

Abbreviations used

Foxp3: Forkhead box protein 3
 GFP: Green fluorescent protein
 GMD: Goat anti-mouse IgD serum
 GST: Glutathione-S-transferase
 IC: Immune complex
 MM: Multiple myeloma
 SLE: Systemic lupus erythematosus
 Treg: Regulatory T

IL-10 production has also been reported for human MM cell lines,²⁹ and high serum IL-10 levels have been found in patients with SLE.³⁰ However, increased IL-10 production has not been described as a cause of immunodeficiency in patients with MM or other conditions exhibiting plasmacytosis.

Defects in neutrophil functions contribute to the general immunodeficiency associated with SLE and MM.^{22,31} Neutrophils are important innate effector cells^{32,33} crucial for protection against bacterial pathogens.^{34,35} Consistently, bacterial infections are frequently observed in patients with SLE and myeloma.^{17,18,22,23} On the other hand, neutrophils drive the acute and chronic inflammation associated with autoimmune diseases.³⁶

The complement fragment C5a, one of the most potent neutrophil chemoattractants, is formed by complement activation in response to immune complexes (ICs).³⁷ For example, local activation of complement and neutrophil infiltration is necessary for the initiation of epidermolysis bullosa acquisita, an antibody-mediated chronic autoimmune skin-blistering disease.^{38,39} Accordingly, C5a receptor signaling in neutrophils drives the inflammatory response in the Arthus reaction, the prototypic local immune reaction mediated by acute IC deposition.^{40,41}

Here we demonstrate that plasmacytosis-associated IL-10 limits neutrophil-mediated inflammation. Additionally, we provide evidence that the price for this reduced inflammation is a deficiency in neutrophil function, causing increased susceptibility to severe bacterial infections.

METHODS**Mice**

C57BL/6 and BALB/c mice were purchased from Charles River (Bar Harbor, Me). IL-10 reporter (Vert-X), BALB/c forkhead box protein 3 (Foxp3) reporter (Foxp3^{GFP}), and CD19 Cre/IL-10 flox/flox mice and their littermate controls were bred at the animal facility of the University of Lübeck. Experiments were performed at the animal facilities of the Universities of Lübeck and Greifswald.

Experimental epidermolysis bullosa acquisita and polyclonal plasmacytosis

Epidermolysis bullosa acquisita was induced by means of subcutaneous immunization and scored, as previously described.⁴² Autoreactive plasma cells were identified by means of flow cytometry, as previously described.⁴² For more information, see the [Methods](#) section in this article's Online Repository at www.jacionline.org. Collagen VII (amino acids 757-967) was produced, as recently described.⁴² Collagen-specific serum antibodies were quantified by means of ELISA. Plates were coated with 500 ng of collagen VII. After blocking, wells were incubated with a 150-fold dilution of the serum samples for 60 minutes. Detection was performed with biotinylated goat anti-mouse IgG antibody (SouthernBiotech, Birmingham, Ala), followed by

streptavidin-coupled alkaline phosphatase (Roche Diagnostics, Mannheim, Germany) and ALP (Roche Diagnostics GmbH). Polyclonal plasmacytosis was induced by means of intraperitoneal injection with 200 μ L of goat-anti mouse IgD. Some groups received anti-IL-10 receptor (clone IBI.3, a generous gift from DNAX, Palo Alto, Calif).

Murine myeloma

As previously described,⁴³ murine myeloma was induced by means of intravenous injections of MOPC315.BM cells (5×10^5 cells) stably transfected with eGFP. MOPC315.BM myeloma-specific anti-DNP IgA antibodies were quantified by means of ELISA. Briefly, plates were coated with 10 μ g/mL DNP-BSA/PBS (1 hour at room temperature). Nonspecific binding was blocked with 1 mg/mL BSA/PBS. Subsequently, sera were incubated for 1 hour at room temperature. Detection was done with biotinylated goat anti mouse IgA (SouthernBiotech), followed by streptavidin-coupled alkaline phosphatase (Roche Diagnostics) and ALP (Roche Diagnostics).

C5a-mediated peritoneal inflammation

Mice were injected intraperitoneally with goat anti-mouse IgD serum (GMD) or goat serum (0.2 mL) and 6 days later were injected (intraperitoneally) with anti-IL-10 receptor antibody (0.5 mg) or rat IgG. One day later, mice were injected with C5a (200 nM, 100 μ L, administered intraperitoneally). After 4 to 5 hours, mice were killed, and neutrophil numbers in peritoneal lavage fluid were determined by means of flow cytometry.

Infection and bioluminescent optical imaging

BALB/c mice with or without plasmacytoma (MOPC315.BM) were infected intranasally with bioluminescent pneumococci (*Streptococcus pneumoniae* D39lux).⁴⁴ For this purpose, pneumococci were cultured to the exponential phase ($A_{600} = 0.35$) in THY medium supplemented with 10% heat-inactivated FBS (Gibco by Life Technologies, Grand Island, NY) and centrifuged, after which the infection dose was adjusted to 5.0×10^8 colony-forming units in 20 μ L. Before intranasal infection, mice were anaesthetized by means of intraperitoneal injection of ketamine (Ketanest S; Pfizer Pharma, Karlsruhe, Germany) and xylazine (Rompun; Provet AG, Lyssach, Germany). The bacterial suspension was administered intranasally. Bioluminescent optical imaging with the IVIS Spectrum Imaging System (Caliper Life Sciences, Hopkinton, Mass) allowed monitoring of pneumococcal dissemination after intranasal infection.^{45,46} At prechosen time intervals after infection, mice were imaged for 1 minute to monitor dissemination of pneumococci. A time series of the images was generated, and the bioluminescent intensity was determined by means of quantification of the total photon emission with the LivingImage 4.1 software package (Caliper Life Sciences).

For more information on antibodies, flow cytometry, ELISA, histology, statistics, and study approval, see the [Methods](#) section in this article's Online Repository.

RESULTS**Polyclonal and neoplastic plasmacytosis is associated with increased IL-10 production**

B lineage cells with a CD138^{hi} plasma cell/plasmablast phenotype can significantly contribute to IL-10 production and thereby control T cell-mediated autoimmune inflammation.²⁸ Here, we first tested the possibility that plasmacytosis increases production of immunosuppressive IL-10. This cytokine was detectable in sera from 6 of 8 patients with advanced myeloma. In contrast, it was present only at a relatively low level in 1 of 7 healthy control subjects and undetectable in patients exhibiting

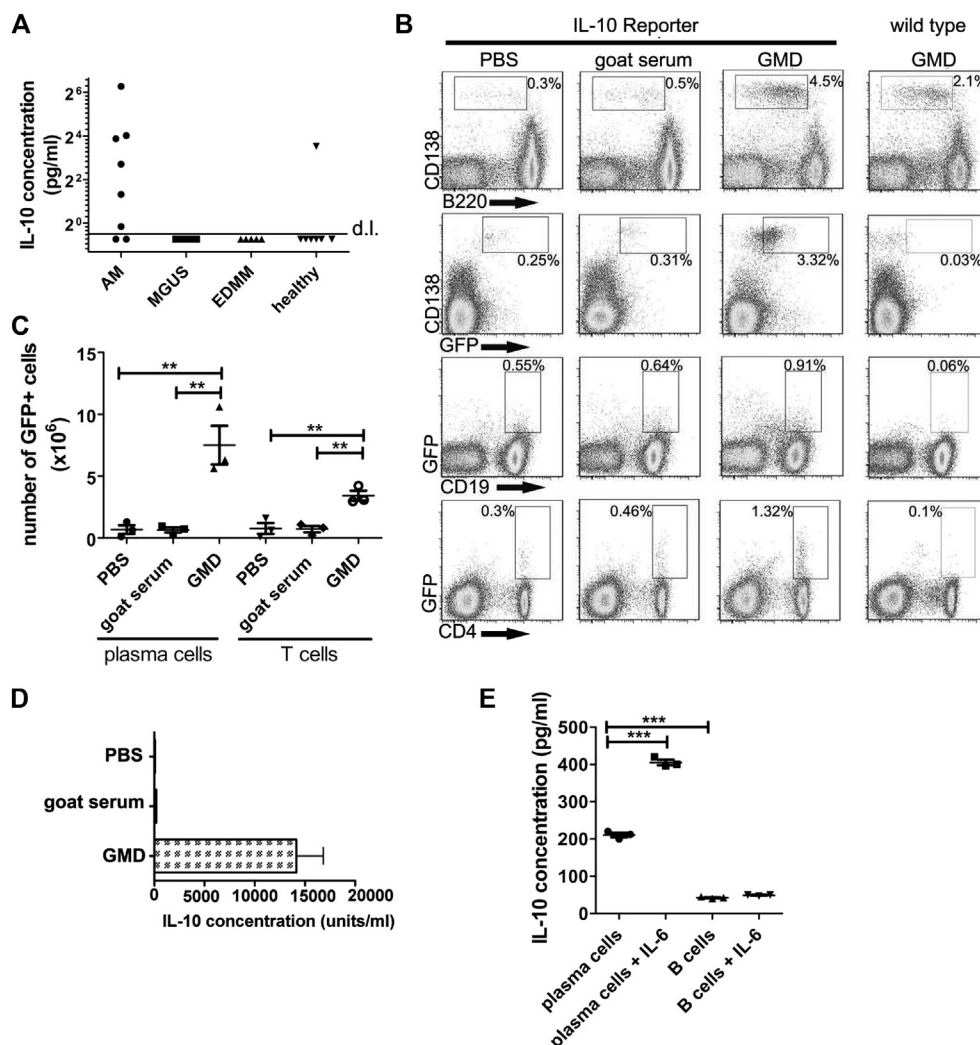


FIG 1. Plasmacytosis is associated with increased IL-10 production. **A**, Serum IL-10 levels of healthy control subjects and patients with monoclonal gammopathy of undetermined significance, early diagnosed myeloma (EDMM), and advanced myeloma (AM). **B**, Cytometric analysis of GMD-, PBS-, or goat serum-treated IL-10 reporter mice. **C**, GFP⁺ cells on day 7. **D**, IL-10 serum levels. **E**, IL-10 concentrations in supernatants from cultures with or without IL-6 (ANOVA). ** $P < .01$ and *** $P < .001$.

monoclonal gammopathy of undetermined significance, a precursor state of MM, and patients with early diagnosed myeloma (Fig 1, A). This finding is in accordance with the notion that IL-10 is produced by a considerable proportion of plasma cell lines derived from patients with myeloma.²⁹

Patients with SLE, the best characterized autoimmune disease involving polyclonal plasmacytosis, also exhibit increased IL-10 levels.³⁰ To investigate IL-10 expression during the course of polyclonal plasmacytosis, we induced a massive polyclonal plasma cell response by injecting Vert-X mice with GMD.⁴⁷ In this mouse model enhanced green fluorescent protein (GFP) is expressed under control of the IL-10 promoter. At day 7, GMD-injected mice had plasmacytosis and showed a considerable population of GFP⁺ cells that had the B220^{low}CD138^{hi} phenotype characteristic of early plasma cells. GMD-injected mice also showed a moderate increase in GFP⁺ cell counts among CD4⁺ T cells. As shown in Foxp3 reporter mice, IL-10⁺Foxp3⁺CD4⁺ and IL-10⁺Foxp3⁻CD4⁺ cell populations

were expanded by means of GMD treatment (see Fig E1 in this article's Online Repository at www.jacionline.org). Hence plasmacytosis is associated with increased IL-10 production by plasma cells and CD4 T cells, including regulatory T (Treg) cells. However, plasma cells accounted for the great majority of the increase in absolute GFP⁺ cell numbers (Fig 1, B and C). Cells other than B or T cells did not show increased GFP expression in GMD-treated mice (data not shown). These data confirm those of Madan et al,⁴⁷ showing that plasma cells are a major source for IL-10 in GMD-treated mice. In accordance with the massive increase in GFP⁺ cell numbers, GMD-induced plasmacytosis was associated with an at least 50-fold increase in serum IL-10 levels (Fig 1, D). Of note, serum IL-10 levels were less than the detection limit of 0.2 ng/mL of our ELISA, even in mice treated with GMD. Therefore serum IL-10 levels were quantified by using an *in vivo* cytokine capture assay.⁴⁸

CD138^{hi} plasma cells generated *in vitro* after LPS stimulation of B cells from Vert-X mice exhibited uniform GFP expression.

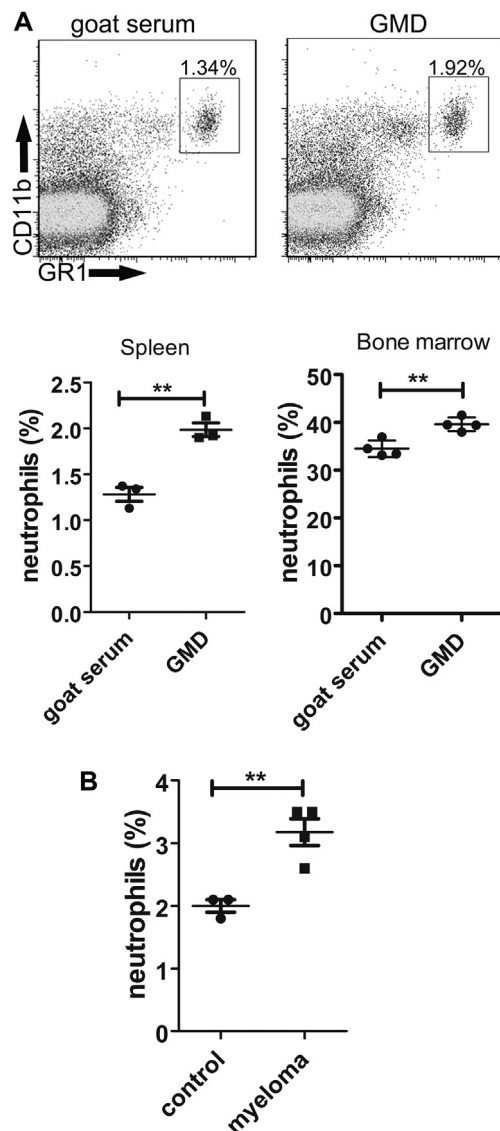


FIG 2. Plasmacytosis is accompanied by changes in neutrophil frequencies. **A**, Neutrophil frequencies at day 7 after induction of GMD-induced plasmacytosis compared with those in goat serum-treated control mice. **B**, Neutrophil frequencies in spleens of mice with MOPC315.BM myeloma compared with those in control mice (Student *t* test). ***P* < .01.

To investigate whether GFP expression correlated with IL-10 protein production, at day 4 of culture, B cells and CD138^{hi} plasma cells were separated by means of fluorescence-activated cell sorting and cultured for another 16 hours. Culture supernatants from CD138^{hi} plasma cells contained approximately 5-fold more IL-10 than supernatants of B cells isolated from the same primary LPS culture. IL-10 production by plasma cells was further increased when IL-6 was added to the cultures (Fig 1, E). Supernatants of the murine plasma cell line MOPC315.BM contained similar IL-10 levels (data not shown).

Together, these data show that IL-10 production is a common feature of normal and neoplastic plasma cells and that IL-10 levels increase with progression of polyclonal and neoplastic plasmacytosis.

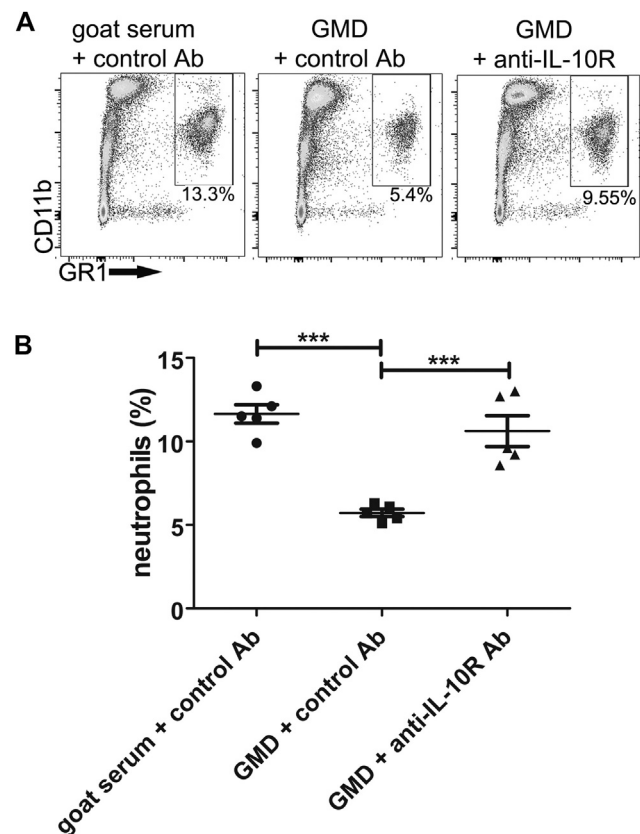
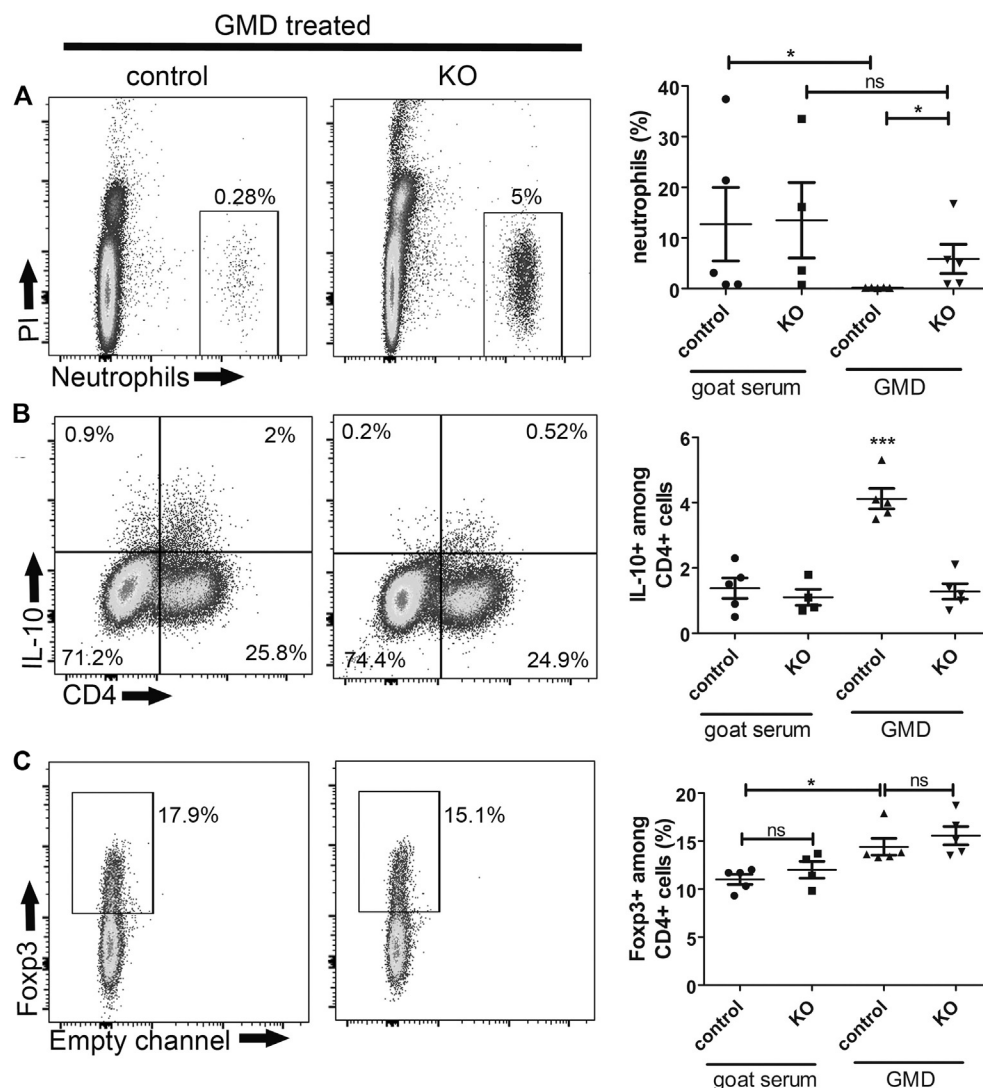


FIG 3. Polyclonal plasmacytosis leads to IL-10-dependent suppression of neutrophil migration toward C5a. Mice treated with GMD or goat serum (control) are shown. One week later, C5a-mediated neutrophil influx was analyzed in the peritoneum. Anti-IL-10 receptor (*IL-10R*) or control antibody was injected 1 day before analysis. **A**, Representative fluorescence-activated cell sorting analysis. **B**, Neutrophils in the peritoneum (ANOVA). Ab, Antibody. ****P* < .001.

Plasmacytosis-mediated suppression of neutrophil migration depends on B lineage-derived IL-10

At the peak of GMD-induced plasmacytosis, an increase in neutrophil populations was observed in the spleen and bone marrow (Fig 2, A). Neutrophil numbers were also increased in these tissues but not in blood (see Fig E2 in this article's Online Repository at www.jacionline.org). Neutrophil frequencies in spleens were also increased in mice inoculated with MOPC315.BM plasmacytoma cells (Fig 2, B). Complement-mediated inflammation was mimicked by injecting the neutrophil attractant C5a into the peritoneum of mice at the peak of GMD-induced polyclonal plasmacytosis to assess the effect of high plasma cell loads on neutrophil migration. As a positive control, C5a was injected into the peritoneum of mice that had been treated with goat serum. As expected, C5a induced massive migration of neutrophils into the peritoneum (Fig 3, A). Neutrophil frequencies in the peritoneum of GMD-treated mice were reduced by approximately 50% compared with those of goat serum-treated control mice. The inhibitory effect in GMD-treated mice was reversed by injecting them with anti-IL-10 receptor antibody (Fig 3, A and B). Neutrophil counts in blood were not altered by IL-10 receptor blockade (see Fig E3 in this article's Online Repository at www.jacionline.org). These results suggest that polyclonal plasmacytosis-associated IL-10 limits



neutrophil migration to sites of complement activation. Importantly, within 1 day, IL-10 receptor blockade reversed the plasmacytosis-associated effects on neutrophils, but within this short period, it had no effect on the GMD-induced increase in Foxp3⁺CD4⁺ Treg cell frequencies (see Fig E4 in this article's Online Repository at www.jacionline.org). This suggests that the observed effects on neutrophil migration were not merely caused by the expansion of Treg cell populations.

GMD-induced plasmacytosis did not block neutrophil accumulation in the peritoneum of B cell-specific IL-10 knockout (CD19 Cre/IL-10 flox/flox) mice (Fig 4, A), indicating that B cell/plasma cell-derived IL-10 is required to suppress neutrophil migration during plasmacytosis. Increased IL-10 production by CD4 T cells was also dependent on B lineage-derived IL-10 (Fig 4, B). In contrast, expansion of Foxp3⁺CD4⁺ Treg cells was not dependent on IL-10 production by B cells (Fig 4, C). These data are consistent with previous findings that B

lineage-derived IL-10 can promote IL-10 production by Foxp3⁺ regulatory type 1 T cells.⁴⁹ Together, these data indicate that under conditions of plasmacytosis, plasma cell-derived IL-10 suppresses neutrophil migration, either directly or through induction of IL-10 production by CD4 T cells. However, the mere expansion of Foxp3⁺ Treg cells did not affect neutrophil migration under these conditions.

Experimental MOPC315.BM plasmacytosis results in an IL-10-mediated complete block of neutrophil migration and increased susceptibility to bacterial infection

Notably, patients with myeloma have increased susceptibility to bacterial infections,¹⁸ and neutrophils are crucial for controlling such infections in healthy subjects.³² Because IL-10 is produced by several human myeloma cell lines and by the

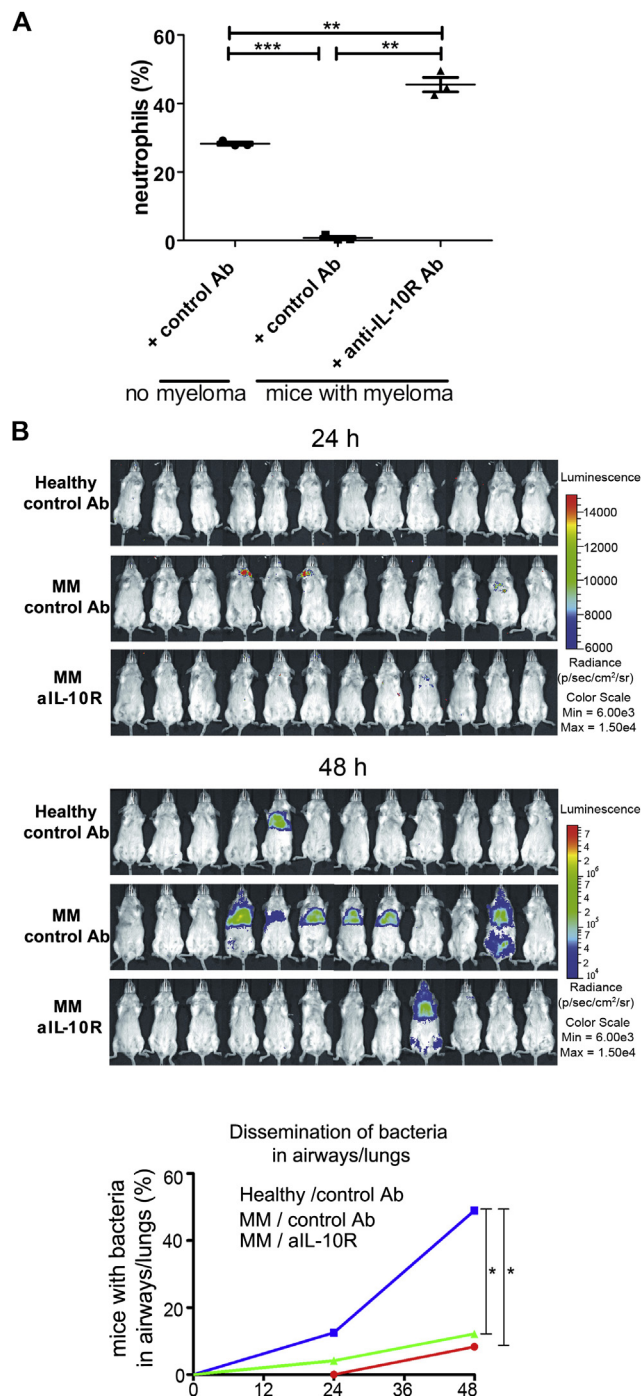


FIG 5. Experimental myeloma results in IL-10-dependent suppression of neutrophil migration and increased susceptibility to pneumococcal pneumonia. **A**, C5a-mediated neutrophil influx in mice with and without MOPC315.BM myeloma treated with anti-IL-10 receptor (*IL-10R*) or control antibody, as indicated. **B**, Dissemination of pneumococci at 24 and 48 hours after infection after treatment with anti-IL-10 receptor antibody or control antibody, as indicated. Ab, Antibody. * $P < .05$, ** $P < .01$, and *** $P < .001$.

murine MOPC315.BM plasmacytoma cells used in this study, we tested whether IL-10 suppresses neutrophil function in experimental plasmacytoma *in vivo*. For this purpose, MOPC315.BM cells were injected into syngeneic BALB/c mice, resulting in a disease that closely resembles MM.^{43,50}

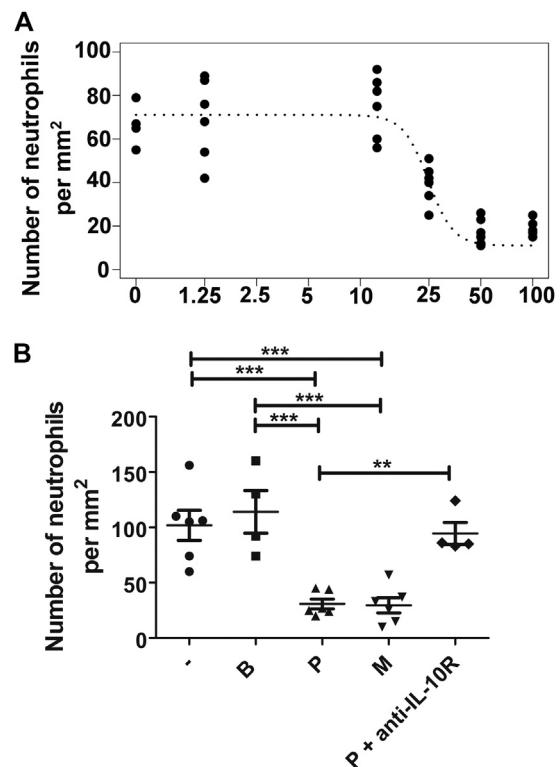


FIG 6. IL-10 can directly suppress neutrophil migration toward C5a. **A**, C5a-mediated neutrophil migration after incubation with various IL-10 concentrations. The dotted line corresponds to a logistic regression curve. Overall dose dependency: $P < .001$ (Jonckheere-Terpstra test). **B**, Migration of neutrophils cultured alone (–) or together with MOPC315.BM cells (M), B cells (B), or plasmablasts (P) with or without anti-IL-10 receptor (*IL-10R*) antibodies, as indicated. ** $P < .01$ and *** $P < .001$.

Mice were distributed into 2 groups showing similar levels of myeloma-specific serum protein and were subsequently injected with either anti-IL-10 receptor or control antibody 1 day before the final analysis. C5a-induced neutrophil influx into the peritoneum was completely abolished in myeloma-bearing mice compared with that seen in control mice that did not receive myeloma cells. Importantly, myeloma-mediated suppression of neutrophil migration was completely restored by IL-10 receptor blockade (Fig 5, A). Interestingly, mice with myeloma treated with anti-IL-10 receptor antibody showed increased neutrophil accumulation compared with mice without myeloma. Moreover, IL-10 blockade also increased C5a-mediated neutrophil accumulation in the peritoneum in mice without myeloma (see Fig E5 in this article's Online Repository at www.jacionline.org). This suggests that IL-10 downmodulates neutrophil migration already under physiologic conditions. However, this effect is much more pronounced during plasmacytosis.

Neutrophils are important for controlling bacterial infections (eg, *S pneumoniae*), which can cause uncontrollable and lethal pneumonia in patients with myeloma. Therefore we assessed whether plasmacytosis-associated IL-10 contributes to increased susceptibility to bacterial pneumonia in the MOPC315.BM myeloma model. Similar to immunocompetent human subjects, otherwise healthy BALB/c mice were relatively resistant to *S pneumoniae*-induced pneumonia. Accordingly, as measured by using bioluminescence,⁴⁴ mice 24 hours after infection without myeloma did not show any visible bacterial load in the

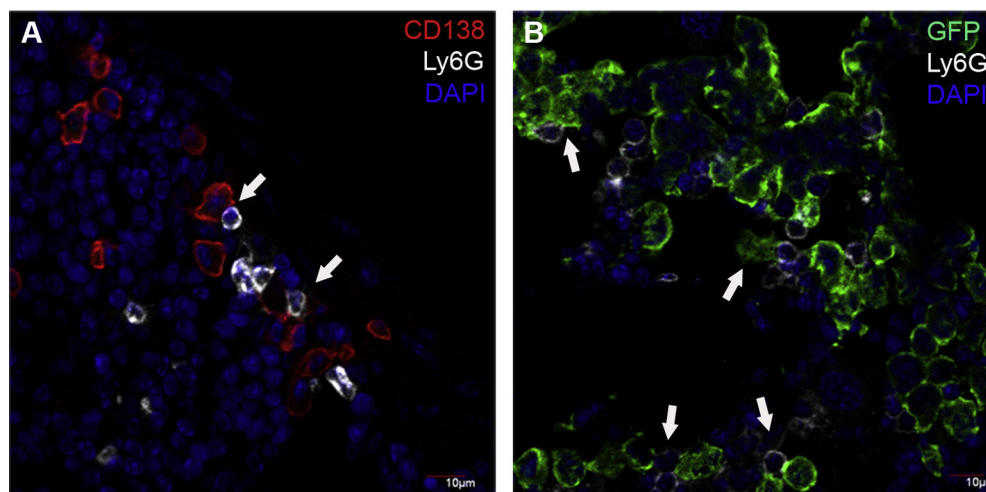


FIG 7. Histology: neutrophils and plasma cells are found in close proximity *in vivo*. **A**, Murine spleens from GMD-treated mice stained for plasma cells (CD138, red), neutrophils (Ly6G, white), and 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI; blue). **B**, Femurs of mice bearing MOPC315.BM GFP⁺ MOPC315.BM myeloma cells stained for neutrophils (Ly6G, white) and DAPI (blue). MOPC315.BM cells were identified by using GFP (green).

airways or lungs (0/12). Two days after infection, these mice still showed little visible pneumonia (1/12; Fig 5, B), suggesting that the moderate inhibition of IL-10-mediated neutrophil migration that was observed already under physiologic conditions does not result in susceptibility to bacterial infection. In contrast, already at 24 hours, mice with myeloma showed an increased incidence of bacterial infection in the airways (3/12), and one of them had some bacteria in the lungs. At 48 hours, airway infections in these 3 mice progressed to severe pneumonia, and in addition, 2 further mice of this group also had pneumonia at this time. These results suggest that mice with MOPC315.BM myeloma exhibit an increased susceptibility to bacterial pneumonia similar to that observed in patients with myeloma. However, injection of an IL-10 receptor-blocking antibody at the time of infection reduced the incidence of pneumonia back to the level seen in the group of mice without myeloma (1/12). Of note, both groups that did not receive IL-10 receptor blocking antibodies received control antibodies instead to control for unspecific effects. Hence increased IL-10 expression significantly contributes to faster progression of bacterial infection in the airways and higher incidence of bacterial load in the lungs as observed in the MOPC315.BM myeloma model. Together, these results indicate that plasmacytosis-associated overexpression of IL-10 can contribute considerably to neutrophil dysfunction and increased susceptibility to infection.

High IL-10 concentrations directly suppress neutrophil migration toward C5a

In the experiments described above, neutrophil migration was directly induced by injection of the neutrophil attractant C5a, excluding indirect effects of IL-10 through reduction of inflammatory chemokine levels. This finding suggests that plasmacytosis-associated IL-10 directly suppresses C5a-mediated neutrophil migration. To investigate this further, we determined the effect of IL-10 on C5a-mediated neutrophil migration *in vitro*. Although IL-10 concentrations of up to 12 ng/mL showed no effect, a concentration-dependent inhibition

of neutrophil migration was observed at higher concentrations (Fig 6, A). Also, neutrophil migration was inhibited after 45 minutes of coculture of neutrophils with either purified plasma cells or murine MOPC315.BM plasmacytoma cells. This effect was reversed by anti-IL-10 receptor antibody treatment (Fig 6, B). These results indicate that IL-10 concentrations in the vicinity of plasma cells reach levels sufficient to inhibit neutrophil migration. Despite this, IL-10 concentrations in plasma cell culture supernatants were less than 0.5 ng/mL, which is insufficient to inhibit neutrophil migration to C5a. Of note, sera from patients with myeloma and sera from mice inoculated with MOPC315.BM plasmacytoma cells also contained concentrations of IL-10 much less than those required to inhibit neutrophil migration (data not shown).

Together, these data might suggest that plasmacytosis-associated inhibition of neutrophil migration *in vivo* takes place in tissues but not in the circulation, most likely in the vicinity of IL-10-secreting cells. Bone marrow and splenic red pulp are important reservoirs for both plasma cells and neutrophils.^{3,33} Histologic observations confirmed that GMD-induced plasma cells, as well as MOPC315.BM murine plasmacytoma cells, often colocalize with neutrophils (Fig 7), as has been described for other experimental systems.^{51,52} These data support a model in which IL-10-producing cells must be close to neutrophils to block complement-mediated neutrophil activation.

IL-10 expressed during polyclonal plasmacytosis suppresses neutrophil-dependent autoimmune skin blistering disease

A recent study showed that plasma cell-derived IL-10 can block T cell-mediated autoimmune inflammation.²⁸ The data presented here led us to speculate that a massive plasma cell response might be able to suppress neutrophil-dependent inflammation through IL-10. C5a generated as a result of IC-mediated complement activation is a major neutrophil chemoattractant and is crucial for the induction of inflammation in many autoantibody-driven diseases, including epidermolysis

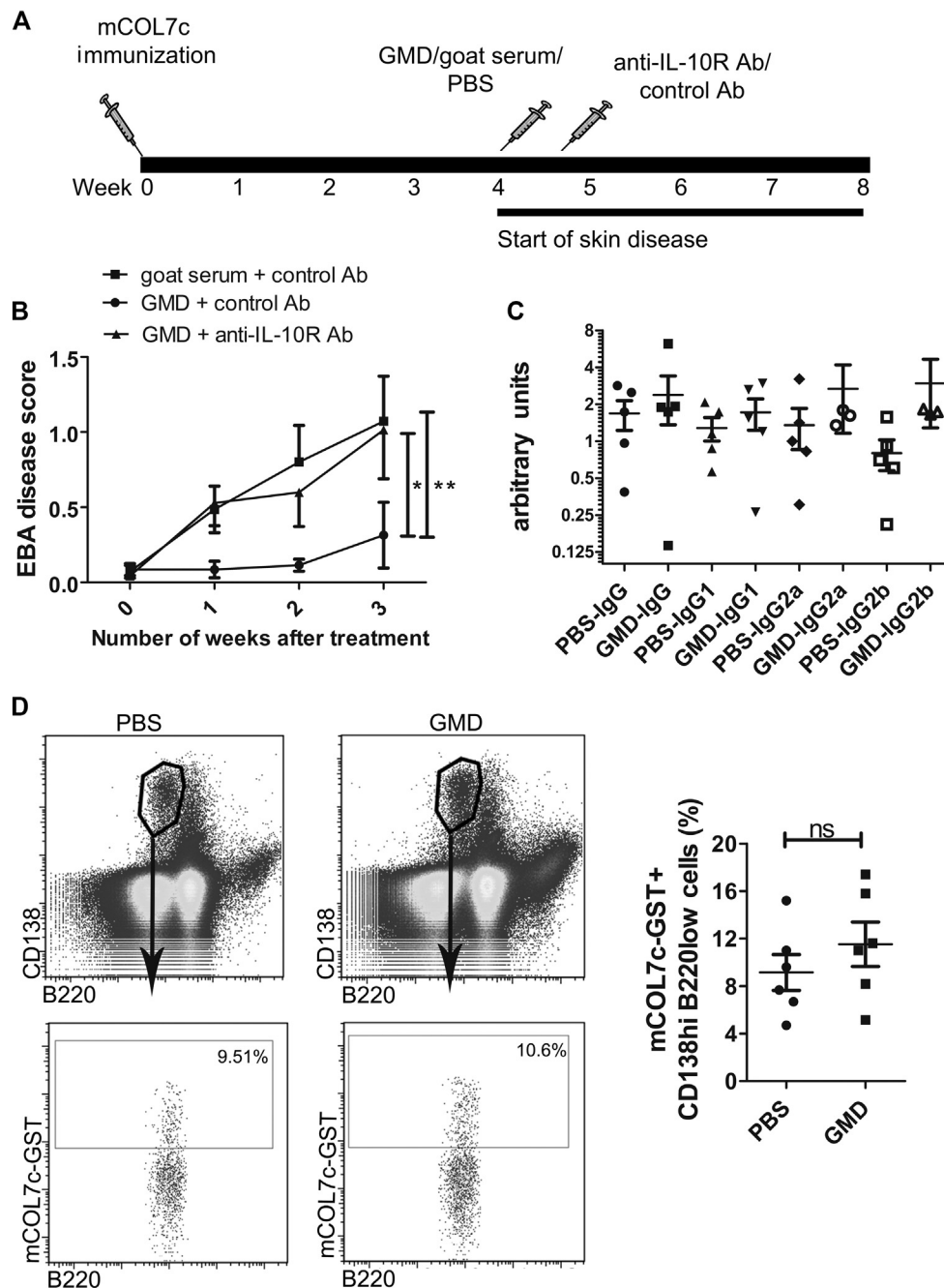


FIG 8. Plasmacytosis-associated IL-10 blocks autoimmune skin inflammation. Epidermolysis bullosa acquisita was induced by collagen VII-GST immunization. At the start of inflammation 4 to 5 weeks later, mice were treated with GMD, anti-IL-10 receptor (*IL-10R*), or control antibody. **A**, Experimental scheme. **B**, Disease score by weeks (ANOVA). **C**, Serum autoantibodies 3 weeks after GMD treatment. **D**, Collagen VII-GST-specific plasma cells in lymph nodes. Ab, Antibody; ns, not significant. * $P < .05$ and ** $P < .01$.

bullosa acquisita, an autoimmune blistering disease of the skin mediated by collagen type VII-specific autoantibodies.⁴² Neutrophils are key players during the effector phase of this disease and are required to induce skin inflammation.³⁸ Experimental epidermolysis bullosa acquisita does not involve plasmacytosis but is driven by an autoreactive plasma cell response restricted to the lymph nodes.⁴² Therefore this model allowed us to test the anti-inflammatory potential of experimentally induced plasmacytosis on antibody-induced and neutrophil-mediated inflammation.

Disease was initiated by a single injection of the autoantigen collagen VII fused to glutathione-S-transferase (GST; collagen VII-GST). The type VII collagen-specific autoreactive plasma cell response reaches its peak level 2 weeks after immunization, but skin inflammation does not start before week 4 to 5 after immunization.^{42,53} After the start of skin inflammation, plasmacytosis was induced by injection of GMD (Fig 8, A). GMD-treated mice were protected from skin inflammation and neutrophil infiltration for at least another 3 weeks. Suppression

of epidermolysis bullosa acquisita skin inflammation was abrogated by means of coinjection of a neutralizing IL-10 receptor antibody (Fig 8, B). Despite its anti-inflammatory effect, GMD treatment neither reduced the numbers of autoreactive collagen VII-GST-specific plasma cells nor type VII collagen-specific autoantibodies that trigger epidermolysis bullosa acquisita skin inflammation (Fig 8, C and D). Hence plasmacytosis-associated IL-10 is sufficient to prevent the development of an autoantibody-initiated and neutrophil-mediated inflammation.

DISCUSSION

Recent studies identified plasma cells as a relevant source of IL-10 that can control T cell-mediated inflammation.^{27,28} The present study extends these findings and demonstrates that polyclonal and neoplastic plasmacytosis-associated IL-10 mediates suppression of effective neutrophil function. Even moderate GMD-induced polyclonal plasmacytosis was sufficient to block neutrophil-mediated inflammation and pathophysiology in an autoimmune setting, whereas neutrophil migration was completely blocked in the MOPC315.BM myeloma model, in which plasma cells persist longer and in greater numbers than in GMD-immunized mice. Moreover, in accordance with the complete block of IL-10-mediated neutrophil migration, myeloma-associated IL-10 mediated increased susceptibility to bacterial pneumonia.

In contrast to the effect of IL-10 on migration to other chemoattractants, its effect on neutrophil migration to C5a was not widely studied. Vicioso et al⁵⁴ found that IL-10 concentrations that block neutrophil migration to IL-8 are not sufficient to block migration to C5a and suggest that IL-10 is generally a modest inhibitor of neutrophil chemotaxis. On the basis of their *in vitro* studies, these authors conclude that the accumulation of phagocytes at infection sites would not be significantly affected by IL-10. Indeed, the present study suggests that relatively high IL-10 concentrations are required to block neutrophil migration to C5a. Accordingly, the moderate effects of IL-10 on neutrophil migration observed under physiologic conditions did not result in susceptibility to bacterial pneumonia. However, increased IL-10 production associated with plasmacytosis led to a considerable dysfunction of neutrophil migration and increased susceptibility to bacterial infection.

We propose that the IL-10-dependent mechanism identified here could be relevant under the following conditions: it can (1) limit antibody/IC-mediated inflammation in autoimmune and infectious diseases that are associated with plasmacytosis, such as autoimmune myelofibrosis, autoimmune hepatitis, SLE,¹⁰⁻¹³ and malaria and schistosomiasis⁵⁵⁻⁵⁷ and (2) contribute to the immunodeficiency of patients with MM.

IC-mediated inflammation frequently occurs in patients with severe chronic infections⁵⁵⁻⁵⁹ as a result of increasing numbers of antibody-secreting plasma cells. In this setting the anti-inflammatory properties of plasmacytosis-associated IL-10 might limit IC/neutrophil-mediated inflammation without affecting protective functions of antibodies, such as microbe immobilization, toxin neutralization, and induction of complement-mediated pathogen opsonization and lysis.

Among the plasmacytosis-associated autoimmune conditions, SLE resembles the best-known disease. Autoantibodies are often present in sera of patients with SLE years before the clinical onset

of disease.⁶⁰ The anti-inflammatory pathway induced by plasmacytosis-associated IL-10 might contribute to delayed onset of lupus immunopathology. Support for this idea comes from Lyn-deficient mice, which exhibit B cell-intrinsic hyperreactivity that leads to uncontrolled plasma cell formation, autoantibody production, and development of a lupus-like disease. In this model B lineage-derived IL-10 was shown to reduce lupus nephritis development,⁶¹ and among B lineage cells, CD138⁺ plasma cells were described as a major source of this cytokine.⁶¹ In contrast, B lineage-derived IL-10 does not inhibit autoimmunity in MRL.Fas(lpr) mice.⁶² However, in contrast to other models and patients with SLE,^{3,63} lupus in the MRL.Fas model is mainly driven by an uncontrolled T-cell response rather than by B-cell hyperreactivity.⁶⁴

Plasmacytosis-induced deficiency in neutrophil function seems also to increase the risk of infection. Indeed, deficiencies in neutrophil functions, including migration, have been reported in patients with diseases associated with plasmacytosis, such as MM and SLE.²² Of note, neutrophils are required to elicit an efficient immune defense against gram-positive bacteria, which are the most frequent cause of severe infections in patients with both diseases.^{17,18,22,23} The results presented in this study might encourage studies aimed at probing blockade of the IL-10 receptor signaling pathway as a novel strategy to improve immune dysfunction and reduce severe infections in patients with MM and other diseases associated with plasmacytosis.

Activated B cells and plasma cells might also modulate inflammation through mechanisms other than IL-10. There is increasing evidence that B cells/plasma cells modulate CD4 T-cell responses, including T follicular helper cell responses, as well as inflammation, through their capacity to present antigen through MHC class II and to produce multiple cytokines, such as IL-6, IL-17, and IL-35, among others, in addition to IL-10.^{25-27,65-67} Hence the GMD-activated B cells and MOPC315.BM myeloma plasma cells used in this study might influence immune responses and inflammatory processes in multiple ways. Nevertheless, our data indicate that IL-10 production is a key immunosuppressive factor and that blockade of the IL-10 signaling pathway might be useful to prevent plasmacytosis-associated susceptibility to infections.

The data presented here describe a novel IL-10-dependent mechanism that inhibits neutrophil migration and shows that this is relevant under conditions of plasmacytosis. IL-10 produced by other cell types during immune responses or even under homeostatic conditions might also control neutrophil function. The data presented here suggest that IL-10 might need to be present in the vicinity of neutrophils rather than acting systemically to suppress neutrophil migration. Hence place and time of IL-10 production might determine its effect on neutrophil function. However, this issue remains to be elucidated.

In addition to the direct inhibitory effect of IL-10 on neutrophil migration, IL-10 has many other anti-inflammatory effects, such as blockade of neutrophil activation and degranulation and inhibition of inflammatory chemokines and T cells,^{68,69} suggesting that plasmacytosis-associated IL-10 can suppress inflammation and protective immunity at multiple levels. Nevertheless, neutrophil infiltration is a prerequisite for local tissue destruction by these important innate effector cells.³³ Hence the direct inhibitory effect of IL-10 on neutrophil

migration toward C5a could be a key mechanism for limiting inflammation but also a mechanism that impairs innate immune defense.

In conclusion, this study provides evidence that “normal” and neoplastic plasma cell responses integrate an immunosuppressive IL-10 response, which can serve as a counterbalancing mechanism to limit harmful antibody/IC-mediated inflammation in case of a strong plasma cell response. This protection comes at the price of the severe immunodeficiency that often accompanies diseases associated with plasmacytosis.

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Clinical implications: Our results might encourage studies aimed at probing blockade of the IL-10 pathway as a novel strategy to reduce severe infections in patients with plasmacytosis-associated diseases.

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METHODS

Flow cytometry

After blockade of Fc receptors with anti-CD16/CD32, single-cell suspensions were incubated for 15 minutes with conjugated antibodies against cell-surface markers. Samples were analyzed with an LSRII (BD Biosciences, San Jose, Calif). Data were analyzed with FlowJo software (TreeStar, Ashland, Ore). Antibodies used were fluorescein isothiocyanate-labeled anti-IL-10 (clone JES5-16E3, eBioscience, San Diego, Calif); phycoerythrin-labeled anti-CD138 (clone 281-2, BD Biosciences Pharmingen) and anti-Foxp3 (clone FJK-16s, eBioscience), Alexa Fluor 647-labeled anti-B220 (clone RA3.B2) and anti-GR1 (clone RB6-8C5, in-house production), APC-labeled anti-Ly6G (clone 1A8; BioLegend, Fell, Germany) and anti-CD19 (clone 1D3, eBioscience), and Alexa Fluor 405-labeled anti-CD4 (clone GK1.5, eBioscience) and anti-CD11b (clone M1/70.15.11, in-house production). Collagen VII (amino acids 757-967) was produced, as recently described.⁴¹ The protein was coupled to Alexa Fluor 700 and Alexa Fluor 488 (Life Technologies, Grand Island, NY), according to the manufacturer's protocols.

IL-10 ELISA and *in vivo* IL-10 capture assay

IL-10 levels in supernatants of the murine B cell/plasma cell cultures were measured with a mouse IL-10 ELISA DuoSet kit (R&D Systems, Minneapolis, Minn). IL-10 levels in patients' serum were quantified by using the highly sensitive Human IL-10 Quantikine HS ELISA Kit (R&D Systems). Low levels of IL-10 in serum were quantified by using an *in vivo* IL-10 capture assay. Mice were injected intraperitoneally with 10 μ g of biotinylated anti-mouse IL-10 mAb (free of endotoxin) in 200 μ L of 1% mouse serum in PBS. Mice were bled 24 hours later, and the complex formed between the antibody and the cytokine was detected from serum by means of ELISA. A 7-step 1:4 serial dilution of 100 ng/mL IL-10/anti-IL-10 antibody was performed on a 96-well microplate. Similar dilution was performed for the serum samples. Another 96-well high-binding microtiter plate was coated with 50 μ L of coating antibody (concentration, 2 μ g/mL in PBS) and incubated at 2°C to 8°C. The plate was washed 4 times with washing buffer. Twenty-five microliters of diluted serum samples and standard was added from the other 96-well plate to the coated plate in duplicates and incubated at room temperature for 30 minutes. The plate was washed 6 times as before, and 25 μ L of streptavidin-horseradish peroxidase (concentration, 50 ng/mL in dilution buffer) was added to each well. The plate was incubated at room temperature for 20 minutes. During this time, streptavidin-horseradish peroxidase binds to the biotinylated anti-IL-10 antibody. The plate was washed 10 times, and development of the signal and data obtained was analyzed by comparing OD values.

Chemotaxis assay

Bone marrow-derived cells were resuspended in chemotaxis medium (Gey's Balanced Salt Solution containing 2% BSA) at a density of 6×10^6 cells/mL. The chemoattractant C5a (10^{-8} mol/L) was diluted in chemotaxis medium, placed in the bottom wells of a Micro Boyden Chemotaxis Chamber (Neuro Probe, Gaithersburg, Md), and overlaid with a 3- μ m

polycarbonate membrane. Then 50 μ L of the cell suspension was placed in the top wells, and the chambers were incubated for 30 minutes at 37°C. Subsequently, membranes were removed, and cells on the bottom side of the membrane were stained with Diff-Quick. Numbers of migrated cells in 5 high-power fields were counted and calculated by using computer-assisted light microscopy.

Histology

Paraformaldehyde-fixed tissues were incubated for 3 days in increasing concentrations of 10%, 20%, and 30% sucrose; embedded in Tissue-Tek medium (Sakura Finetek, Torrance, Calif); and snap-frozen in liquid nitrogen. Tissue sections (8 μ m) were prepared with a microtome. Nonspecific binding was blocked by PBS/10% rat serum and anti-CD16/CD32 (clone 2.4G2). Staining with antibodies and secondary reagents was performed at room temperature for 30 to 60 minutes. Staining reagents were goat anti-GFP-fluorescein isothiocyanate (Rockland Immunochemicals Inc, Limerick, Pa), donkey anti-goat IgG-Alexa Fluor 488 (Molecular Probes, Eugene, Ore), APC-labeled anti-Ly6G (clone 1A8, BioLegend), and phycoerythrin-labeled anti-CD138 (clone 281-2, BD). Sections were analyzed by means of confocal microscopy with an Olympus IX81 microscope (Olympus, Center Valley, Pa).

Statistics

GraphPad Prism software (GraphPad Software, La Jolla, Calif) was used for statistical analysis. Experiments with 2 groups were analyzed by using an unpaired 2-sided Student *t* test. Comparisons involving multiple groups were analyzed in a 2-stage procedure by means of 1-way ANOVA. If the ANOVA indicated a significant difference between groups ($P < .05$), all groups were further compared pairwise by using the Tukey multiple comparison test. Nonparametric data were analyzed by using the Kruskal-Wallis test. For bar graphs, data are expressed as means $\pm$ SEMs, as indicated in the figure legends.

Study approval

Human blood samples were acquired from anonymized patients of the University Clinics of Ulm. Patients' consent forms were approved by the appropriate institutional review board of the University Clinics of Ulm.

The animal experiments conducted in this study were done in strict accordance with the German regulations of the Society for Laboratory Animal Science and the European Health Law of the Federation of Laboratory Animal Science Associations. Animal experiments performed at the Universities of Lübeck or Greifswald were approved by the appropriate local Committee on the Ethics of Animal Experiments of the state Schleswig-Holstein or local Committee on the Ethics of Animal Experiments of the state of Mecklenburg-Vorpommern, respectively.

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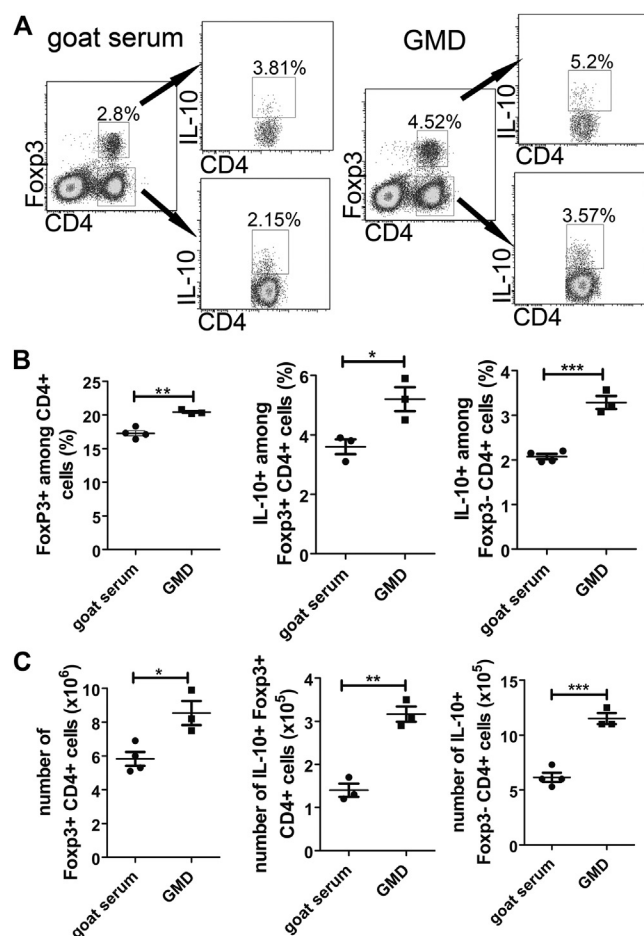


FIG E1. Polyclonal plasmacytosis augments IL-10 production in Foxp3⁺ and Foxp3⁻ CD4 T cells. Foxp3^{EGFP} mice were treated with GMD or goat serum. Seven days later, Foxp3⁺CD4⁺ cells, IL-10⁺Foxp3⁺CD4⁺ cells, and IL-10⁺Foxp3⁻CD4⁺ cells were analyzed in spleens. **A**, Representative flow cytometric data. **B**, Graphs representing frequencies of Foxp3⁺ Treg cells among CD4⁺ T cells (left), IL-10⁺ cells among Foxp3⁺CD4⁺ Treg cells (middle), and IL-10⁺ cells among Foxp3⁻CD4⁺ T cells (right). **C**, Graphs representing total numbers of Foxp3⁺CD4⁺ T cells (left), IL-10⁺Foxp3⁺CD4⁺ cells (middle), IL-10⁺Foxp3⁻CD4⁺ cells (right) in spleens. Representative data are shown for one of 2 experiments. * $P < .05$, ** $P < .01$, and *** $P < .001$, 2-tailed unpaired Student t test. Each dot represents data from an individual mouse. Data are expressed as means $\pm$ SEMs.

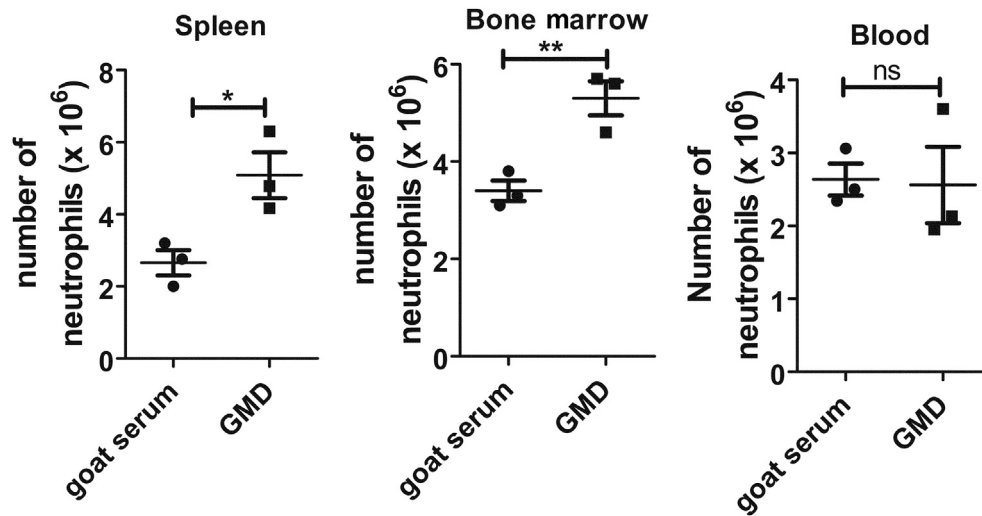


FIG E2. Polyclonal plasmacytosis augments neutrophil counts in spleen and bone marrow. C57BL/6 mice were treated with GMD or goat serum. Seven days later, neutrophils were analyzed in spleen and bone marrow by means of flow cytometry, and absolute numbers were calculated from frequencies and total cell counts per organ. Blood neutrophil counts were obtained with a Hemavet 950 FS cell counter (Drew Scientific, Miami Lakes, Fla). * $P < .05$ and ** $P < .01$, 2-tailed unpaired Student t test. *ns*, Not significant. Each dot represents data from an individual mouse. Data are expressed as means $\pm$ SEMs.

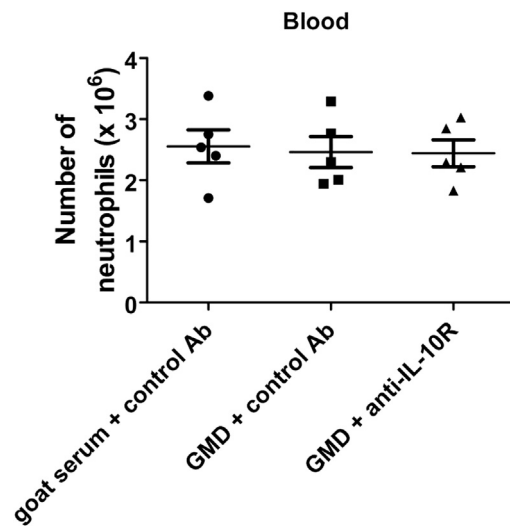


FIG E3. IL-10 receptor blockade does not alter neutrophil counts in blood. Mice received a single injection of GMD or goat serum (control). One week later, C5a was injected into the peritoneum, and neutrophil influx was analyzed 3 to 4 hours later. Anti-IL-10 receptor (*anti-IL-10R Ab*) or control antibody (*control Ab*) was injected 1 day before analysis. Neutrophil counts in blood were analyzed with a Hemavet (Drew Scientific). Each *dot* represents data from an individual mouse. *Error bars* indicate means $\pm$ SEMs.

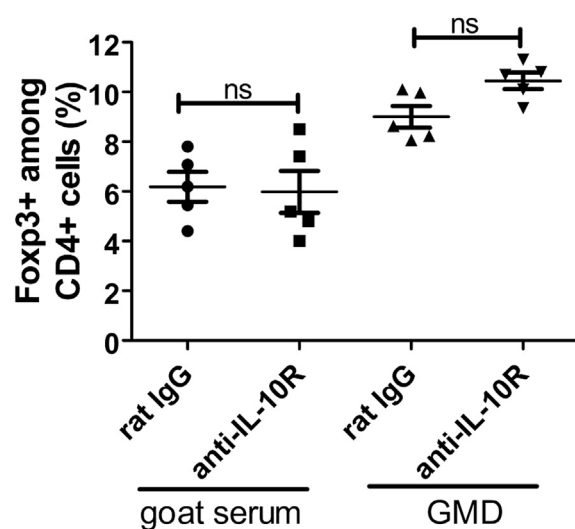


FIG E4. Short-term IL-10 receptor blockade does not reverse plasmacytosis-induced Treg cell expansion. Mice received a single injection of GMD or goat serum (control). Anti-IL-10 receptor (*anti-IL-10R Ab*) or control antibody (*control Ab*) was injected 1 day before analysis. Cells from spleens were stained for Foxp3 and CD4 and analyzed by means of flow cytometry, as shown in Fig 4. Treg cell frequencies among CD4 T cells (ANOVA). Each dot represents data from an individual mouse. Error bars indicate means $\pm$ SEMs.

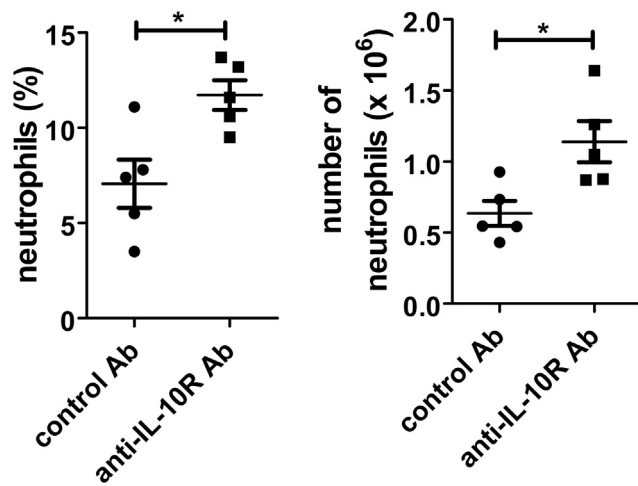


FIG E5. IL-10 modulates C5a-mediated neutrophil accumulation in the peritoneum in the absence of plasmacytosis. BALB/c mice received single injections of anti-IL-10 receptor (*anti-IL-10R Ab*) or control antibody (*control Ab*), as indicated. One day later, C5a was injected into the peritoneum, and neutrophil influx was analyzed 3 to 4 hours after injection. Frequencies (*left*) and absolute neutrophil counts (*right*) are shown. Data are expressed as means $\pm$ SEMs. * $P < .05$, 2-tailed unpaired Student t test. Each *dot* represents data from an individual mouse. Data are expressed as means $\pm$ SEMs.