

# Granulocyte macrophage colony-stimulating factor and the intestinal innate immune cell homeostasis in Crohn's disease

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**Däbritz J.** Granulocyte macrophage colony-stimulating factor and the intestinal innate immune cell homeostasis in Crohn's disease. *Am J Physiol Gastrointest Liver Physiol* 306: G455–G465, 2014. First published February 6, 2014; doi:10.1152/ajpgi.00409.2013.—Current literature consolidates the view of Crohn's disease (CD) as a form of immunodeficiency highlighting dysregulation of intestinal innate immunity in the pathogenesis of CD. Intestinal macrophages derived from blood monocytes play a key role in sustaining the innate immune homeostasis in the intestine, suggesting that the monocyte/macrophage compartment might be an attractive therapeutic target for the management of CD. Granulocyte macrophage colony-stimulating factor (GM-CSF) is a hematopoietic growth factor that also promotes myeloid cell activation, proliferation, and differentiation. GM-CSF has a protective effect in human CD and mouse models of colitis. However, the role of GM-CSF in immune and inflammatory reactions in the intestine is not well defined. Beneficial effects exerted by GM-CSF during intestinal inflammation could relate to modulation of the mucosal barrier function in the intestine, including epithelial cell proliferation, survival, restitution, and immunomodulatory actions. The aim of this review is to summarize potential mechanistic roles of GM-CSF in intestinal innate immune cell homeostasis and to highlight its central role in maintenance of the intestinal immune barrier in the context of immunodeficiency in CD.

pathogenesis; immune response; immunodeficiency; epithelial barrier; cytokine autoantibodies; myeloid-derived suppressor cells; dendritic cells

CROHN'S DISEASE (CD) is a relapsing systemic inflammatory bowel disease (IBD), affecting the gastrointestinal tract from mouth to perianal area. CD has a rising incidence and prevalence with a highest reported annual incidence of 5.0 to 20.2 per 100,000 person-years and a highest prevalence of 319 to 322 per 100,000 persons in different regions of the world (5, 84). The disease is characterized by a transmural inflammation and by skip lesions. Additionally, prolonged diarrhea with abdominal pain, with or without gross bleeding, fatigue, and weight loss are the hallmarks of CD. The typical course in patients with CD involving the small and/or large intestine is one of intermittent exacerbations of symptoms followed by periods of remission. The transmural inflammatory nature of CD often leads to fibrosis, obstructive clinical presentations, (micro)perforations, and fistulas. Moreover, extraintestinal manifestations of CD include arthritis, eye and skin disorders, and biliary tract involvement. Current treatment options for CD include enteral nutrition, antibiotics, immunosuppressants

(e.g., steroids), immunomodulators, biological response modifiers [e.g., anti-tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) agents], and surgery.

Although the pathogenesis of CD is not well understood, studies of humans and animal models suggest that genetically determined factors contribute to IBD susceptibility. Genome-wide association studies (GWAS) revealed that innate and adaptive immune responses play key roles in the pathogenesis of CD (69). The following two basic themes emerge: 1) dysregulation of the innate and adaptive immune system directed against luminal bacteria or their products; and 2) inappropriate immune responses against organisms in the intestine that normally do not elicit a response, possibly due to intrinsic alterations in mucosal barrier function (78).

Phagocytic cells within the lamina propria [e.g., macrophages, dendritic cells (DCs), and neutrophils] and epithelial barrier represent the central components of the intestinal innate immune system. Intestinal epithelial cells (IECs) provide an anatomical barrier between the intestinal microbiota and the lamina propria and regulate intestinal permeability. In addition, IECs secrete antimicrobial peptides (e.g.,  $\alpha$ -defensins produced by Paneth cells) in response to commensal-derived signals and regulate the commensal-host cell cross talk via the

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recruitment, maintenance, and regulation of effector functions of various intestinal immune cells (39, 46, 60). Antigens reaching the lamina propria activate innate immune cells followed by a response of T lymphocytes, which represent the key cell population of adaptive immunity. In this regard, the innate and the adaptive immunity arms are tightly cross-regulated serving to uphold intestinal homeostasis (66, 107).

Macrophages represent the most abundant mononuclear immune cell population of the intestine and derive from blood monocytes. They play an important role in intestinal antigen presentation to other immune cells in the lamina propria and in sustaining immune homeostasis in the intestine (33, 85). Functional properties of intestinal macrophages are controlled by a multitude of factors (e.g., cytokines) produced by a range of immune and nonimmune cells (20). Importantly, dysregulation of host-microbe interactions can lead to increased intestinal inflammation and disease susceptibility (1). In this regard, altered innate immunity has been strongly implicated as playing a causative role in IBD. This includes a diminished responsiveness (loss of function) but also hyperresponsiveness (gain of function) of innate immune cells upon recognition of microbial ligands (118). In addition, regulatory myeloid cells prevent intestinal inflammation by inhibiting T cell proliferation (65). Intestinal homeostasis is maintained in part by the actions of resident noninflammatory CX<sub>3</sub>CR1<sup>+</sup> macrophages (derived from highly plastic Ly6C<sup>+</sup> blood monocytes) that have enhanced phagocytic and bactericidal activity and decreased production of proinflammatory cytokines. TNF- $\alpha$ - and interleukin (IL)-1 $\beta$ -secreting Ly6C<sup>hi</sup> monocytes are recruited in the initial phase of microbial challenge or tissue injury, whereas reparative IL-10-, transforming growth factor- $\beta$ -, and vascular endothelial growth factor-secreting Ly6C<sup>lo</sup> monocytes are mobilized during the resolution phase of inflammation (69). CX<sub>3</sub>CR1<sup>int</sup> cells are Ly6C<sup>+</sup> monocyte-derived, like the resident CX<sub>3</sub>CR1<sup>+</sup> macrophages, and express proinflammatory cytokines. These CX<sub>3</sub>CR1<sup>int</sup> cells are critical proinflammatory effector monocytes that drive inflammation. Ly6C<sup>hi</sup> monocytes can differentiate into CX<sub>3</sub>CR1<sup>int</sup>Ly6C<sup>lo</sup>MHCII<sup>+</sup>CD11c<sup>+</sup> cells, which differ from the acute Ly6C<sup>hi</sup> effector monocytes and downregulate expression of proinflammatory effectors and pathogen sensors and harbor some T cell stimulation potential (33). Collectively, these findings and the pathophysiological role of monocytes in CD (128) suggest that the monocyte/macrophage compartment might be an attractive target for the management of CD (91, 94, 122, 129). Dual function of monocytes in CD pathogenesis include on one hand the impaired monocyte function initiating disease and on the other the overactivation of monocytes and adaptive immunity maintaining the disease. Importantly, these defective innate immune mechanisms in CD seem to play a role in the (inflamed) intestinal mucosa rather than in peripheral blood (99).

Emerging evidence suggests a central role for granulocyte macrophage colony-stimulating factor (GM-CSF) in the pathogenesis of CD. Beneficial effects of GM-CSF treatment in patients with CD might be explained by specific monocyte activation that combines innate immune activation in the gut and a simultaneous regulatory function serving to limit adaptive immune activation and excessive intestinal inflammation (19, 123, 124). This review focuses on the potential mechanistic role of GM-CSF in intestinal innate immune cell homeostasis in CD in humans and experimental colitis in murine

models (Fig. 1). The review also highlights the utility of neutralizing endogenous serum GM-CSF auto-antibodies (Ab) as biomarkers for disease activity in CD and for the early prediction of disease relapses.

*CD as an immunodeficiency.* The concept of CD arising in the setting of immunodeficiency was suggested several decades ago in association with known genetic syndromes involving neutrophil dysfunction (71). However, the hypothesis was discarded principally because of emerging immunosuppressive therapies and the predominance of theories focusing on CD as a proinflammatory disorder due to abnormal T cell responses. Recent developments, however, have further consolidated the view of CD as a form of immunodeficiency and continue to highlight underlying defects in innate immunity in CD (12, 35, 44, 70, 71, 79, 81, 82, 92, 103, 127), which will not be covered in depth in this review. Briefly, it has been suggested that the development of CD is caused by a mucosal innate immunodeficiency characterized by a variety of genetic defects [e.g., gene mutations of the pattern recognition receptor protein nucleotide-binding oligomerization domain-containing protein 2 (NOD2)] (64, 69) as well as a dysfunction of granulocytes, macrophages, and IECs (70, 127).

The concept of CD as an immunodeficiency is based on three temporally distinct stages (103). The first is characterized by the penetration of foreign material of the fecal stream into the bowel wall. The translocation of luminal content into underlying bowel tissues is facilitated by environmental factors [e.g., viral or bacterial infection, nonsteroidal anti-inflammatory drugs (NSAID), trauma, etc.] or an increased intestinal permeability due to inherent defects in the mucosal barrier of patients with CD (79). In the second stage, a defective acute inflammatory response results in impaired clearance of the foreign material from the bowel wall. The underlying innate immunodeficiency is characterized by relatively weak inflammatory responses due to delayed neutrophil recruitment and function (71, 101) and impaired secretion of proinflammatory cytokines by macrophages from CD patients (80, 99, 109), caused by premature trafficking of protein to lysosomes, compared with robust bacterial killing under normal conditions in healthy controls (79, 109). This is supported by the observation that a number of congenital disorders of neutrophil function (disorders of neutrophil production or migration, disorders of lysosome trafficking or respiratory burst) result both in immunodeficiency and noninfectious bowel inflammation that is indistinguishable from CD (44, 71, 92). However, data on this subject are rather controversial with respect to altered neutrophil function vs. a defective neutrophil recruitment in CD. Congenital phagocyte disorders are caused by arrested myelopoiesis (neutropenias), reduced leucocyte migration into tissues (leukocyte adhesion deficiency-1), impaired delivery of digestive enzymes (disorders of lysosome trafficking), or defective respiratory burst or lack of substrate for respiratory burst (disorders of respiratory burst). This concept of CD pathogenesis is consistent with data from animal models of IBD and from GWAS that highlighted genes related to innate immune function, phagocyte biology, and cytokine release (35, 69, 79, 81, 82). Finally, the impaired neutrophil influx and clearance of foreign material leads to the third stage, which is characterized by a compensatory adaptive immune response and chronic granulomatous inflammation. In this model, T cell-mediated granuloma formation and lymphocytic infiltra-

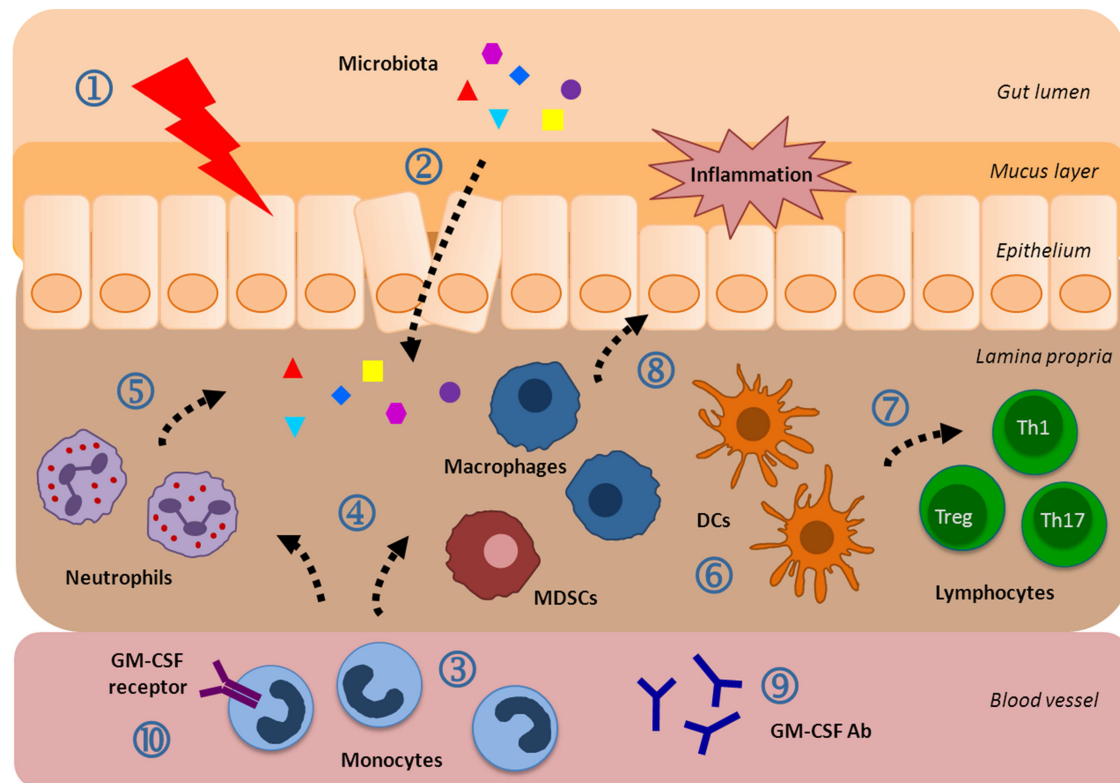


Fig. 1. Potential roles of granulocyte macrophage colony-stimulating factor (GM-CSF) in innate and adaptive immune responses in the intestine of Crohn's disease (CD) patients. 1, Genetically defined factors contribute to CD susceptibility due to an underlying dysregulation of mucosal innate immunity. 2, Inherent defects in the mucosal barrier of CD patients increase the intestinal permeability followed by translocation of luminal content (bacteria, viruses, pathogens, foreign material) into underlying bowel tissue. GM-CSF improves the gut barrier function and resistance to bacterial translocation. 3, Increased levels of GM-CSF during inflammation lead to the mobilization of monocytes from the bone marrow to the blood and prime monocytes for an increased antimicrobial response. 4, GM-CSF promotes recruitment, survival, activation, and differentiation of macrophages and neutrophils as well as the expansion of myeloid-derived suppressor cells (MDSCs). Macrophages are the principal GM-CSF-responsive cell type, and intestinal macrophages derived from blood monocytes play a key role in sustaining the innate immune homeostasis in the intestine. 5, GM-CSF accelerates the accumulation of neutrophils, which have important antimicrobial functions, including phagocytosis, oxidative burst, and bacterial killing (bacterial clearance). 6, GM-CSF also mediates the recruitment, maturation, and retention of CD11b<sup>+</sup>CD103<sup>+</sup> dendritic cells (DCs) during microbial infection. 7, DCs are characterized by their potential to present antigens and to initiate/polarize adaptive immune responses. GM-CSF can influence the interactions between DCs and T cells and in this manner determine a pro- vs. anti-inflammatory adaptive immune response via T helper 1 (Th1) and T helper 17 (Th17) cell responses or the induction of regulatory T cells (Treg). 8, GM-CSF promotes tissue repair via increased intestinal epithelial cell proliferation/survival (mucosal healing) and via bone marrow-derived cells (e.g., intestinal macrophages) as effectors of wound healing. 9, GM-CSF bioactivity varies inversely with increasing serum levels of endogenous neutralizing GM-CSF auto-antibodies (Ab). Increased GM-CSF Ab levels are associated with alterations of epithelial barrier function, bowel permeability, bacterial translocation, neutrophil antimicrobial functions, and epithelial cell survival and proliferation. High GM-CSF Ab levels are correlated with disease activity, aggressive disease behavior, and a higher risk of disease relapse in CD patients. 10, Decreased expression of the GM-CSF receptor  $\alpha$ -subunit (CD116) and accordingly reduced GM-CSF signaling in circulating monocytes and granulocytes of CD patients affect mucosal integrity, bacterial killing, and neutrophil migration, proliferation, and survival.

tion occur days to weeks after the translocation of foreign luminal content and represent compensatory mechanisms for the initial failure of innate immune mechanisms. Secondary macrophage activation results in a second wave of proinflammatory cytokine production, lymphocyte recruitment, and polarization.

Challenges to the immunodeficiency hypothesis of CD and apparent contradictions with clinical experience relate to the absence of a susceptibility to severe or recurrent infection and the efficacy of immunosuppressive medications in patients with CD (81). The absence of an obvious phenotype of recurrent infection can be explained by the fact that the abnormality in CD is partial compared with congenital neutrophil disorders. Therefore, in CD patients, innate immune responses may be sufficient to suppress most exogenous infections but becomes overwhelmed by the enormous microbial load in the gastrointestinal tract (12).

Nonetheless, a subset of CD patients reportedly shows increased susceptibility to gastroenteritis, appendicitis, post-operative infections, or urinary tract infections (12, 81). A systemic deficiency of macrophages is also consistent with the occurrence of extraintestinal manifestations in CD. Furthermore, the efficacy of immunosuppressants in CD is mainly limited to the treatment of established inflammatory reactions (secondary/chronic phase of the disease) and less potent in maintaining remission (early/acute phase of the disease) (21, 81). On the contrary, there are positive data from the use of GM-CSF as an immunostimulant in patients with CD (discussed later). Furthermore, another example of organ-specific manifestation of an inherited, systemic macrophage deficiency is provided by patients with pulmonary alveolar proteinosis (PAP), where impaired surfactant destruction and host defense functions by alveolar macrophages are caused by a loss of GM-CSF signaling (11).



*GM-CSF biology and its role in the human gastrointestinal tract.* GM-CSF is a hematopoietic growth factor that also promotes survival and activation of macrophages, neutrophils, and eosinophils. GM-CSF also mediates the maturation of DCs and is crucial for the differentiation of alveolar macrophages and invariant natural killer T cells (53). GM-CSF release is characteristic of the host response to infection or injury, broadly showing dose-dependent, proinflammatory effects by enhancing myeloid cell survival, proliferation, and differentiation (49). Effects of GM-CSF on myeloid cell function are of pivotal importance in pathogen-exposed tissues, including the lung, intestinal lamina propria, and skin. Increased levels of GM-CSF during inflammation lead to the mobilization of monocytes and other myeloid populations from the bone marrow to the blood and prime monocytes for an increased *in vitro* response to other stimuli (e.g., lipopolysaccharides) (19, 52). GM-CSF is produced by a range of cell types, including fibroblasts, smooth muscle cells, endothelial cells, stromal cells, macrophages, and osteoblasts (52). Macrophages are the principal GM-CSF-responsive cell type; however, other myeloid lineages and nonhematopoietic lineages (e.g., keratinocytes, endothelial cells, epithelial cells, smooth muscle cells) express the GM-CSF receptor (52). The GM-CSF receptor is a heterodimer composed of a specific ligand-binding ( $\alpha$ ) subunit and a common signal transduction ( $\beta$ ) subunit. Activation of the GM-CSF receptor stimulates at least three different signaling pathways: janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway, the mitogen-activated protein kinase (MAPK) pathway, and the phosphoinositide 3-kinase (PI3K) pathway (53, 57).

The biological functions of GM-CSF in the human intestine are relatively unexplored, and, until recently, little attention had been given to the *in vivo* regulatory role of GM-CSF in immune and inflammatory reactions in the intestine (31). Several studies have indicated deficiencies in certain growth factors in patients with IBD (74) (and references cited therein), and one possible explanation for the difficulty in treating CD is that immunosuppressive therapies fail to address impaired epithelial repair as another key pathophysiological aspect of CD. Recent mechanistic studies showed the importance of GM-CSF in the growth and differentiation of hematopoietic precursors and the maintenance of the innate immune barrier of the intestine (25, 74). GM-CSF receptors are expressed on both myeloid and IECs throughout the gastrointestinal tract (90, 117). Therefore, GM-CSF may contribute to intestinal homeostasis by acting via differentiation of DCs and by maintaining the integrity of the intestinal epithelium (31). It has been reported that peripheral blood mononuclear cells of patients with CD show an impaired GM-CSF secretion via NOD2-dependent and -independent pathways and display an impaired NOD2-dependent downregulation of TNF- $\alpha$  secretion (8).

Several types of DCs form a further central part of the functional mucosal barrier of the intestine and play an important role in IBD pathogenesis (10). Recent advances have highlighted a fundamental role of DCs in intestinal innate immune homeostasis (113). DCs are critical for initiation of innate immune responses during microbial invasion and inflammation (65). Gut lamina propria DCs are divided into several subsets possessing different immune homeostatic functions. This includes enhancing or suppressing T cell responses, including T helper 1/T helper 17 polarization or induction of

forkhead box P3-positive (FoxP3<sup>+</sup>) regulatory T cells (Treg). Lamina propria-resident CD11b<sup>+</sup>CD103<sup>+</sup> DCs play an important role in the presentation of antigen captured from the gut lumen to T cells. The development of this subset of DCs requires GM-CSF (33). Thus, GM-CSF is involved in DC development and function, and GM-CSF is indeed often used to generate DC populations *in vitro* (52). It has been suggested that GM-CSF controls monocyte-derived DC differentiation *in vivo*, and it has been shown that the absence of GM-CSF receptor impairs the development of a specific lamina propria DC population (7). However, it has also been shown that, in contrast to the current understanding that GM-CSF mainly controls the development of inflammatory DCs *in vivo*, GM-CSF is a steady-state cytokine that controls the induction of cytotoxic CD8<sup>+</sup> T cell immunity to particulate antigens through the regulation of nonlymphoid tissue-resident DC survival and homeostasis. Thus, inflammatory DCs that accumulate in injured tissues develop independently of GM-CSF receptor signaling (48). In contrast, the GM-CSF-induced CD103<sup>+</sup> DCs are able to prime naïve T cells after migrating to the draining mesenteric lymph nodes (42). These CD103<sup>+</sup> DCs have many unique properties, including Treg polarization and gut-homing T cell imprinting, which make these cells attractive targets for modulating the intestinal immune response (100). Thus, the balance of lamina propria DC subsets is important for gut homeostasis. DCs can both restrain and originate intestinal inflammation by activating T cells and secreting cytokine mediators. Future research will further elucidate the role of GM-CSF for the development and function of mucosal CD103<sup>+</sup> DCs during intestinal inflammation *in vivo* (3, 7, 15, 106, 119) and identify other attractive targets for manipulating the intestinal immune response.

Myeloid-derived suppressor cells (MDSCs) are a heterogeneous group of immature cells that includes precursors of macrophages, granulocytes, DCs, and myeloid cells at earlier stages of differentiation. Mouse MDSCs are defined by their coexpression of Gr1 and Cd11b and their ability to suppress T cell responses via arginase 1 activity and production of reactive oxygen species and nitric oxide. MDSCs regulate immune responses and tissue repair in healthy individuals, expanding rapidly during inflammation, infection, and cancer. MDSC accumulation is regulated by several factors that are released by tumor cells, tumor stromal cells, activated T cells and macrophages, apoptotic tumor cells, bacterial and viral agents, and pathogen-infected cells. These factors (including GM-CSF) trigger several different signaling pathways in MDSCs that mainly involve the STAT family of transcription factors. Murine MDSCs consist of two main subsets: monocytic MDSCs, which have a Cd11b<sup>+</sup>Gr1<sup>mid</sup>Ly6C<sup>hi</sup>Ly6G<sup>-</sup>Cd49d<sup>+</sup> phenotype, and granulocytic (polymorphonuclear) MDSCs, which have a Cd11b<sup>+</sup>Gr1<sup>hi</sup>Ly6C<sup>low</sup>Ly6G<sup>+</sup>Cd49d<sup>-</sup> phenotype (37). Human cells do not express a marker homologous to mouse Gr1, and human MDSCs are most commonly defined as CD11b<sup>+</sup>CD33<sup>+</sup>HLA-DR<sup>neg/low</sup> cells that can be divided into granulocytic CD14<sup>-</sup> and monocytic CD14<sup>+</sup> subtypes (36, 47). In most tumor models, it is predominantly (70–80%) the granulocytic subset of MDSCs that expands, whereas only monocytic MDSCs can differentiate into mature DCs and macrophages *in vitro* (36). Until today, only limited data are available on human MDSCs in nontumor settings (47). However, MDSCs have been recently identified as a new immune

regulatory pathway in IBD (18, 51, 108, 111). Although the in vivo mechanisms behind the beneficial/suppressive effects of MDSCs during intestinal inflammation are not well defined, these assays can be used to determine the in vivo function of immunoregulatory MDSCs (50). It has been suggested that accumulation of MDSCs requires two different signal transduction pathways. One signal is predominantly responsible for MDSC expansion and the second pathway for driving MDSC activation. The first process is induced by various cytokines and growth factors (e.g., GM-CSF), whereas a second activating signal is provided by proinflammatory molecules (e.g., IFN $\gamma$ , IL-1 $\beta$ ). This could explain why acute inflammation, associated with the release of proinflammatory factors in the absence of sustained upregulation of growth factors, does not result in accumulation of MDSCs (14). Consequently, MDSCs can be generated in vitro using different protocols that vary in the dose and time of GM-CSF culture and the combination with other (proinflammatory) cytokines (93). However, it has been shown that GM-CSF may not be absolutely required for the development of MDSCs, since cells with a surface phenotype similar to that of MDSCs (i.e., Gr1 and Cd11b coexpression) are readily detectable in *Gm-csf*<sup>-/-</sup> mice (28). Nevertheless, the immunosuppressive strength among MDSC subsets is determined by GM-CSF. GM-CSF silencing alters MDSC subset ratios and phenotypes and results in perturbation of MDSC subset distribution in the spleens of tumor-bearing mice and reduced tumor-induced tolerance (27). Furthermore, whether the Gr1<sup>+</sup>Cd11b<sup>+</sup> cells identified in *Gm-csf*<sup>-/-</sup> animals are true MDSCs possessing immune-suppressive function is unclear. Future studies are needed to evaluate the possible role of GM-CSF not only in MDSC expansion but also in skewing/shaping MDSC composition and function during intestinal inflammation.

**Treatment of CD with GM-CSF.** Current treatment approaches in patients with (refractory) CD target the inhibition of the adaptive immune system and the blockade of proinflammatory cytokines using biological agents such as monoclonal antibodies against TNF- $\alpha$  (5, 21). However, inflammatory changes of a CD phenotype have been described in primary immunodeficiencies. In addition, advances in the understanding of the role of the innate immune system in the pathogenesis of CD have resulted in the development of therapies that enhance innate immunity and defense (23). These new approaches are based on the immunodeficiency hypothesis and address dysregulation of the innate immune response observed in patients with CD. Stimulators of the innate immune system that have been tested so far include granulocyte colony-stimulating factor (G-CSF) and GM-CSF (4). Colony-stimulating factors (CSFs) are often deployed therapeutically to increase neutrophil prevalence in the context of chemotherapy and bone marrow transplantation. In contrast to many models of inflammation and autoimmunity in which depletion of CSFs is beneficial (52), GM-CSF has a protective role in intestinal immune responses (53). The controversy with regard to the clinical trials was that GM-CSF is classically viewed as a proinflammatory factor, and so, while it might boost bacterial killing and barrier function, it might also boost adverse myeloid cell inflammatory functions. Indeed, patients receiving placebo experience fewer adverse events than GM-CSF-treated patients with CD. However, a recent Cochrane systematic review showed no statistically significant differences in the

proportions of patients who experienced at least one adverse event or serious adverse events (95).

The first report on treatment of CD with CSFs (published in 1999) described a case of beneficial and safe use of G-CSF in the treatment of rectal fistulas (120). Further open-labeled studies have proven the positive effects of G-CSF in CD (22, 72). The clinical use of sargramostim, a recombinant human form of GM-CSF, for CD treatment was initially reported in 2002 in a small open-label pilot study of patients with moderate to severe CD (24). A subsequent large placebo-controlled trial performed from 2001 to 2003 showed that GM-CSF therapy decreased disease severity, induced mucosal healing, and improved the quality of life in patients with active CD (73). However, this study failed to confirm the high response rate seen in the aforementioned pilot study. A large international phase III multicenter study, presented at the Digestive Disease Week in 2007, also failed to document clinical superiority of GM-CSF over placebo in patients with active CD (34). However, a secondary composite remission/response analysis showed GM-CSF to be numerically better than placebo at the end of treatment or at 30-day follow-up and at any time during the study. Considerable heterogeneity in the rate of placebo response was observed between the nine countries but may have been due to differences between countries in patient populations and practice patterns that influenced the placebo response. Nevertheless, GM-CSF has been shown to be more effective than placebo for inducing steroid-free remission in a phase II trial conducted between 2003 and 2005 in patients with corticosteroid-dependent CD (117). The first pediatric trial of sargramostim in active CD was published in 2010 and confirmed the encouraging efficacy and safety data observed in some of the adult studies (67). Additional small/case studies of sargramostim in CD suggest that medical therapy with GM-CSF is both a safe long-term treatment option (125) and also decreases clinical disease activity. On the other hand, this approach might have only limited efficacy in the induction of clinical remission (112) or the treatment of fistulas (77). A recent Cochrane review demonstrated that sargramostim does not appear to be more effective than placebo for remission induction in patients with CD (95, 96). However, there was a nonsignificant trend ( $P = 0.06$ ) toward benefit of GM-CSF treatment for a 100-point (Crohn's disease activity index score) clinical response (secondary outcome). Only three randomized trials were assessed as meeting eligibility criteria for inclusion in this systematic review (95). Furthermore, the overall quality for the primary and secondary outcomes was low to moderate at best due to the lack of data and heterogeneity in results between trials. The authors concluded that the results should not be seen as the final verdict on this hypothesis, but rather a preliminary step (96).

Importantly, most of the recent clinical trials of biological therapy in CD have not been superior to placebo, and even anti-TNF- $\alpha$  is only successful in a subgroup of patients (21). This may reflect substantial heterogeneity in pathogenic mechanisms, and so GM-CSF as a therapy may also only be appropriate for a subgroup of patients that, for example, does not respond to other forms of biological therapy. It is possible that IL-17A immunoneutralization may counteract the protective effects of IL-17A in the intestine. Although current literature suggests an important role of IL-17A in perpetuating chronic inflammation, protective roles have also been postu-

lated based on T cell-dependent (88) and -independent experimental models of colitis (89). Results from a small proof-of-concept study (59 patients with active CD) suggest no benefit from blockade of IL-17A in patients with moderate to severe CD (62). Importantly, the results also suggest that IL-17A blockade may have adverse outcomes in a subgroup of patients with objective evidence of inflammation. On the contrary, a small open label uncontrolled study indicates that blockade of IL-17A (and IFN $\gamma$ ) may be useful in maintaining clinical remission in patients with IBD (58). Thus, it is conceivable that an effect of IL-17A in protecting barrier integrity and Treg function is crucial in IBD compared with other inflammatory diseases. However, IL-17 modulation certainly warrants further investigation as a strategy for IBD therapy.

Therefore, further studies are needed to elucidate the effects of GM-CSF as a treatment option in CD to confirm a true underlying effect on the primary outcome and for different patient subsets. Based on the immunodeficiency hypothesis of CD and the potential effects of GM-CSF during the initial phase of (impaired) innate immunity, it will be particularly important to verify the efficacy of GM-CSF not only in patients with active CD (induction of remission) but also in those with quiescent CD (maintenance of remission). Furthermore, future research is also needed to provide robust mechanistic data for the treatment of CD with GM-CSF.

*The role of GM-CSF in mouse models of intestinal inflammation.* To further explore the mechanisms by which GM-CSF can alter the course of intestinal inflammation, the effects of CSFs have been investigated in chemically [with dextran sulfate sodium (DSS) or 2,4,6-trinitro benzene sulfonic acid (TNBS)] induced or T cell-dependent mouse models of intestinal inflammation and/or mice deficient in *Gm-csf* (6, 16, 19, 32, 43, 59, 68, 75, 76, 83, 97, 110, 123, 124, 126). The first such study showed that administration of a neutralizing anti-M-CSF antibody significantly inhibited DSS-induced colitis (83). Consistent with the improvement in clinical signs of disease (body weight loss, diarrhea, fecal blood, anal bleeding) the authors reported reduced colon shortening, mucosal ulceration, inflammatory cell infiltration of the colon, and decreased levels of proinflammatory cytokines in the supernatant of colon cultures in mice treated with anti-M-CSF. Another study showed that DSS-induced colitis was ameliorated by repetitive subcutaneous injections of G-CSF, verified by suppression of mucosal inflammation, epithelial damage, and apoptosis of IECs (75).

The effect of GM-CSF has also been studied in murine models of DSS-induced colitis, which can be ameliorated by administration of GM-CSF (6, 97). For example, *Gm-csf*<sup>-/-</sup> mice are more susceptible to acute DSS-induced colitis (126) but do not manifest spontaneous intestinal inflammation. Additionally, *Gm-csf*<sup>-/-</sup> mice displayed higher bacterial load than wild-type controls following DSS challenge. It has previously been shown that GM-CSF improves survival in gut-derived sepsis by improving the gut barrier function and resistance to bacterial translocation (43). Reduced bacterial clearance in gastrointestinal tract infections has also been reported in *Gm-csf*<sup>-/-</sup> mice following bacterial challenge with *Salmonella typhimurium* (16) and *Citrobacter rodentium* (59). Based on morphological changes of monocytes/macrophages, the function of infiltrating intestinal macrophages may be compromised in DSS-exposed *Gm-csf*<sup>-/-</sup> mice, leading to additional release

of cytokines and chemokines (126). GM-CSF treatment in DSS-exposed wild-type mice led to decreased TNF- $\alpha$  and IL-1 $\beta$  expression, but plasmacytoid DC prevalence and type 1 IFN release were increased (97). Surprisingly, *Gm-csf*<sup>-/-</sup> mice were shown to be protected from experimentally induced peritonitis due to reduced secretion of TNF- $\alpha$  and IL-6 by peritoneal macrophages (110).

GM-CSF administration in DSS-induced colitis also promotes colon tissue repair (mucosal healing) via accelerated ulcer epithelialization rather than by modulating mouse susceptibility to DSS-induced mucosal damage (6). Likewise, colon crypt epithelial cell proliferation and ulcer healing in vivo was shown to be significantly decreased in *Gm-csf*<sup>-/-</sup> mice at early times after DSS injury via changes of expression of epithelial genes associated with cell proliferation, which could be reversed by exogenous GM-CSF (32). In addition, GM-CSF injections in mice with DSS colitis resulted in a marked accumulation of splenic CD11b<sup>+</sup> myeloid cells. Likewise, intravenous transfer of splenic in vivo (but not in vitro) GM-CSF-induced CD11b<sup>+</sup> myeloid cells during DSS exposure significantly improved disease severity. Interestingly, the transferred cells localized to the colon at the bottom of crypts adjacent to epithelial cells, whereas GM-CSF-expanded CD11b<sup>+</sup> myeloid cells promoted wound closure and epithelial cell proliferation in vitro (6). GM-CSF therapy maintained its beneficial effects on DSS colitis when nonhematopoietic cells were unresponsive to GM-CSF in a bone marrow transplant approach. GM-CSF-induced promotion of wound healing was associated with altered composition of myeloid cell populations in DSS-induced colonic inflammatory infiltrates, characterized by reduced neutrophil numbers and increased accumulation of different monocytic cell subsets (6). Accordingly, it has been shown that the transfer of in vitro GM-CSF-activated monocytes into wild-type mice ameliorates DSS-induced chronic colitis via anti-inflammatory properties of derived cells that predominantly migrated to the inflamed intestine (123, 124). It has also been shown that, in antigen-induced peritonitis, the number of peritoneal monocytes/macrophages is reduced in *Gm-csf*<sup>-/-</sup> mice or wild-type mice treated with a neutralizing antibody to GM-CSF (76). Neutrophil accumulation has also been reported to be significantly attenuated in the colon of anti-GM-CSF-treated mice in a model of TNBS-induced colitis (68) and in the peritoneum of *Gm-csf*<sup>-/-</sup> mice in a model of antigen-induced peritonitis (76). Furthermore, it has been shown that the number of CD11c<sup>+</sup> DCs in the colon is significantly reduced in DSS-treated *Gm-csf*<sup>-/-</sup> mice compared with wild-type mice (32) and in *Gm-csf*<sup>-/-</sup> mice infected with a mouse enteric bacterial pathogen (*C. rodentium*) (59). Impaired recruitment and retention of mucosal DCs was related to a GM-CSF-dependent failure of IECs to produce the DC chemoattractant CCL22. The abnormal host response was mimicked by selective DC depletion from control mice and rescued by GM-CSF administration to *Gm-csf*<sup>-/-</sup> mice (59). The results suggest that GM-CSF-mediated improvement of DSS-mucosal damage is driven by bone marrow-derived cells as effectors of colonic mucosal healing rather than by direct action of GM-CSF on colon epithelial cells. In fact, macrophages are important regulators of tissue repair (86), and blood monocytes are the exclusive source of macrophages in inflamed intestinal mucosa (105, 128). In addition, GM-CSF



seems to be especially important for mucosal DCs and their tight regulation of tolerance and immunity in the intestine (31).

Interestingly, bone marrow chimera and DC depletion experiments revealed that nonhematopoietic cells, not myeloid cells, are the cellular source of GM-CSF required for early colon epithelial proliferation and repair of DSS-injured colonic mucosa, which required activation of the STAT5 pathway (32). DSS-induced colon lesions in wild-type mice show an increased production of GM-CSF (26). Freshly isolated murine IECs produce GM-CSF, and the proliferation processes of crypt cells in vitro are dependent on the GM-CSF concentration in the culture medium (102). Similarly, loss of GM-CSF signaling in nonhematopoietic cells increases ileal barrier dysfunction and IEC apoptosis in a murine model of NSAID-induced ileal injury (54). However, restoration of wild-type bone marrow improved NSAID-induced ileitis in *Gm-csf*<sup>-/-</sup> mice, which indicates that hematopoietic cells also have a role in the protection against injury of the small intestine and that hematopoietic cells may be the main source of GM-CSF in the intestine (54). The therapeutic effects of GM-CSF were considered to be independent of T and B cells because GM-CSF is also effective in severe combined immunodeficient (*Rag1*<sup>-/-</sup>) mice treated with DSS (97). However, it has been shown that the transfer of in vitro GM-CSF-activated monocytes into colitic *Rag1*<sup>-/-</sup> mice ameliorates intestinal inflammation in the T cell transfer colitis model through specific activation of innate immune functions and by regulation of adaptive immunity (19). Local release of GM-CSF in the intestinal mucosa is controlled by the proinflammatory cytokine IL-17 (derived from activated lymphocytes) that enhances TNF- $\alpha$ -induced secretion of GM-CSF from subepithelial myofibroblasts via both transcriptional and posttranscriptional mechanisms (2). IL-17 is significantly increased in *Gm-csf*<sup>-/-</sup> mice following DSS challenge, but not in wild-type mice (126).

**GM-CSF autoantibodies.** Cytokine autoantibodies play an important role in the pathogenesis of various inflammatory and autoimmune diseases (121). High serum levels of neutralizing GM-CSF Ab play a mechanistic role in autoimmune PAP (116). PAP is a human disease characterized by myeloid dysfunction resulting in pulmonary surfactant accumulation and respiratory insufficiency. In healthy individuals, GM-CSF is tightly maintained at very low but functionally significant levels, and GM-CSF Ab levels are normally present at low concentrations (116). In PAP, GM-CSF bioactivity varies inversely with increasing GM-CSF Ab levels. Above a critical threshold sufficient to neutralize GM-CSF bioactivity, GM-CSF-dependent myeloid cell functions, including phagocytosis, adhesion, oxidative burst, and bacterial killing, are impaired, and the risk of developing PAP increases (114, 121).

Because GM-CSF administration can reduce disease activity in CD and experimental colitis, studies have determined whether GM-CSF Ab influence neutrophil function and intestinal homeostasis in CD. In this regard endogenous GM-CSF Ab have been identified as a risk factor that reduces neutrophil antimicrobial functions and increases the likelihood of aggressive ileal CD requiring surgery (55). Administration of GM-CSF Ab to *Nod2*<sup>-/-</sup> mice reduced ileal epithelial barrier function leading to transmural ileitis following NSAID exposure (55). These results reveal a pivotal role for GM-CSF in regulating intestinal barrier function and homeostatic responses to gut injury. GM-CSF signaling in nonhematopoietic cells is

required for the maintenance of ileal homeostasis by direct regulation of apoptosis and proliferation of IECs. In this regard, it has been shown that increased GM-CSF Ab levels in ileal CD are associated with dysregulation of IEC survival and proliferation and that GM-CSF signaling in nonhematopoietic cells regulates 1) susceptibility to NSAID-induced ileitis; 2) ileal barrier function; 3) intestinal epithelial survival response to injury; and 4) IEC proliferation (54). Thus, the stimulation of intestinal barrier function via exogenous GM-CSF administration might be beneficial in restoring mucosal homeostasis in a subset of CD patients showing reduced GM-CSF bioactivity due to the presence of endogenous neutralizing Ab. It has been shown that neutralization of GM-CSF bioactivity leads to an increased expression of ileal CCL25 and peripheral and lamina propria CCR9 in CD and in the abovementioned experimental model of murine ileitis (98). The chemokine CCL25 and its cognate receptor CCR9 maintain lymphocyte trafficking and localization to the small intestinal lamina propria, and alterations of CCL25/CCR9 have been described in small bowel CD. Furthermore, GM-CSF neutralization experiments using GM-CSF Ab revealed GM-CSF-dependent expansion of IL-10-producing DCs in the draining mesenteric lymph nodes and induction of Tregs after NSAID exposure (98). GM-CSF may sustain Treg homeostasis and enhance their suppressive functions through modulation of DC pathways (13, 38, 41). These GM-CSF-dependent homeostatic mechanisms may play a role in prevention of disease in a subset of patients with CD.

The defective mucosal barrier in CD is associated with an abnormally increased intestinal permeability (61). Neutralization of GM-CSF increases intestinal permeability and bacterial translocation in mice (55), which is consistent with findings in *Gm-csf*<sup>-/-</sup> mice where *Gm-csf* deficiency was associated with increased bowel permeability and bacterial translocation (16, 54, 55, 59). Likewise, CD patients with elevated serum GM-CSF Ab levels exhibit increased endotoxin exposure and bowel permeability, independent of the degree of mucosal inflammation, compared with CD patients with lower serum levels of GM-CSF Ab (87). In addition, GM-CSF Ab are enriched in affected/strictured ileal tissue, and neutrophil bacterial killing is reduced in CD patients with elevated serum levels of GM-CSF Ab (63). Accordingly, serum GM-CSF Ab levels in CD are correlated with aggressive (stricturing/penetrating) disease behavior, a higher incidence of surgery, disease activity, location, and extent (17, 30, 40). More importantly, elevated serum GM-CSF Ab levels (>1.7  $\mu$ g/ml) are associated with clinical disease relapse in CD. Regular (longitudinal) measurements of serum GM-CSF levels can predict the course of the disease and detect relapses with a high sensitivity and specificity at an early stage 2–6 mo before the flare becomes clinically apparent (17). Therefore, GM-CSF Ab levels are elevated before the onset of significant intestinal inflammation and might therefore signify the commencement of disrupted intestinal homeostasis that typically precedes disease relapse later on. This concept underpins the potential mechanistic role of GM-CSF in CD pathogenesis, where endogenous serum GM-CSF Ab levels could reflect intestinal permeability, bacterial translocation, neutrophil dysfunction, and reduced antimicrobial activity. Importantly, it might be possible that GM-CSF Ab are the result of cross recognition of microbial antigens, and not GM-CSF itself. However, a recent report showed

that, at least in PAP, GM-CSF Ab clearly recognize several epitopes on human GM-CSF (115).

**Leukocyte GM-CSF receptor expression.** GM-CSF functions through a heterodimeric receptor composed of an  $\alpha$ -subunit that binds GM-CSF with low affinity and a subunit that is shared with the receptors for IL-3 and IL-5, the  $\beta$  common chain. The  $\beta$ -subunit binds cytokine very poorly by itself, but converts low-affinity cytokine binding by the  $\alpha$ -subunit to a high-affinity interaction and is the principal signal-transducing subunit. Assembly of the active GM-CSF receptor complex initiates signaling through JAK2 transphosphorylation. This leads to an activation of the JAK/STAT and MAPK pathways that signal for cell proliferation and the PI3K/Akt pathways that promote cell survival (56). A recent study in adult CD patients revealed decreased expression of the circulating GM-CSF receptor  $\alpha$ -subunit (CD116) on granulocytes and monocytes compared with healthy controls and subjects with irritable bowel syndrome or rheumatoid arthritis (45). Both CD116 expression and GM-CSF signaling via STAT3 were found to be reduced in CD patients. STAT3-mediated signaling in granulocytes has been shown to promote bacterial killing, guide neutrophil migration, and enhance neutrophil proliferation/survival (52). Reduced expression of GM-CSF-dependent molecular mediators in phagocytes leads to impaired mucosal integrity. In this regard, two possible candidates have been suggested: milk fat globule epidermal growth factor-8 (MFG-E8) and peroxisome proliferator-activated receptor- $\gamma$  (PPAR- $\gamma$ ) (29). MFG-E8, produced by lamina propria macrophages, plays a crucial role in maintenance and repair of the intestinal epithelium (9). Furthermore, mice with macrophage-specific *Ppar- $\gamma$*  genetic deficiency are highly susceptible to DSS-induced colitis (104). Therefore, the defective expression and function of CD116 might play a central role in CD, thus implicating an associated defect in innate immune responses toward GM-CSF. However, defective CD116 expression is not necessarily a distinguishing feature of CD, since further studies have shown normal CD116 expression and function in circulating granulocytes (63) and monocytes (99) isolated from CD patients. Further studies are clearly required to determine the role of CD116 expression in CD and also to elucidate whether CD116 might serve as a relevant biomarker for (a subset of) patients with CD.

**Conclusions and outstanding questions.** The benefits of GM-CSF therapy observed in a subset of CD patients and in murine models could be explained by GM-CSF-dependent modulation of intestinal barrier function, which includes effects on epithelial cell proliferation, survival, restitution, and modulation of immune responses. Immunoneutralization of GM-CSF increases intestinal permeability and bacterial translocation while reducing neutrophil bacterial killing and antimicrobial activity. Elevated serum GM-CSF Ab levels are associated with disease activity and relapses in CD. It might therefore be concluded that GM-CSF might play a novel mechanistic role in the pathogenesis of CD (Fig. 1). However, further human and animal studies are needed to comprehensively characterize the in vivo functions of GM-CSF with emphasis on the immunomodulatory role in the intestine. It will be important to define pathways, mechanisms, and myeloid and nonmyeloid cell interactions in the intestinal tract through which GM-CSF regulates the maintenance of intestinal mucosal integrity and mucosal defense mechanisms. Future

research will need to address whether neutrophil and monocyte function, as well as intestinal inflammation, varies with longitudinal serum GM-CSF Ab levels and whether compromised myeloid cell function precedes flares/relapses in parallel with increased GM-CSF Ab levels. Further studies are also needed to address the question if GM-CSF Ab are an acquired phenomena or integral to disease pathogenesis. In addition, further studies are required to determine the role of defective GM-CSF receptor (CD116) expression in CD. To elucidate the effects of GM-CSF as a treatment option in CD and to confirm a true underlying clinical effect on the primary outcome, it is particularly important to verify the efficacy of GM-CSF for the maintenance of remission in patients with quiescent CD.

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#### DISCLOSURES

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