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Protection from Collagen-Induced Arthritis in Granulocyte-Macrophage Colony-Stimulating Factor-Deficient Mice¹

Ian K. Campbell,^{2*} Melissa J. Rich,* Robert J. Bischof,* Ashley R. Dunn,[†] Dianne Grail,[†] and John A. Hamilton*

The involvement of granulocyte-macrophage CSF (GM-CSF) in collagen-induced arthritis (CIA) was examined using GM-CSF-deficient mice. Although CIA is generally considered to be restricted to mice of the H-2^q or H-2^r haplotypes, we examined the role of GM-CSF in the CIA model using GM-CSF-deficient (-/-) and wild-type (+/+) mice on a C57BL/6 (H-2^b) background. Mice were immunized by intradermal injection at the base of the tail with chick type II collagen followed by a repeat injection 21 days later. We found, based on both clinical and histologic assessments, that wild-type mice on this background developed severe CIA, while the GM-CSF-deficient mice had virtually no disease. Mice that were heterozygous for the GM-CSF gene (+/-) collectively displayed an intermediate response between those of the GM-CSF^{+/+} and GM-CSF^{-/-} groups, suggesting a gene dosage effect. GM-CSF^{+/+} and GM-CSF^{+/-} mice exhibited CIA responses ranging from mild (single digits) to severe swelling of all four paws, while in the few GM-CSF^{-/-} mice that developed CIA the disease was confined to single digits. Despite the putative role of GM-CSF in dendritic cell development, GM-CSF-deficient mice exhibited both humoral and cellular (delayed-type hypersensitivity) responses to type II collagen; however, the cellular response was significantly reduced in the GM-CSF-deficient mice compared with the wild-type controls. These findings suggest that GM-CSF is required for CIA development in mice and support the idea that GM-CSF is a key cytokine in inflammatory joint disease. *The Journal of Immunology*, 1998, 161: 3639–3644.

Granulocyte-macrophage CSF (GM-CSF)³ is a glycoprotein traditionally viewed as a growth and differentiation factor necessary for the development of hemopoietic progenitor cells into granulocytes, macrophages, and dendritic cells (1, 2). However, in view of its diverse actions on mature hemopoietic cells, it has been suggested that GM-CSF may also be a proinflammatory cytokine (3–5). Notably, GM-CSF has been reported to have the following actions: induction of class II MHC expression and urokinase-type plasminogen activator production by monocyte-macrophages (3, 5), enhancement of granulocyte and monocyte cellular adherence (4, 6), augmentation of macrophage APC function (7), priming of monocytes for cytokine production (8, 9), stimulation of phagocytosis and superoxide production by neutrophils (10, 11), and neutrophil chemotaxis (12).

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease for which there is evidence that GM-CSF may be involved. GM-CSF has been found at elevated levels in RA lesions (13) and is produced in vitro by resident joint cells (chondrocytes

and synovial fibroblasts) following their stimulation with inflammatory cytokines, such as IL-1 and TNF- α (14, 15). The latter observations led to the CSF network hypothesis (16) that sought to explain the chronicity of rheumatoid joint disease in terms of a positive feedback between joint cell CSF secretion and monokine production. GM-CSF has also been implicated in the adherence of neutrophils to cartilage and its subsequent degradation by these cells (17). Finally, GM-CSF has been reported to cause a flare-up of existing RA when administered (for correction of neutropenia) to patients with Felty's syndrome or following chemotherapy treatment (18, 19).

Collagen-induced arthritis (CIA) in the mouse (20) is an autoimmune model of RA that is dependent upon both humoral and cellular immune responses to type II collagen (CII) (21); it is considered to be restricted to mouse strains bearing the H-2^q or H-2^r haplotypes and is generally performed in DBA/1 mice (22). We recently reported that GM-CSF, when injected i.p. into DBA/1 mice suboptimally primed to develop CIA, exacerbated the disease symptoms (23), suggesting a proinflammatory role for GM-CSF in this model. Although highlighting the importance that elevated circulating levels of GM-CSF could have on the course of disease, this study did not indicate whether endogenous GM-CSF was a necessary component of the CIA response.

Therefore, in the present study we have examined the role of endogenous GM-CSF in the CIA model using GM-CSF-deficient mice (24). For this purpose we have taken the unique approach of establishing the model in mice of a non-H-2^q/non-H-2^r background, thereby eliminating the need for backcrossing the GM-CSF-deficient mice onto the DBA/1 (H-2^q) strain. We report that GM-CSF-deficient mice are relatively resistant to the induction of CIA compared with their littermate wild-type control mice. This study implicates GM-CSF as a key proinflammatory cytokine pivotal to the development of CIA in mice and adds further support to the idea of the involvement of GM-CSF in inflammatory joint diseases (e.g., RA).

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³ Abbreviations used in this paper: GM-CSF, granulocyte-macrophage CSF; RA, rheumatoid arthritis; CIA, collagen-induced arthritis; CII, collagen type II; DTH, delayed-type hypersensitivity.

Materials and Methods

Mice

Mice heterozygous for a disrupted GM-CSF gene (GM-CSF^{+/-}) were provided by the Ludwig Institute for Cancer Research (Royal Melbourne Hospital, Melbourne, Australia) and bred at the Department of Medicine animal house. The derivation was previously reported (24). Briefly, chimeric mice were generated by microinjection of 129/OLA-derived ES cells (H-2^b) with a disrupted GM-CSF gene into C57BL/6 (H-2^b) host blastocysts. Germline transmitters of the mutated GM-CSF allele were crossed with C57BL/6 mice for 11 generations, giving GM-CSF^{+/-} mice that were interbred to yield the GM-CSF^{-/-}, GM-CSF^{+/-}, and GM-CSF^{+/+} mice used for the experiments. In some experiments (see text) mice from the first C57BL/6 cross were interbred and used; these were confirmed as homozygous H-2^b by FACS analysis of spleen cells using H-2^b-specific mAb (I. K. Campbell and P. M. Hogarth, unpublished observations). GM-CSF genotype status was determined by PCR analysis of tail DNA as previously described (24). Animals were fed standard rodent chow and water ad libitum and were housed with same sex littermates in sawdust-lined cages. Mice of both sexes were consigned to experiments at 8 to 15 wk of age.

Collagen-induced arthritis

An emulsion was formed by dissolving 2 mg/ml chick CII (Sigma, St. Louis, MO) overnight at 4°C in 10 mM acetic acid and combining it with an equal volume of CFA containing 5 mg/ml heat-killed *Mycobacterium tuberculosis* (H37 Ra, Difco, Detroit, MI). Mice were injected intradermally at several sites into the base of the tail with a total of 100 µl of emulsion containing 100 µg of CII; this was repeated as a boost 21 days later. Mice immunized without the CII component did not develop arthritis during the period of investigation in this study.

Clinical and histologic assessment of arthritis

Animals were assessed for redness and swelling of limbs, and a clinical score was allocated for each mouse two to three times per week for up to 60 days as previously described (23). The maximum score per mouse was 12. At termination, the rear paws of the mice were removed, fixed, decalcified, and paraffin embedded as previously described (23). Frontal sections (5 µm) were stained with hematoxylin and eosin and evaluated without knowledge of the treatment groups, based on the histologic assessment of Williams et al. (25).

ELISA for detection of Abs to CII

ELISA assays were performed for the detection of Abs to CII by coating 96-well flat-bottom plates (Immunoplate Maxisorp, Nunc, Copenhagen, Denmark) with 50 µl of CII (2 µg/ml in PBS) overnight at 4°C. The wells were then blocked by 1-h incubation at 37°C with 200 µl of PBS containing 1% (w/v) BSA. Next, 50 µl of serial fourfold dilutions (beginning at 1/1000 dilution) of mouse sera in PBS supplemented with 0.05% (v/v) Tween-20 were applied and incubated for 2 h at 37°C. Horseradish peroxidase-conjugated goat anti-mouse whole IgG (Sigma) or isotype-specific (IgG1, IgG2a, IgG2b, and IgG3; Southern Biotechnology, Birmingham, AL) antisera (50 µl) were next applied for 2 h at 37°C followed by *o*-phenylenediamine dihydrochloride substrate (Sigma) in phosphate-citrate buffer (50 µl), and color development was monitored after a standard period by measurement in a microplate reader (model 450, Bio-Rad, Richmond, CA) at 450 nm. Three washes in PBS/0.05% (v/v) Tween-20 were applied between all steps. Standard curves were constructed as follows: for total IgG, protein G-Sepharose affinity-purified mouse anti-CII IgG fraction (serial 1/4 dilutions beginning at 7 µg/ml); for others, mouse anti-CII sera (serial 1/4 dilutions beginning at 1/1000). In each case the anti-CII sera were derived from a pool obtained from CII-hyperimmunized DBA/1 mice. Arbitrary units were assigned to the standards, such that 1 U/ml gave an OD of 0.5 with the different antisera.

Delayed-type hypersensitivity (DTH) reaction

Mice were immunized, as before, by intradermal injection into the base of the tail with chick CII in CFA. Ten days later the mice were anesthetized and injected s.c. into the right hind footpad with 20 µl of a solution containing 2 mg/ml CII in PBS; the left footpad received the same volume of vehicle. Immediately before injection and 24 and 48 h thereafter, the thicknesses of the left and right footpads were measured using spring callipers (Mitutoyo, Tokyo, Japan) accurate to 0.01 mm. The Ag-specific DTH response was determined as the increase in right footpad thickness minus the increase in left footpad thickness at the given time points. Injection of CII into the paws of naive mice produced negligible swelling. Following the 48 h measurement, the mice were sacrificed, and the hind footpads were

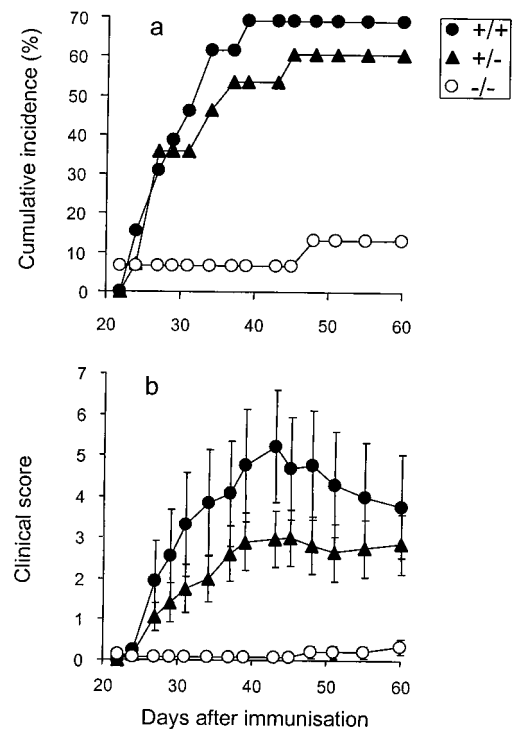


FIGURE 1. CIA development in GM-CSF-deficient mice and littermate controls. The incidence of arthritis (shown as cumulative percentage; *a*) and the mean clinical scores (\pm SEM; *b*) of GM-CSF^{-/-} ($n = 15$), GM-CSF^{+/-} ($n = 28$), and GM-CSF^{+/+} ($n = 13$) mice are shown with time following primary immunization with CII. The final incidence of arthritis was significantly lower in the GM-CSF^{-/-} mice than in the GM-CSF^{+/+} and GM-CSF^{+/-} mice ($p < 0.005$ for each, by χ^2 test). For statistical analysis of clinical scores see Table I.

removed and processed for histologic analysis as described above, except that decalcified specimens were halved in the sagittal plane, and paraffin-embedded sections were cut in this plane from the center of the footpad outwards. Sections were stained with hematoxylin and eosin to confirm cellular infiltration into the dermis and s.c. regions.

Statistics

For clinical scores the Mann-Whitney two-sample rank test was used to determine the level of significance between means of groups. For data pertaining to Ab levels in serum samples and the DTH reaction, Student's *t* test for the difference between two means was employed, while the paired *t* test was used for comparison of mouse weights over the course of the experiments. The incidence of arthritis between different groups and the proportion of joints in different histologic categories were assessed by the χ^2 test. For each test $p < 0.05$ was considered statistically significant.

Results

Reduced incidence and severity of CIA in GM-CSF-deficient mice

To evaluate the requirement for endogenous GM-CSF for the development of CIA, GM-CSF-deficient mice (GM-CSF^{-/-}) and their littermate controls (GM-CSF^{+/+} and GM-CSF^{+/-}), each on the C57BL/6 (H-2^b) background, were primed to develop CIA by intradermal immunization with chick CII in CFA followed by a repeat of the primary injection 21 days later. This immunization schedule successfully elicits CIA in the wild types of these and certain other non-H-2^d/non-H-2^e mouse strains with an incidence only slightly lower than that in DBA/1 mice (our manuscript in preparation).

Figure 1 shows results from the pooled data of two experiments performed using 8- to 14-wk-old mice from the 11th C57BL/6

Table I. Response of GM-CSF $^{+/+}$, $^{+/-}$, and $^{-/-}$ mice to CIA

Parameter	GM-CSF Genotype		
	$^{+/+}$	$^{+/-}$	$^{-/-}$
Clinical assessment ^a			
Average clinical score (d22-60) ^b	3.4 ± 1.0	2.1 ± 0.5	0.1 ± 0.1 ^c
Severity median (range) ^c	9 (1-12)	6 (2-12)	2 (1-2)
Anti-CII IgG (mg/ml) ^d	1.60 ± 0.32	2.04 ± 0.35	1.71 ± 0.29

^a Summary of data in Figure 1.

^b Values show mean ± SEM of the clinical scores of individual mice averaged over days 22 to 60.

^c Values show arthritis severity as the median (and range) of maximum clinical scores obtained for the responsive mice of each genotype.

^d Values are mean ± SEM concentrations of anti-CII total IgG in the mouse sera at day 60.

^e $p < 0.005$ and $p < 0.001$, compared to GM-CSF $^{+/+}$ and GM-CSF $^{+/-}$ groups, respectively.

backcross (99.95% C57BL/6); in these experiments the entire litters of GM-CSF $^{+/-}$ cross-matings were tested. Immunized mice were examined regularly over a 60-day period for signs of redness and/or swelling of the paws, and a clinical score (maximum of 12/mouse) was assigned as previously described (23). The majority of the GM-CSF $^{+/+}$ mice exhibited clinical signs of CIA, with 9 of 13 mice having swollen joints beginning on days 24 to 39 (Fig. 1*a*) and the mean clinical score peaking on day 43 (Fig. 1*b*). In Table I, which summarizes the data from Figure 1, it is evident that the GM-CSF $^{+/+}$ mice demonstrated a broad range of responses, as we and others have observed using DBA/1 mice in this model (23, 26); these varied from minor swelling of digits to severe swelling and ankylosis in all four paws (severity range, 1-12; median, 9).

In contrast, only 2 of 15 GM-CSF $^{-/-}$ mice responded by day 60, one beginning later than day 45, a significantly lower CIA incidence than for the GM-CSF $^{+/+}$ mice ($p < 0.005$; see Fig. 1*a*). The arthritis in the GM-CSF $^{-/-}$ mice was restricted to the swelling of single digits on the affected limbs. The mean clinical score was consistently lower in the GM-CSF $^{-/-}$ mice than in the GM-CSF $^{+/+}$ mice throughout the course of the experiment (Fig. 1*b*), and this was reflected in the significantly lower average clinical scores of the mice over the period from days 22-60 ($p < 0.005$; see Table I). The lower clinical scores of the GM-CSF $^{-/-}$ mice compared with those of the GM-CSF $^{+/+}$ mice were thus a combination of reduced incidence of arthritis as well as considerably lessened severity of disease in responsive mice, shown by the restricted range of 1 to 2 (Table I).

Interestingly, overall the GM-CSF $^{+/-}$ mice exhibited an intermediate response between the other two genotypes for both incidence and clinical score (see Fig. 1) with 17 of 28 mice developing signs of arthritis, although the severity range was comparable to that in the GM-CSF $^{+/+}$ mice (Table I).

As an additional measure of CIA severity, the weights of the GM-CSF $^{+/+}$ and GM-CSF $^{-/-}$ mice were compared over the period from CII boost (day 21) to sacrifice (day 60). Over this time frame the GM-CSF $^{+/+}$ mice failed to gain weight, while the weights of the GM-CSF $^{-/-}$ mice increased by an average of 5% ($p < 0.0001$, by paired *t* test), suggesting reduced morbidity and severity of disease in the latter genotype.

A further experiment was performed using age- and sex-matched GM-CSF $^{+/+}$ and GM-CSF $^{-/-}$ mice ($n = 12$) of mixed C57BL/6 and 129/OLA background (single C57BL/6 cross; see *Materials and Methods*). Using this cohort, CIA, manifested as a solitary swollen digit, was observed in only one GM-CSF $^{-/-}$ mouse. Again, the severity (based on maximum clinical score) of

Table II. Histopathological assessment of joints from GM-CSF $^{+/+}$ and GM-CSF $^{-/-}$ mice^a

Histological Grade	GM-CSF Genotype ^b	
	$^{+/+}$	$^{-/-}$
Total joints	57	57
Normal	7 (12)	52 (91) ^c
Mild	3 (5)	1 (2)
Moderate	6 (11)	2 (4)
Severe	41 (72)	2 (4) ^c

^a Joints of clinically positive mice in the GM-CSF $^{+/+}$ ($n = 4$) and GM-CSF $^{-/-}$ groups ($n = 2$, plus two other mice) were examined histologically.

^b Data show the number (%) in parentheses of joints distributed within each histological severity grade for the two genotypes.

^c $p < 0.001$, compared to GM-CSF $^{+/+}$ group.

the responsive GM-CSF $^{+/+}$ mice had a broad range from 1 to 12 (median 9); the means (\pm SEM) of the clinical scores averaged over days 23-60 differed significantly for GM-CSF $^{+/+}$ and GM-CSF $^{-/-}$ mice (2.18 ± 0.79 and 0.02 ± 0.02 , respectively; $p < 0.01$).

Histologic assessment

To confirm the clinical assessments, at sacrifice the clinically positive hind paws of the two responding GM-CSF $^{-/-}$ mice as well as the hind limbs of two other GM-CSF $^{-/-}$ mice and those of four clinically positive GM-CSF $^{+/+}$ mice from the experiments detailed in Figure 1 were removed and processed for paraffin embedding and sectioning. Histologic grading of hematoxylin- and eosin-stained sections was performed for each joint based on the procedure of Williams et al. (25), and the results are summarized in Table II. The GM-CSF $^{-/-}$ mice exhibited a significantly reduced proportion of joints in the severe histopathologic category compared with that in the GM-CSF $^{+/+}$ mice (4 vs 72%, respectively; $p < 0.001$) and were more frequently normal in histologic appearance (91% compared with 12% for GM-CSF $^{+/+}$ mice; $p < 0.001$). The GM-CSF $^{-/-}$ mouse joints were typically normal in appearance (Fig. 2*a*), while inflammatory cell infiltrate of granulocytes and mononuclear cells, subsynovial inflammation and hyperplasia, as well as cartilage and bone degradation resulting in loss of joint architecture were common features of the GM-CSF $^{+/+}$ mouse joints (Fig. 2, *b* and *c*).

In the majority of GM-CSF $^{-/-}$ mouse joints examined (52 of 57) there was a complete absence of pathology (Fig. 2*a*). However, the interphalangeal and metatarsal-phalangeal joints of the digits showing clinical signs of arthritis in some instances (2 of 57 joints) exhibited histopathology comparable to that observed in the most severely affected GM-CSF $^{+/+}$ mice. Inflammatory cell infiltrate, synovial hyperplasia, pannus formation, and associated cartilage and bone degradation were evident with loss of joint architecture.

Humoral response to CII of GM-CSF-deficient mice

Since CIA development is dependent on both B and T cell responses (21), the almost complete absence of CIA in the GM-CSF $^{-/-}$ mice could be due either to their inability to develop Abs to CII or to a weakened cellular response to CII. To address the first possibility, sera were collected from all mice at sacrifice and examined by ELISA for Abs to CII. The total IgG Abs to CII were comparable for the different mouse genotypes (Table I). Since the IgG2a and IgG2b isotypes, rather than the total IgG, are considered to be of prime importance in the Ab response to CII (27), sera from GM-CSF $^{-/-}$ and GM-CSF $^{+/+}$ mice were also examined for possible differences in the IgG isotype responses to CII. This further

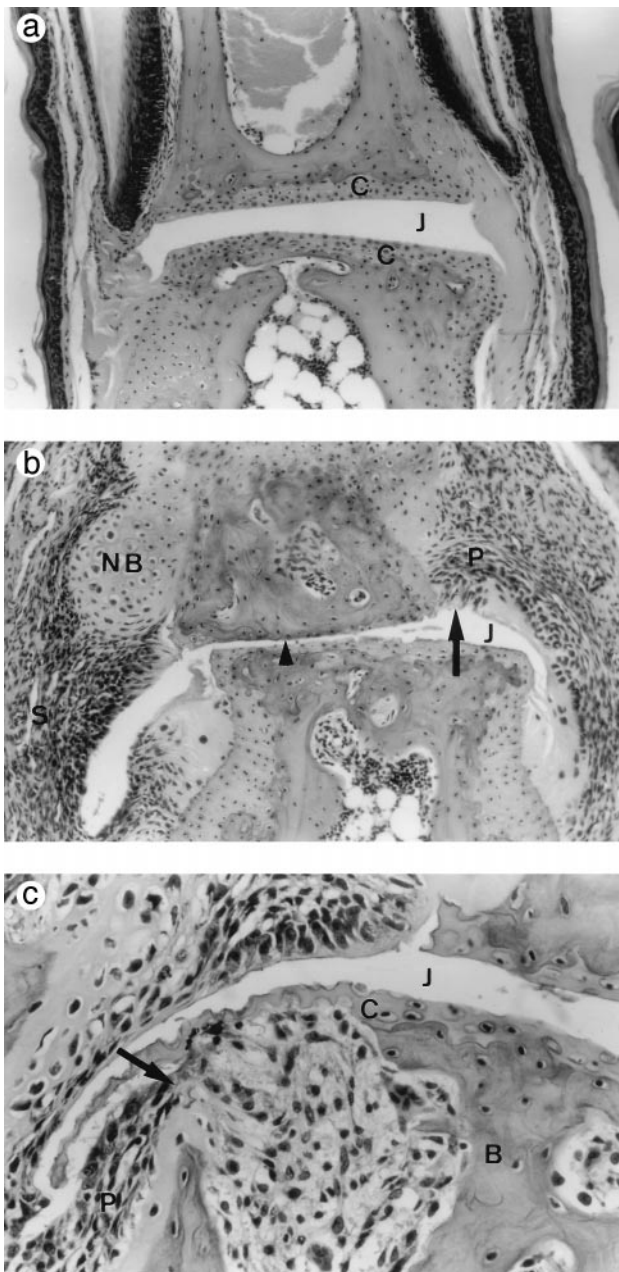


FIGURE 2. Histopathology of joints of GM-CSF^{-/-} and GM-CSF^{+/+} mice. At 60 days post-primary immunization with CII, mice were sacrificed, their hind limbs were removed, and the paws were processed for histology as described in *Materials and Methods*. Frontal sections of the interphalangeal joints of representative GM-CSF^{-/-} (a) and GM-CSF^{+/+} (b and c) mice are shown. The majority of GM-CSF^{-/-} mouse joints examined were normal in appearance, with smooth intact articular cartilage (C) and the absence of inflammatory cell infiltrate. The joints of GM-CSF^{+/+} mice most frequently showed severe pathology, with cartilage erosion down to the subchondral bone (arrowhead), synovial inflammation (S), and formation of invasive pannus (P) resulting in severe cartilage and bone degradation (arrows). New bone (NB) formation and remodeling were also evident in late stage disease. The pannus (see closeup in c) comprised a mixture of monocyte/macrophages, polymorphonuclear leukocytes, and fibroblast-like cells. J, joint space; B, bone. Hematoxylin and eosin stain; original magnification, $\times 100$ for a and b and $\times 250$ for c.

level of investigation did not reveal any statistically significant differences between the two groups of mice in the Ab responses to CII (Fig. 3).

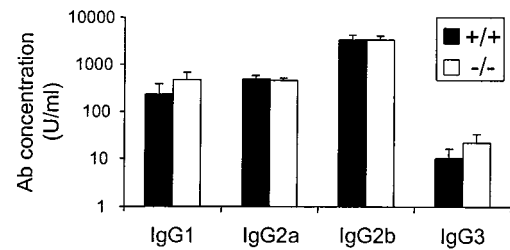


FIGURE 3. Humoral response to CII in GM-CSF^{-/-} and GM-CSF^{+/+} mice: IgG isotypes. Circulating levels of CII-specific Abs (IgG1, IgG2a, IgG2b, and IgG3 isotypes) were determined in individual sera from GM-CSF^{-/-} ($n = 15$) and GM-CSF^{+/+} ($n = 13$) mice at 60 days postimmunization with CII. Results show the mean \pm SEM (arbitrary units per milliliter; see *Materials and Methods* for definition). No significant difference was observed between the two mouse genotypes for levels of any of the anti-CII IgG isotypes (by Student's *t* test).

Cellular response to CII of GM-CSF-deficient mice

The cellular response to CII was next investigated by the DTH reaction based on footpad swelling. While GM-CSF^{-/-} mice were capable of a DTH response to CII, as indicated by the significantly greater degree of swelling in their CII-injected footpads vs that in vehicle-injected footpads ($p < 0.005$, by paired *t* test; data not shown), there was a significant reduction ($p < 0.05$) in its magnitude compared with that in GM-CSF^{+/+} mice at 24 and 48 h after Ag challenge (Fig. 4). Histologic assessment of footpad sections from each group of mice confirmed that the swelling observed was indeed due to mononuclear cell infiltration into the dermis and not simply edema.

Discussion

CIA has been widely used as a means of examining the roles of cytokines and other inflammatory mediators in arthritis progression. The approaches used have involved direct injection of cytokines, neutralizing Abs, or receptor antagonists of the cytokines. We previously employed the first of these approaches to demonstrate that GM-CSF exacerbates CIA in DBA/1 mice (23), thereby illustrating the effects of increased circulating GM-CSF levels on arthritis development. Numerous studies have employed the second approach (neutralizing Abs) in attempts to demonstrate the role of targeted endogenous cytokines in CIA, but this approach

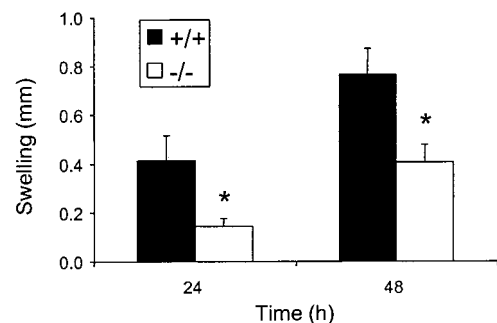


FIGURE 4. Cellular (DTH) response to CII in GM-CSF^{-/-} and GM-CSF^{+/+} mice. Age- and sex-matched GM-CSF^{-/-} and GM-CSF^{+/+} mice were immunized by intradermal injection of CII and challenged 10 days later by s.c. injection into the rear footpad with either CII or vehicle control. Results show the DTH response measured as the difference in millimeters between the increase in thickness of the right (CII-treated) and the left (vehicle-treated) footpads at 24 and 48 h postchallenge. Values are the mean \pm SEM for six mice per group. *, $p < 0.05$ compared with the GM-CSF^{+/+} group.

can have the following limitations: 1) the Ab may not be accessible to the site of action of the cytokine; and 2) there may be reduced efficacy of the neutralizing Ab due to an immune response to this foreign protein in the mice. One way to avoid these potential inadequacies is to employ gene knockout mice, which do not express the mediator of interest. Recently, several studies have appeared in the scientific literature using this approach in the CIA model with various target genes (28–32), and these have invariably involved backcrossing the knockout mice onto the CIA-susceptible DBA/1 (H-2^d) strain for up to five generations.

In the present study, GM-CSF knockout mice on a C57BL/6 (H-2^b) background were employed to test the requirement for endogenous GM-CSF in the development of CIA. We found that 70% of wild-type mice on this background developed CIA within 40 days of primary CII immunization (Fig. 1*a*, GM-CSF^{+/+}). This discovery dispensed with the putative requirement to backcross the GM-CSF null mice onto the DBA/1 background to attain a CIA-susceptible strain. By comparing, in the CIA model, GM-CSF-deficient and wild-type control mice on this background we showed that the absence of the GM-CSF gene product protected against disease development. Only 2 of 15 GM-CSF-deficient mice developed very mild clinical symptoms of CIA, and this was shown histologically to be confined to the interphalangeal and metatarsal-phalangeal joints of the digits. In contrast, the GM-CSF-competent mice showed a significantly greater incidence of disease and clinical responses ranging from mild (score 1) to severe (score 12), with swelling and ankylosis of all four paws.

Histologic analyses of arthritic paws confirmed the clinical assessments: the joints of the GM-CSF-deficient mice were most often normal in histologic appearance, whereas those of the wild-type mice were frequently severely disrupted, with pannus tissue formation and associated cartilage and bone loss (Table II and Fig. 2). Interestingly, in 2 of the 57 joints examined from the GM-CSF-deficient mice the degree of joint damage was comparable to that observed in the most severely affected wild-type mice. It would therefore appear that GM-CSF is not absolutely required for the development of CIA and that in a small number of individuals disease within isolated joints can proceed to the end stage in its absence. Rather, GM-CSF may be needed for the rapid systemic progression of CIA toward polyarthritis, since during the course of this study (up to 60 days) arthritis appeared to be confined to isolated digits on the affected limbs of GM-CSF-deficient mice. In further delineating the role of GM-CSF in this model it would be of interest to compare the effects of systemic and local GM-CSF reconstitution in the GM-CSF null mice.

Investigations were undertaken to examine why GM-CSF-deficient mice were resistant to CIA induction. Given the importance of GM-CSF in dendritic cell development *in vitro* (2) and the reported dependence of the CIA model on both humoral and cell-mediated immunities to CII (21), GM-CSF-deficient and wild-type mice were compared for their immune responses to CII. No differences were observed between the two genotypes in the serum levels of either the total IgG or IgG subclass responses to CII at 60 days postprimary immunization, suggesting a normal humoral response to this Ag in the GM-CSF-deficient mice. Since complement fixing Abs of the IgG2 subclass are considered critical for CIA development (27), the latter result precluded the possibility that, although the total IgG levels were comparable between the two genotypes, other IgG subclasses may have been generated by the GM-CSF^{-/-} mice at the expense of the IgG2 subclasses, thereby accounting for their reduced CIA incidence. The further possibility that a temporal difference between the humoral responses to CII of the two genotypes may account for the subsequent differences in CIA development is unlikely given that one of

the two GM-CSF-deficient mice that developed CIA was the first mouse to show signs of joint swelling (on day 22). Instead, in accordance with the work of others (21, 33), it would appear that the capacity of mice to raise Abs to CII is alone insufficient to result in CIA.

In contrast, while the GM-CSF-deficient mice were capable of eliciting cell-mediated immunity to CII, as determined by the DTH response, it was at a significantly reduced level compared with that in the wild-type control mice (Fig. 4), suggesting suppression of T cell function. Whether this reduction is sufficient to account for the minimal arthritic response in these mice remains uncertain. Recent studies (34) examining the T cell function of immunized GM-CSF-deficient mice reported a reduced CD4⁺ proliferative response to specific Ags; the mechanism was thought to involve a GM-CSF-induced dendritic cell-derived factor capable of enhancing the T cell proliferative response, rather than an intrinsic malfunction in the T cells. The reported incapacity of dendritic cells to either process or present CII (35) suggests that another explanation may be needed for the reduced cellular response to CII in the present study. For instance, it could reflect the inability of the GM-CSF-deficient mouse T cells to activate macrophages through GM-CSF production. Finally, it must be acknowledged that these studies compared the immune responses to chick CII; the possibility remains that differences exist between the GM-CSF-deficient and wild-type mice in their abilities to develop an autoimmune reaction to murine CII.

The absence of GM-CSF in the knockout mice could, based on *in vitro* studies, have ramifications for granulocyte, monocyte-macrophage, or dendritic cell responses in the CIA model. Interestingly, GM-CSF-deficient mice have normal hemopoiesis up to 12 wk of age (24) and normal levels of both myeloid-related and lymphoid-related dendritic cells in the major lymphoid organs (spleen, lymph node, and thymus) (36). However, these are under steady state conditions where mice have not been elicited to develop autoimmune inflammatory disease, such as CIA. Local or systemic differences between the GM-CSF^{+/+} and GM-CSF^{-/-} mice in either the numbers or levels of activation of any of the three cell lineages listed above may still account for the observations reported herein. Recent functional studies with the GM-CSF-deficient mouse have demonstrated an increased tolerance to endotoxin-mediated septic shock that was related to reduced circulating levels of the cytokines IFN- γ , IL-1 α , and IL-6 (37). Moreover, LPS-stimulated peritoneal macrophages from GM-CSF^{-/-} mice produced less IL-1 α and nitric oxide than those from wild-type mice. Thus, while GM-CSF knockout mice have normal steady state levels of circulating monocytes, they may be impaired in their ability to respond to certain stimuli. Studies of the local (joint) and systemic cytokine levels of CII-immunized GM-CSF-deficient mice may provide further insight into how they are protected from CIA development.

The results of this study taken together with our previous report of exacerbation of the CIA model by exogenous GM-CSF (23) support the idea of GM-CSF as a proinflammatory mediator and provide a strong argument for a pivotal role for GM-CSF in CIA development and inflammatory joint disease.

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