Immunology and Cell Biology (2012) 90, 553–558

www.nature.com/icb

ORIGINAL ARTICLE

Protection against *Nippostrongylus brasiliensis* infection in mice is independent of GM-CSF

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Granulocyte macrophage-colony stimulating factor (GM-CSF) is a cytokine with the capacity to promote inflammation in a wide variety of infectious and inflammatory diseases. These conditions include allergic airway inflammation, which is driven by T-helper 2 (Th2) cells. Because of the importance of Th2 cells in parasite infections, we have investigated the role of GM-CSF in mice infected with the nematode *Nippostrongylus brasiliensis*. The effect of primary and secondary infection was investigated in mice lacking functional genes for GM-CSF (*CSF2* genes) (ΔGM-CSF mice), and in mice lacking the cytokine receptor common β chain (Δβ mice), the latter being unable to signal in response to GM-CSF and interleukin (IL)-5. ΔGM-CSF mice showed no significant defect in parasite immunity, measured by larval numbers in the lungs, worm numbers in the intestine or egg numbers in the faeces, in either primary or secondary infection. By contrast, the Δβ mice showed increased parasite burden, with higher numbers of lung larvae after secondary infection and higher numbers of intestinal worms and faecal eggs after both primary and secondary infection. Unexpectedly, there were increased numbers of circulating eosinophils in the ΔGM-CSF mice, associated with significantly reduced larval numbers in the lungs. These results indicate that GM-CSF is redundant in protection against *N. brasiliensis* infection, and that the increased susceptibility of Δβ mice to infection is likely to be attributed to the lack of IL-5 signalling in these mice. The results suggest that clinical use of agents that neutralise GM-CSF may not be associated with increased risk of parasite infection.
Immunology and Cell Biology (2012) **90,** 553–558; doi:10.1038/icb.2011.69; published online 16 August 2011

Keywords: GM-CSF; *CSF2*; helminth; IL-5; *Nippostrongylus brasiliensis*; parasite infection

Granulocyte macrophage-colony stimulating factor (GM-CSF) is a cytokine with a wide array of functions ranging from hematopoiesis to inflammation.[1] Many studies have established a role for GM-CSF in the proliferation, differentiation and survival of granulocytes and macrophages.[1] Despite this, mice lacking GM-CSF do not have major defects in steady-state hematopoiesis.[2,3] In the absence of inflammation, the major abnormality in mice lacking functional *GM-CSF* genes is a condition known as pulmonary alveolar proteinosis.[2,3] In this condition, surfactant protein is deposited in the alveoli because of the inability of the alveolar macrophages to clear it. Patients with pulmonary alveolar proteinosis were found to have increased levels of autoantibodies to GM-CSF, thereby indicating the importance of GM-CSF in the maintenance of normal lung homeostasis.[4,5]

GM-CSF has been shown to be essential for inflammatory responses in various mouse disease models associated with T-helper 1 (Th1) and/or Th17 responses, including collagen-induced arthritis,[6] experimental allergic encephalomyelitis[7] and recently, experimental autoimmune myocarditis.[8] These studies indicate that GM-CSF is a proinflammatory agent and provide support for trialling agents that target GM-CSF in treatment of clinical diseases such as rheumatoid arthritis and multiple sclerosis.[1] In addition to inflammatory conditions associated with Th1 and Th17 responses, GM-CSF is also required for Th2-associated allergic inflammation. Recent findings by Su *et al.*[9] have shown that GM-CSF is required for the development of bronchial eosinophilia, a prominent feature of allergic airway inflammation in mice. These findings complement clinical studies demonstrating overproduction of GM-CSF in allergic inflammatory disorders such as allergic asthma.[10]

Numerous studies have demonstrated a central role for Th2 cells and their corresponding cytokines in allergic inflammation and in helminthic infections.[11,12] Allergic airway inflammation is generally viewed as an inappropriate Th2 response to allergen,[13] and it has been hypothesised that Th2 responses evolved to protect against helminth infections.[14] Helminthic parasites elicit robust Th2 responses in the host that may help to resolve the infection and provide protection against re-infection.[15] Th2 responses normally involve eosinophils, elevated immunoglobulin E (IgE) production, smooth muscle hypercontractility and goblet cell metaplasia with enhanced mucus

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Received 20 April 2011; revised 5 July 2011; accepted 13 July 2011; published online 16 August 2011

production, all of which are dependent on production of the typical Th2 cytokines, interleukin (IL)-4, IL-5, IL-9 and IL-13.[11,12] The importance of IL-5 in eosinophil responses is illustrated by experiments in mice lacking or overproducing IL-5.[16–18] During infection with *Nippostrongylus brasiliensis*, mice lacking IL-5 produce few eosinophils in response to the parasite and exhibit increased parasite burdens.[17]

Eosinophils, an important component of the Th2 response, have been shown to provide protection against some parasitic helminth species, but not others.[19,20] Because mice lacking GM-CSF have a defect in eosinophilia in allergic airway inflammation, it is possible that they also have defective eosinophil responses in, and increased susceptibility to, helminth infection. If mice lacking GM-CSF have a defect in protection against helminth infection, patients treated with therapeutic agents that block GM-CSF could be at risk of increased susceptibility to such infections.

The biological activity of GM-CSF is mediated by its binding to a specific α chain and the common β chain (βc) that is shared with the receptors for IL-3 and IL-5.[21] The mouse has an alternative β chain receptor for IL-3, allowing mice lacking βc to maintain responsiveness to IL-3, while being unable to respond to the other two cytokines.[22] In this study, we describe the consequences of loss of GM-CSF function during infections with the parasitic helminth *N. brasiliensis*. We assessed protection against infection and Th2 responses in mice deficient for the ligand, GM-CSF (ΔGM-CSF) and mice lacking βc and therefore unable to respond to either GM-CSF or IL-5.

RESULTS

Determination of peak lung larval numbers and peak intestinal worm numbers

Mice were infected with *N. brasiliensis* on day 0 and killed at various time points from day 0 to day 13, and relevant tissues were collected for enumeration of larvae or worms. It was found that maximal number of lung larvae were present in the mice at day 2, whereas the highest number of intestinal worms were found at day 6 post infection (p.i.; Supplementary Figure 1) On the basis of these data, we collected lung tissue at day 2 p.i. and intestinal tissue at day 6 p.i. for all related analysis.

Lung phase of infection

To investigate the potential importance of GM-CSF in resistance to primary and secondary *N. brasiliensis* infections, the ability of parasites to migrate to the lungs and the resulting inflammatory infiltrate were examined. After primary infection, there was comparable larval recovery in the lungs of all groups of mice, suggesting that all strains were equally susceptible at this stage of infection (Figure 1). Larval numbers in lungs of all three strains were markedly lower in secondary than primary infections (Figure 1). During secondary infections, larval numbers were significantly lower in ΔGM-CSF mice compared with wild-type (WT) mice, whereas numbers in Δβc mice were significantly higher than ΔGM-CSF, and higher that WT, although the latter did not reach statistical significance (Figure 1).

In WT mice, cell numbers in the bronchoalveolar lavage (BAL) fluid were similar in both primary and secondary infection. However, cell numbers were much higher in both ΔGM-CSF and Δβc than WT mice during primary infection, although the results with ΔGM-CSF did not reach statistical significance (Figure 2a). This increase of cell numbers after the first infection was possibly because of the pulmonary alveolar proteinosis in the mice caused by lack of GM-CSF or GM-CSF signalling. After the primary infection, neutrophils were abundant in all groups and significantly higher in Δβc than WT mice (data not shown), and this is likely to be associated with pulmonary haemorrhaging. Although the cell numbers differed between the groups, the parasite load was similar in all groups (Figure 1), indicating that cell numbers in the BAL fluid in mice do not correlate with parasite numbers. By contrast, eosinophils were low in all groups, although eosinophils were detectable in the Δβc mice (Figure 2b).

After the secondary infection, total cell numbers were lower in all groups. Nevertheless, parasite numbers were much lower in the secondary infection, consistent with previous findings that most parasites are eliminated at the site of injection in secondary infection.[17] Eosinophils were higher in secondary-infected ΔGM-CSF mice than in WT mice, although this difference did not reach statistical

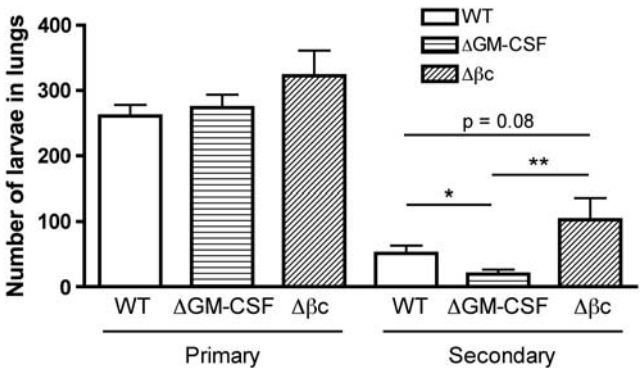

Figure 1 Lung larval numbers. At 2 days after primary or secondary infection with *N. brasiliensis*, larvae were recovered from lungs. Data represent mean lung larval burden/mouse ± s.e.m., n=10–20. $^{*}P<0.05$ and $^{**}P<0.01$.

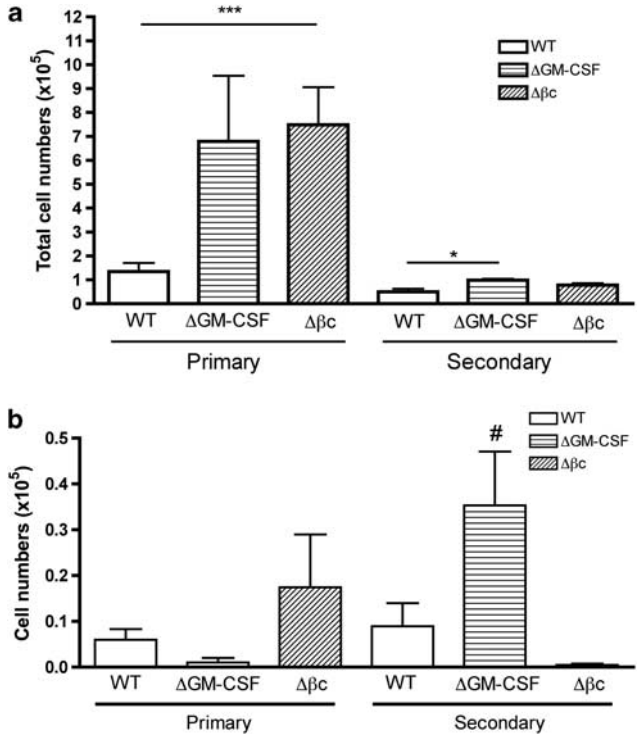

Figure 2 Cell numbers in BAL fluid. (**a**) Total cell numbers in BAL fluid (**b**) Eosinophil numbers in BAL fluid taken from mice at respective time points. n=5–13. $^{*}P<0.05$ and $^{***}P<0.001$; $^{\#}P=0.0871$.

significance, but eosinophils were almost undetectable in Δβc mice (Figure 2b). As a measure of Th2 responses during the lung phase of the infection, IL-4 and IL-5 levels were assessed in BAL fluid from infected mice at different time points. IL-4 and IL-5 levels did not indicate any significant differences between all three groups at any of the time points measured (data not shown).

Intestinal phase of infection and eosinophil responses

Worm numbers and egg production were assessed at 6 days after primary or secondary infection with *N. brasiliensis*. There was a significant decrease in intestinal worm burden in secondary infection compared with primary infection in all groups (Figure 3a). In both primary and secondary infection, the worm counts in ΔGM-CSF mice were not significantly different from those in WT mice. The Δβc mice had higher worm numbers than the other groups in both primary and secondary infections, although the difference only reached statistical significance in the secondary infections (Figure 3a). These results suggest that protection against *N. brasiliensis* infections in Δβc mice could be impaired during migration of larvae from lung to intestine or in the intestinal stage. By contrast, there was no defect in ΔGM-CSF mice.

During primary infections, egg production in ΔGM-CSF mice was lower than in WT mice, but the difference did not reach statistical significance. Egg production in Δβc mice was significantly greater compared with the other groups (Figure 3b). When mice were re-infected, as was the case with intestinal worm numbers, parasite egg counts were significantly lower in all groups compared with primary infection. In both WT and ΔGM-CSF groups, eggs were undetectable in all mice, but were still detectable in Δβc mice. The data on egg production are consistent with the findings on intestinal worm burden, in that there was a defect in immunity in the Δβc mice but no apparent impairment in ΔGM-CSF mice.

At day 6 after primary infection, WT mice had higher numbers of eosinophils in the intestinal wall than ΔGM-CSF and Δβc mice (Figure 4). On day 13, eosinophil numbers in all groups were decreased and remained so even when a secondary infection was given. Eosinophils were very low at all time points in Δβc mice, but eosinophils in ΔGM-CSF mice were not significantly different from WT mice on days 13 or 27 (Figure 4).

Blood eosinophilia and serum IgE

Blood samples were obtained from mice at different time points and analysed for eosinophil numbers. Samples taken at day 2 p.i. indicated that blood eosinophil levels in WT, ΔGM-CSF and Δβc mice were very low. In response to the primary infection, blood eosinophil levels in WT mice peaked at day 6 and remained relatively high until day 13, then fell to a very low level by 2 days after the secondary infection (day 23 overall). By contrast, in ΔGM-CSF mice, blood eosinophil levels rose more slowly and only peaked at day 13, when they were significantly higher than in WT mice. Eosinophils remained moderately high compared with WT mice on day 2 after secondary infection. However, between day 2 and day 6 after secondary infection, eosinophil levels in WT mice increased to a much greater extent compared with ΔGM-CSF. In contrast, blood eosinophil levels in Δβc mice

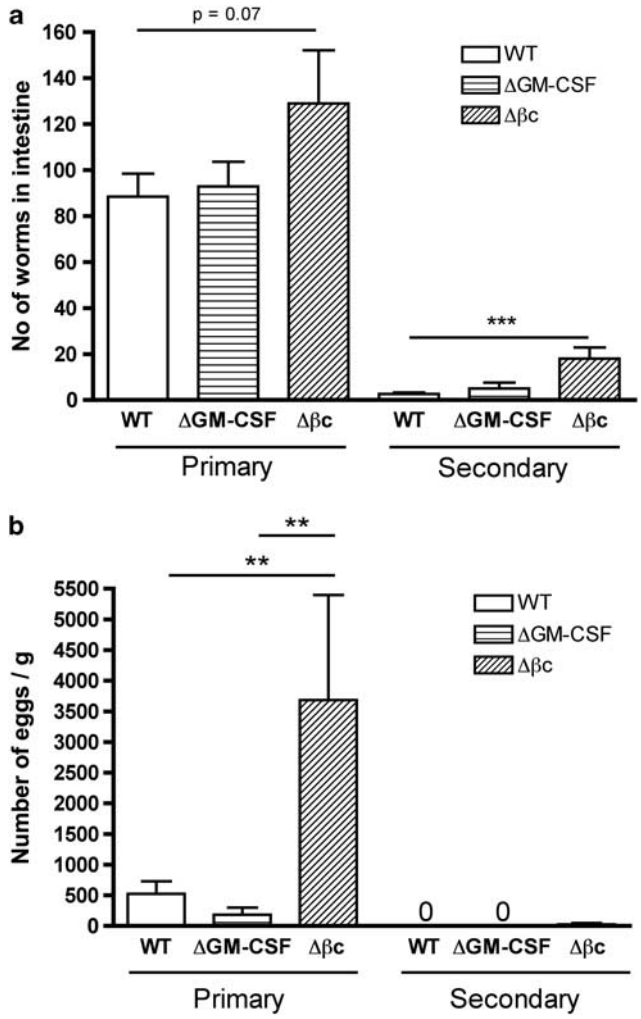

Figure 3 Intestinal worm and faecal egg counts. Data were determined at 6 days after primary and secondary infections with *N. brasiliensis*. (**a**) Intestinal worm counts. (**b**) Faecal egg production. Egg numbers are presented as number of eggs g^{-1} of faeces. Data represent mean counts ± s.e.m., n=15. $^{**}P<0.01$ and $^{***}P<0.001$.

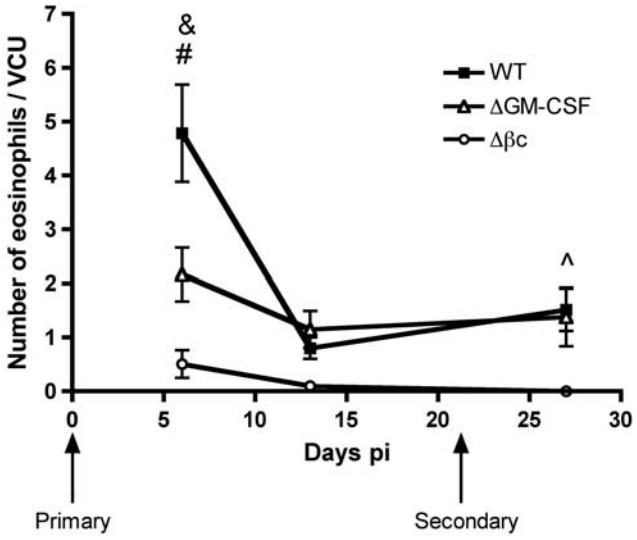

Figure 4 Intestinal eosinophilia. Mean number of eosinophils per villus crypt unit (VCU) ± s.e.m. in sections of small intestine extracted at different time points throughout infection. n=5–10. $^{\#}P<0.05$ between WT and ΔGM-CSF, $^{\&}P<0.01$ between WT and Δβc, $^{\wedge}P<0.05$ between WT and Δβc, other differences not significant.

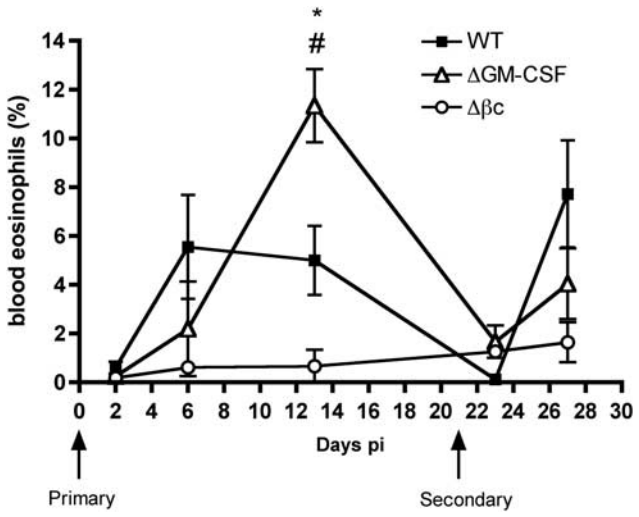

Figure 5 Blood eosinophilia. Blood eosinophils (% of leukocytes) measured over a period of 27 days p.i. Data represent mean blood eosinophil data ± s.e.m., n=5–7. $^{*}P<0.05$ compared between WT and ΔGM-CSF, $^{\#}P=0.0575$ compared between WT and Δβc.

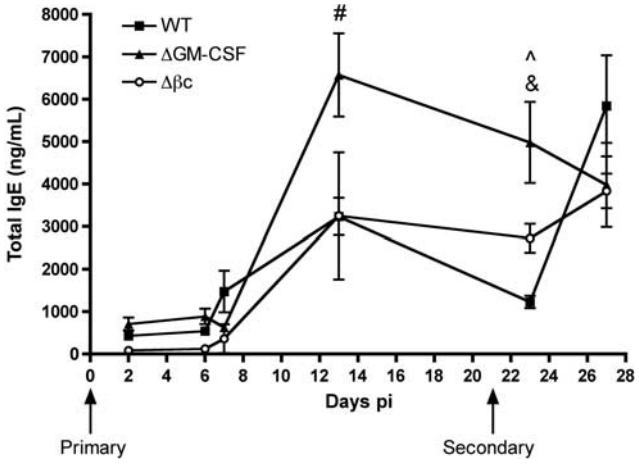

Figure 6 Total serum IgE. Blood was collected from mice at various times after primary and secondary *N. brasiliensis* infections. Data represent mean total IgE levels ± s.e.m., n=5–10. $^{\#}P<0.05$ between WT and ΔGM-CSF, $^{\&}P<0.001$ between WT and ΔGM-CSF, $^{\wedge}P<0.001$ between WT and Δβc.

remained consistently low throughout the course of primary and secondary infections (Figure 5).

Total serum IgE levels peaked in all groups at day 13 after the primary infections (Figure 6). The highest levels at this time were found in the ΔGM-CSF mice. IgE remained high in the ΔGM-CSF mice after the commencement of the secondary infection. This pattern resembled that for blood eosinophil numbers, in which the ΔGM-CSF mice also had the highest values at day 13 and 23. An anamnestic response was seen in WT mice, whereas a more sustained response was evident in the Δβc mice, consistent with a more prolonged infection.

Host weight changes during infection

Mice were weighed regularly following primary and secondary infection. All groups lost weight during the first 2 days, after which they began to gain weight (Figure 7). After the infection had been cleared

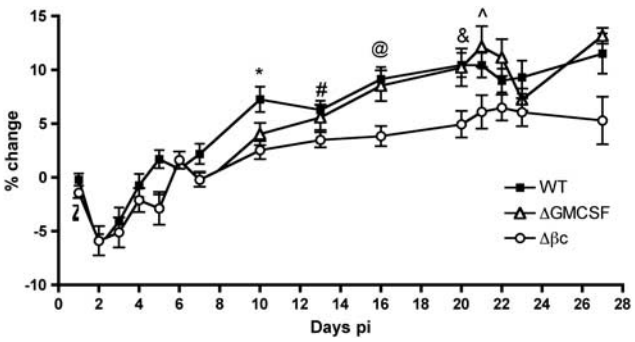

Figure 7 Effect of primary and secondary infection on body weight. Data represent mean % change from initial weight ± s.e.m., n=12–17. $^{*}P<0.01$ between WT and Δβc, $^{\#}P<0.05$ between WT and Δβc, $^{@}P<0.01$ between WT and Δβc, $^{\&}P<0.01$ between WT and Δβc, $^{\wedge}P<0.05$ between WT and Δβc.

by day 11, ΔGM-CSF mice gained weight at a similar rate to WT mice, whereas Δβc mice gained weight at a significantly slower rate and remained lighter throughout the secondary infection (Figure 7). This is consistent with the finding that Δβc mice had a higher parasite burden in secondary infections. The weights of the other groups were not significantly affected by the second infection.

DISCUSSION

The aim of this study was to determine the importance of GM-CSF in mediating resistance against *N. brasiliensis* infections. GM-CSF has a key part in mediating inflammation in various conditions,[1] including allergic inflammation,[9] however, its role in resistance against parasite infections had not been described. We subjected WT mice, ΔGM-CSF mice and Δβc mice to both primary and secondary infection with *N. brasiliensis*. ΔGM-CSF mice were compared with Δβc mice to establish any differences between the absence of GM-CSF alone and absence of function of both GM-CSF and IL-5.

In primary *N. brasiliensis* infection, there were no significant differences between the ΔGM-CSF and WT mice in terms of parasite burden in the lung or intestine, whereas the Δβc mice had increased egg production and a trend towards increased intestinal worm burden. When mice were given a second infection with *N. brasiliensis*, all groups showed decreased worm burden in both the lung and intestinal phases, and decreased egg production, compared with primary infection. These results indicate the development of protective immunity, which has been reported as a feature of *N. brasiliensis* and certain other parasite infections.[17,23,24] In the secondary infection, there was no increase in parasite burden in the ΔGM-CSF mice compared with the WT mice. In fact, during early secondary infection (lung phase), parasite numbers were significantly lower in ΔGM-CSF mice than in WT mice. However, the Δβc mice exhibited increased worm burden and egg production. These experiments indicate that GM-CSF is not required for the protection against primary or secondary infection with *N. brasiliensis*, whereas mice lacking both GM-CSF and IL-5 have impaired resistance to infection with this parasite. These findings imply that IL-5 is required for resistance to infection, and are consistent with our earlier report that mice lacking IL-5 are more susceptible to primary and secondary *N. brasiliensis* infections.[17]

In the ΔGM-CSF mice, there was a marked peak in peripheral blood eosinophils at day 13 of primary infection, followed by relatively high eosinophil numbers in BAL fluid 2 days after the secondary infection. Previous studies in mice with IL-5 transgene-induced

eosinophilia and mice with defects in eosinophilopoiesis have demonstrated that eosinophils contribute to anti-parasite resistance during primary and secondary infection with *N. brasiliensis*.[19,20] The eosinophilia in the ΔGM-CSF mice may have contributed to the reduced lung larval burden seen relative to that in WT mice. The finding of increased blood and tissue eosinophils in the ΔGM-CSF mice is surprising, because GM-CSF has a key role in mediating tissue eosinophilia in allergic inflammation.[9] However, it seems that in the case of *N. brasiliensis* infection, GM-CSF is not essential for eosinophil recruitment or survival.

GM-CSF has been shown to have a role in eosinophil recruitment to tissues,[9,25] and so it is possible that the high blood eosinophil levels at day 13 in ΔGM-CSF mice could arise as a result of a defect in eosinophil recruitment to the sites of inflammation. However, our studies in the two key sites of eosinophil accumulation, the lung and the intestine, did not reveal any reduction in eosinophil numbers in the tissues of ΔGM-CSF mice, suggesting recruitment was not impaired. Despite increased eosinophil numbers in the blood and the tissues, no significant changes were detected in IL-5, the key cytokine in eosinophil production. Although we were not able to demonstrate increased IL-5 production, it is possible that ΔGM-CSF mice may overproduce this cytokine at certain times or locations that were not assessed. It is also possible that the elevated eosinophilia in the ΔGM-CSF mice may have arisen because of complex interactions between host and parasite that might arise from GM-CSF inducing a negative regulator of eosinophilia. Alternatively, there might have been changes in other cytokines involved in Th2 responses, such as IL-25, IL-33 and thymic stromal lymphopoietin.[26–28]

In the Δβc mice, the number of eosinophils in the blood and intestine was very low throughout primary and secondary infection with *N. brasiliensis*. These findings confirm and extend earlier work by Nishinakamura *et al*.,[29] although their study did not report parasite burden. The reduction in eosinophil numbers is likely to explain the increased parasite numbers that we observed in the Δβc mice. Although eosinophils were markedly reduced in Δβc mice, the presence of small numbers of eosinophils, especially in the lungs in primary infection, suggests an alternative pathway of generation. IL-3 is a candidate to stimulate such a pathway, because in these mice, IL-3 can act via the alternative β-chain receptor for IL-3,[22] and IL-3 can act as an eosinophil growth factor.[30] However, eosinophils were reduced or undetectable in the intestine in primary and secondary infection, and in the lungs in secondary infection, suggesting that such an alternative pathway of generation is most apparent in the presence of the very high parasite load in the primary infection in the lungs.

The present experiments add weight to the argument that IL-5 is the most important cytokine for eosinophil production. Although GM-CSF can stimulate eosinophil production *in vitro*,[31] it is not an essential growth factor that precedes the action of IL-5 in eosinophilopoiesis. There is no evidence for defective eosinophil production in ΔGM-CSF mice under steady state conditions,[2] in allergic airway inflammation[9] or in parasite infection as indicated in the present report. By contrast there is evidence for a requirement for GM-CSF for tissue eosinophilia in allergic airway inflammation[9] and in peritoneal inflammation.[32] However, the current studies demonstrate that during infection with *N. brasiliensis*, there are sufficient stimuli to induce tissue eosinophilia in the absence of GM-CSF.

Serum IgE levels in ΔGM-CSF mice exhibited a delayed response, which by day 13 was elevated compared with WT mice. This delayed peak is similar to that seen with blood eosinophil counts in the ΔGM-CSF mice. Despite the increase in serum IgE levels, no increase in IL-4, a key cytokine required for IgE production in mice, was detected in the BAL fluid of infected ΔGM-CSF mice.[33] Serum IgE levels in Δβc mice rose after the initial infection, and remained elevated. Despite the increased parasite burden in Δβc mice, there were no apparent differences in IgE or IL-4 levels in these mice compared with WT mice.

This study has shown clearly that GM-CSF, although necessary for induction of inflammation in Th2-associated allergic airway inflammation, does not have a similar role in the Th2 response to *N. brasiliensis* infection. The findings are relevant to the clinical use of biological agents that block GM-CSF activity. A number of such reagents are undergoing clinical trials.[1] Our studies on ΔGM-CSF mice suggest that patients treated with agents that inhibit GM-CSF may not be at increased risk of infection with helminthic parasites.

METHODS

Animals

CSF2 gene-knockout mice (ΔGM-CSF) and common β-chain receptor knockout (Δβc) mice, derived on a C57BL/6 background, were provided by the Ludwig Institute for Medical Research (Melbourne, Australia).[2] ΔGM-CSF mice were bred at the Garvan Institute of Medical Research and then maintained as a closed line at the Australian BioResources facility in Moss Vale, New South Wales, Australia. C57BL/6 WT mice were purchased from the Animal Resource Centre (Perth, Western Australia, Australia). All mice were housed in the specific pathogen-free animal facility at the Garvan Institute of Medical Research and were closely age-matched within experiments. All procedures were approved by the Garvan Institute of Medical Research/St Vincent's Hospital Animal Ethics Committee and were conducted according to the Animal Welfare Guidelines of the National Health and Medical Research Council of Australia.

N. brasiliensis infection of mice

Infective third-stage larvae (L3) of *N. brasiliensis* were prepared at the University of Adelaide as described previously[34] and transported to the Garvan Institute. On arrival, L3 were washed extensively in sterile Dulbecco's phosphate buffered saline (DPBS) and 500 live L3 in 100 μl DPBS were injected subcutaneously into each mouse. L3 were injected over the abdomen using a 21-gauge needle. Some mice were given a secondary infection, by subcutaneous injection with 500 live L3 at day 21 after the first injection.[34]

Recovery of lung larvae

Lungs were removed at day 2 p.i., washed in phosphate-buffered saline, finely minced with scissors and incubated in phosphate-buffered saline at 37 °C for at least 4 h to promote migration of larvae out of the lung fragments. The larvae were counted using a dissecting microscope.

Intestinal worm recovery

The small intestine was removed at day 6 p.i., rinsed in DPBS, split longitudinally and placed in DPBS. Samples were incubated at 37 °C for at least 4 h to encourage worm detachment from the wall of the tissue. Worm numbers that emerged from intestinal tissue were determined using a dissecting microscope. The small intestine was also examined by stereomicroscopy to ensure that remaining worms still attached to the wall were included in the counts.

Faecal egg counts

Egg counts were performed at day 6 p.i. by extracting well formed faecal pellets from the rectum of each mouse. Faecal pellets were weighed and then homogenised in 400 μl of DPBS for 10–15 min before disaggregation. To promote egg floatation, 400 μl of saturated sodium chloride solution was added to each sample. A Whitlock Universal slide chamber (JA Whitlock & Co, Eastwood, New South Wales, Australia) was loaded with the whole sample and total egg counts were performed at ×10 magnification using a compound light microscope.

Serum IgE and cytokine ELISA

Total serum IgE was detected by using the Mouse IgE ELISA Set (BD Biosciences Pharmingen, San Diego, CA, USA) according to the manufacturer's instructions. Absolute concentrations were determined with the manufacturer's

IgE standard. The lower detection limit was 5 ng ml^{-1}. Sera were diluted 1:50 with phosphate-buffered saline. Cytokine concentrations in BAL fluids and culture supernatants were measured by mouse IL-4 and IL-5 ELISA sets (BD Biosciences Pharmingen) according to the manufacturer's instructions, with lower detection limits of 7.8 and 15.6 pg ml^{-1}, respectively. BAL fluids used for cytokine detection were taken from the first lavage.

Analysis of cell subpopulations in blood

Blood samples were obtained by cardiac puncture at different days p.i. Blood was collected in syringes containing 20 µl of 0.005 M EDTA and thoroughly mixed. Blood samples were analysed by an automated Abbott Cell Dyn Haematology Analyzer (Abbott Diagnostics, Abbott Park, IL, USA).

Statistical analysis

Data from experiments were analysed by Student's *t*-test with significance achieved when $P<0.05$. Statistical tests were performed using GraphPad Prism software (La Jolla, CA, USA).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

The work was supported by the Co-operative Research Centre for Asthma, by an Australian Postgraduate Award to DSCS, and by the Faculty of Medicine, University of NSW.

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