periods after IgG injection. As expected, wild-type mice (n = 5) injected with pathogenic IgG developed subepidermal blisters 12 h after IgG injection (Table I), while Mϕ-deficient mice (n = 5) injected with pathogenic IgG did not show any sign of skin blistering (Table I). Toluidine blue staining showed a slightly (but not significantly) higher degree of MC degranulation in the skin of Mϕ-sufficient as compared with Mϕ-deficient mice injected with pathogenic IgG at 2 and 12 h post-IgG injection (Fig. 4A, a and c). As expected, MC degranulation in the skin of Mϕ-sufficient and Mϕ-deficient mice co-injected with pathogenic IgG and cromolyn was minimal (Fig. 4A, b and d). We also failed to see any significant difference in total number of MCs and percentage of MC degranulation in paired groups of mice (Mϕ-sufficient vs Mϕ-deficient group and cromolyn-treated Mϕ-sufficient vs cromolyn-treated Mϕ-deficient mice) across all time points (Fig. 4B). These results suggest that depletion of Mϕ does not affect MC degranulation.
We found that Mϕ-deficient mice reconstituted with neutrophils become susceptible to experimental BP either by i.d. injection of pathogenic IgG plus 5 × 10⁵ mouse neutrophils or i.d. injection of pathogenic IgG plus IL-8. Twelve hours after IgG injection, the injected Mϕ-deficient mice (n = 5) developed subepidermal blisters (Table I). As a control, Mϕ-deficient mice injected with IL-8 alone exhibited neutrophil infiltration in the skin but no skin lesions (Table I). Taken together, these results suggest that Mϕ mainly participate in PMN recruitment in experimental BP.

**Mϕ-mediated neutrophil recruitment is MC-dependent**

To determine whether Mϕ-mediated neutrophil infiltration depends on MCs, wild-type, MC-deficient, and Mϕ-deficient mice with or without pretreatment of carrageen or cromolyn were injected i.d. with pathogenic IgG. Twelve hours later, neutrophil infiltration in the skin of the injected animals was quantified by MPO assay. We found that MC-deficient mice (n = 5) with and without Mϕ depletion showed similar MPO activities (0.34 ± 0.04 for untreated vs 0.39 ± 0.05 for carrageen-treated mice; Fig. 5A). In contrast, Mϕ-deficient mice pretreated with cromolyn exhibited a significant reduction in tissue MPO activity as compared with Mϕ-deficient mice without cromolyn treatment (0.68 ± 0.11 for untreated vs 0.05 for cromolyn-treated mice).

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**FIGURE 3.** Relative contribution of inflammatory cells in experimental BP. Neonatal wild-type (WT; bar 1), neutrophil (PMN)-deficient (bar 2), MC-deficient (bar 3), Mϕ-deficient (bar 4), T cell (T)-deficient (bar 5), and T and B cell (T&B)-deficient mice (bar 6) received 2.64 mg/g body weight pathogenic anti-mBP180 IgG. Tissue MPO activities (mean ± SEM) in the injection sites were determined 12 h after the IgG injection. n = 8 for each group. *, p < 0.05; **, p < 0.01. Student t test for paired samples (bar 1 vs 2, 3, or 4). The MPO values shown were corrected for control IgG controls. Each group of mice injected with control IgG yielded an average MPO activity of ~0.1 OD₄₆₀/mg protein.

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**FIGURE 4.** Mϕ deficiency has no effects on MC degranulation. Mϕ-sufficient (Mϕ⁺) and Mϕ-deficient (Mϕ⁻) mice were pretreated with the MC degranulation blocker cromolyn sodium (CS) for 2 h and injected i.d. with pathogenic IgG R530. The injected animals were examined at different time points after IgG injection. A. Toluidine blue staining. At 12 h post IgG injection, Mϕ⁺, but not Mϕ⁻, CS-treated Mϕ⁺, and CS-treated Mϕ⁻ mice developed blisters (Table I). At 2 h post IgG injection (peak of MC degranulation), Mϕ⁺ (a) and Mϕ⁻ (c) mice showed extensive MC degranulation in Toluidine blue-stained skin sections. In contrast, CS-treated Mϕ⁺ (b) and CS-treated Mϕ⁻ (d) mice exhibited background levels of MC degranulation. B. Time course (0–12 h postinjection) of MC degranulation. The MCs in the dermis were counted and classified as degranulated (>10% of the granules exhibiting fusion or discharge) or normal (see Materials and Methods). Toluidine blue-stained sections showed a similar pattern of progression of MC degranulation between comparable groups of mice: Mϕ⁺ vs Mϕ⁻ and CS-treated Mϕ⁺ vs CS-treated Mϕ⁻.
Each group of mice injected with control IgG yielded an average MPO > 0.1 OD460/mg protein. Thus, MΦ does not totally impair neutrophil infiltration, we first tested whether MΦ deficiency could impair MC activation/degranulation. We found that 1) MΦ did not affect MC degranulation (Fig. 4); 2) MC-deficient mice and mice deficient in both MCs and MΦ exhibit the same disease scores and similar levels of neutrophils (Fig. 5A); and 3) inhibition of MC degranulation further reduced neutrophil infiltration in MΦ-depleted mice (Fig. 5B). Thus, MCs act upstream of MΦ in the inflammatory cascade in experimental BP.

MCs can produce a variety of inflammatory mediators such as leukotrienes, platelet-activating factor, and cytokines that contribute directly or indirectly to neutrophil recruitment (46, 47). In fact, high levels of histamine, leukotriene B4, IL-1, -2, -5, -6, and TNF-α are present in BP blister fluids (13–20). It is likely that some of these mediators are released from MCs and are involved in the recruitment of neutrophils directly by themselves and indirectly by activating MΦ. Both activation and degranulation of MCs are associated with matrix degradation (47). MC-specific serine protease MCP-4 (chymase) also activates gelatinase B (48, 49). Therefore, MCs could also contribute to tissue damage in experimental BP directly by cleaving structural proteins in the DEJ or indirectly by activating gelatinase B. Similarly, MΦ are also capable of releasing a variety of proinflammatory mediators, proteolytic enzymes, and reactive oxygen species (50), and could participate in either recruiting neutrophils or directly damaging BMZ. How MCs regulate MΦ pathological functions in experimental BP is currently under investigation.

Lymphocytes are found in the lesional/perilesional skin of human BP (1) and the skin of experimental BP (25). However, our data show that mice deficient in T cells and T and B cells are susceptible to experimental BP, suggesting that these cells do not participate, at least during the early stages of the disease induced by passive transfer of pathogenic anti-BP180 IgG. Interestingly, T cell-deficient mice show more infiltrating neutrophils in the dermis and more severe disease activity relative to wild-type mice (Fig. 2). T cells contain α1-proteinase inhibitor, the physiological inhibitor of neutrophil elastase (51). We have shown that α1-proteinase inhibitor is critical to the down-regulation of subepidermal blistering (52). Therefore, infiltrating T lymphocytes may secrete this inhibitor at the skin site to block neutrophil elastase activity, which subsequently inhibits neutrophil recruitment (44). It is also worth noticing that eosinophils are absent in the lesional skin of mice during the first 24 h of blister development, although they are the predominant cells in the inflammatory infiltrate in human BP. The difference in predominant cell infiltration between human BP and mouse BP could be due to the difference in their disease stages: skin biopsies of mouse BP are obtained at 12 or 24 h post-IgG injection, while most BP patient biopsies are obtained more than days or weeks after the initial onset of clinical disease activity. In fact, eosinophils are identified in the lesional skin of mouse BP when the mouse model is maintained long term (>96 h; our unpublished observations). It is also possible that eosinophils are not directly involved or play only a secondary role in the initiation of human BP. Because a pathogenic role of eosinophils in human BP remains to be proved, we cannot rule out the possibility that there are critical differences in the immunopathology between human and mouse BP. Whether eosinophils play a role in the late stages of the disease process, e.g., wound healing after tissue damage, needs further investigation.

To determine the functional relationship between MCs and MΦ in mediating neutrophil infiltration, our previous study demonstrated that neutrophils, MCs, and MΦ, but not T and B lymphocytes, are required for full expression of the disease phenotype in experimental BP. We also established the relationship between MCs and MΦ in the development of the cutaneous disease. MCs play a major role in recruiting neutrophils, while MΦ amplify the neutrophil infiltration in a MC-dependent fashion. Based on immunohistological evidence, it has long been hypothesized that anti-BMZ autobody-triggered subepidermal blister formation in BP is mediated by inflammatory cells (43). Our previous and current findings provide in vivo evidence that DEJ separation is initiated by anti-BP180 IgG (25, 44) and dependent on neutrophils (28), MCs (27), and MΦ (Fig. 2). We have previously established a causal relationship between neutrophil infiltration into the skin and subepidermal blistering in experimental BP (28). Depletion of circulating neutrophils completely abolishes the skin disease. Neutrophil elastase and gelatinase B mediate BMZ tissue damage and DEJ separation (42, 45). We further showed that neutrophil recruitment triggered by anti-BP180 Abs depends mainly on MCs (27). MC-deficient mice are resistant to experimental BP and exhibit ~70% reduction of neutrophil infiltration into the skin.

The fact that MC deficiency does not totally impair neutrophil recruitment in experimental BP suggests that there are at least two neutrophil recruitment pathways, MC-dependent and MC-independent pathways. Our present data show that MΦ are also involved in anti-BP180 IgG-triggered neutrophil infiltration in mice.
BP and have significant implications for therapeutic intervention for this disease.

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