G-CSF suppresses allergic pulmonary inflammation, downmodulating cytokine, chemokine and eosinophil production

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A B S T R A C T

Aims: Granulocyte Colony-Stimulating Factor (G-CSF), which mobilizes hemopoietic stem cells (HSC), is believed to protect HSC graft recipients from graft-versus-host disease by enhancing Th2 cytokine secretion. Accordingly, G-CSF should aggravate Th2-dependent allergic pulmonary inflammation and the associated eosinophilia. We evaluated the effects of G-CSF in a model of allergic pulmonary inflammation.

Main methods: Allergic pulmonary inflammation was induced by repeated aerosol allergen challenge in ovalbumin-sensitized C57BL/6j mice. The effects of allergen challenge and of G-CSF pretreatment were evaluated by monitoring: a) eosinophilia and cytokine/chemokine content of bronchoalveolar lavage fluid; pulmonary interstitium, and blood; b) changes in airway resistance; and c) changes in bone-marrow eosinophil production.

Key findings: Contrary to expectations, G-CSF pretreatment neither induced nor enhanced allergic pulmonary inflammation. Instead, G-CSF: a) suppressed accumulation of infiltrating eosinophils in bronchoalveolar, peribronchial and perivascular spaces of challenged lungs; and b) prevented ovalbumin challenge-induced rises in airway resistance. G-CSF had multiple regulatory effects on cytokine and chemokine production: in bronchoalveolar lavage fluid, levels of IL-1 and IL-12 (p40), eotaxin and MIP-1α were decreased; in plasma, KC, a neutrophil chemoattractant, was increased, while IL-5 was decreased and eotaxin was unaffected. In bone-marrow, G-CSF: a) prevented the increase in bone-marrow eosinophil production induced by ovalbumin challenge of sensitized mice; and b) selectively stimulated neutrophil colony formation.

Significance: These observations challenge the view that G-CSF deviates cytokine production towards a Th2 profile in vivo, and suggest that this neutrophil-selective hemopoietin affects eosinophilic inflammation by a combination of effects on lung cytokine production and bone-marrow hemopoiesis.

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Introduction

Granulocyte Colony-Stimulating Factor (G-CSF) (Demetri and Griffin 1991) promotes expansion and maturation of neutrophil populations, further increasing their effector capacity and lifespan (Shochat et al. 2007). G-CSF mobilizes hemopoietic stem cells (HSC) to peripheral blood, which is increasingly used as a source of HSC for transplantation (Pusic and DiPersio 2008; Nervi et al. 2006). G-CSF has numerous additional immunoregulatory effects (Rutella et al. 2005; Xiao et al. 2007). Unexpectedly, G-CSF use in HSC mobilization decreases the incidence of severe acute graft-versus-host disease (GVHD), a major complication of HSC transplantation in both humans and mice (Bensinger et al. 2001; Berger et al. 1999; Ji et al. 2002). Because acute GVHD is mediated by Th1 lymphocytes (Ferrara 1998), and Th2 lymphocytes prevent the disease (Fowler et al. 1994), it has been suggested that G-CSF promotes Th2 responses (Pan et al. 1995), an effect believed to underlie its beneficial effects in other autoimmune and inflammatory diseases (Hadaya et al. 2005; Hommes et al. 1996; Zavala et al. 2002). However, G-CSF suppresses production of both Th1 and Th2 cytokines by activating neutrophil granulocytes (Vasconcelos et al. 2003), which possibly accounts for its protective activity against GVHD in humans and mice (Vasconcelos et al. 2006). This prompted us to reexamine the assumption that G-CSF acts in vivo to promote Th2-mediated responses, using a murine model for allergic pulmonary inflammation. This model is highly dependent on Th2 lymphocytes,
which secrete IL-4, IL-5 and IL-13 (Townley and Horiba 2003). IL-5 maintains production of eosinophils in the bone-marrow (Denburg et al. 1997), promotes their mobilization and migration into challenged sites (Rosenberg et al. 2007) and increases their lifespan in tissues (Rothenberg and Hogan 2006). Accordingly, bone-marrow and blood eosinophilia, and lung eosinophil infiltration depend on IL-5. Both eotaxin and IL-13 enhance eosinophil production in the presence of IL-5 (Queto et al. 2010). Eotaxin and IL-13 also interact with IL-5 to induce lung eosinophilic inflammation and airway hyperreactivity (Townley and Horiba 2003; Effros and Nagaraj 2007). These Th2 effects are effectively counteracted by Th1 responses (Effros and Nagaraj 2007).

Due to its sensitivity to both Th1 and Th2 influences, allergic pulmonary inflammation in mice should provide useful information about the immunomodulatory effects of G-CSF in vivo. We describe here, for the first time, a strong inhibitory effect of G-CSF on multiple parameters of allergic pulmonary inflammation, especially down-modulation of inflammatory cytokine, eosinophil-selective chemokine and eosinophil production.

Methods

Animal procedures

CS7BL/6j male and female mice, aged 8 week, provided by CECAL (FIORUZ, Rio de Janeiro, Brazil) were immunized with two s.c. 0.4 ml injections of 100 μg ovalbumin (OVA), mixed with 4 mg/ml Al(OH)₃ in 0.9% NaCl, at 7 day intervals (Gaspar Elsas et al. 1997), as approved by the institutional ethics committee (CEUA-FIOCRUZ license #PO107-02). Beginning 5 days after the second injection, mice were challenged on two consecutive days with aerosolized OVA (5 g/100 ml in saline, 1 h), or saline (SAL) as a negative control, and sacrificed 24 h after the last challenge. Mice received rhG-CSF (Bioptintética, São Paulo, Brazil), 1.2 μg/g body weight/day s.c. (OVA-G; SAL-G), for 5 days, beginning 3 days before challenge and ending before last challenge, or vehicle as a negative control (OVA, SAL).

Pulmonary inflammation and function studies

Bronchoalveolar lavage fluid (BALF) collected after flushing the lungs with 0.5 ml chilled PBS containing 1% FCS through a tracheal cannula was used for total and differential cell counts and cytokine quantification. For histological analyses, lungs were fixed 48 h in buffered formalin (10%), inclusion (LEICA EG1160), sectioning (LEICA RM2155) and H&E staining or PAS staining. Mucus-producing (goblet) cells were stained with PAS. Tissue sections were examined under 100-× magnification, in a Leica DMLS microscope, and photographed using a Leica DMLS 300F camera (1300 × 1300 pixels). Pulmonary function was evaluated 24 h after the last challenge. Air flow and transpulmonary pressure were recorded in individual mice with a Finepointe RC (Buxco Research System) under anesthesia (Nembutal 60 μg/kg), neuromuscular blockade (bromide pancuronium 1 mg/kg) and mechanical ventilation, following tracheostomy, cannulation and connection to a pneumotachograph. Mice were exposed to aerosolized methacholine (3–27 mg/ml, for 2.5 min) or PBS after 5 min stabilization. Airway resistance (cm H2O/ml/s) and dynamic lung compliance (ml/cm H2O) were calculated and digitized per breathing cycle. Increases in enhanced pause (Penh) were also recorded during 5 min in conscious, unrestrained mice preexposed for 2.5 min to methacholine (6–25 mg/ml), using barometric whole-body plethysmography (Buxco Research System, Wilmington, NC). Where indicated, mice of all groups (n = 3 in all cases) were used as a source of lung-infiltrating CD4+ and CD8+ T lymphocytes, following the procedures of Maximiano et al. 2005. Lungs were washed free of blood, excised, minced and digested in 2 ml Iscove Modified Dulbecco's Medium containing 24 mg/ml collagenase (Sigma C0130, St. Louis, MO) and 0.125 mg/ml DNAase (Sigma AMPD1) for 20 min, at 37 °C. The resulting cell suspension was filtered in a cell strainer (Falcon 2360) and separated on a discontinuous Percoll gradient, with lymphocytes being recovered from the 60–75% Percoll interface. After staining with FITC-conjugated anti-mouse CD4 antibody (Cat: 11-0041-82, ebioscience, San Diego, CA) and PE-conjugated anti-mouse CD8 antibody (Cat: 12-0083-82, ebioscience), lymphocytes were analyzed for FSC, SSC, FL-1 (CD4+) and FL-2 (CD8+) in a FACScanTO II (BD Biosciences Pharmingen, San Diego, CA), with the Flowing Software 1.60. Isotype controls were Rabbit (sc-3871) IgG–PE and Rabbit (sc-3870) IgG–FITC (Santa Cruz Biotech, Santa Cruz, CA).

Cytokine assays

BAL fluid and plasma samples from each mouse were obtained 24 h after last challenge for multiplex cytokine quantifications (Bio-Rad Luminex Kit, Life Science, CA, USA), using 23-Plex Mouse cytokines (eotaxin, G-CSF, GM-CSF, IFN-γ, IL-10, IL-12 p40, IL-12 p70, IL-13, IL-17, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, KC, MCP-1, MIP-1α, MIP-1β, RANTES and TNF-α) in BALF (50 μl) or serum (10 μl) according to the manufacturer’s instructions. Data acquired in a Luminex 200 Total System Instrument were analyzed by xPONENT 3.1 software, which enables simultaneous detection of multiple cytokines from a single sample.

Antibody quantitation

Ovalbumin (Cat: 950512, ICN Biomedicals Aurora, OH), 20 mg/ml in 0.1 M sodium phosphate buffer, pH 8, was used to coat NUNC MaxiSorbTM 96- well plates, 50 μl per well, overnight, at 4 °C, before washing with PBS-Tween 20 (0.1%) and quenching for 2 h with PBS containing 1% BSA (100 μl per well). 100 μl serial dilutions of plasma in PBS/BSA, were added and incubated overnight at 4 °C before further washing. For IgG quantitation, 100 μl of goat anti-mouse IgG (H+L)-HRP-conjugated antibody (Cat: 1031-05, Southern Biotechnology, AL), diluted 1:000 in PBS/BSA were then added to each well for 2 h, before washing and developing with 100 μl of OPD (0.5 mg/ml, in Perborate buffer of 12.15 mg/ml) for 2 min. The reaction was stopped and OD490 was determined. For IgG quantitation, 100 μl of rat anti-mouse IgG (Cat: 1130-01, Southern Biotechnology) diluted 1:1000, as a primary antibody, and 100 μl of rabbit anti-IgG, biotinylated, as a secondary antibody, were used sequentially, for 2 h and 1 h respectively. After washing out unbound IgG, 100 μl avidin-conjugated HRP (Cat: 004100-EN2, ebioscience), diluted 1:250, were added for 1 h. Developing was as above.

Hematological procedures

Blood sampled from vena cava was used for total cell haemocytometer counts, and Wright–Giemsa-stained smears for differential leukocyte counts. Bone-marrow cells were obtained by flushing the two femurs of each mouse with RPMI 1640 medium containing 1% FCS. Liquid cultures were used to evaluate the response of lineage–committed eosinophil and neutrophil precursors (Gaspar Elsas et al. 1997). Briefly, 10⁵ bone-marrow cells were seeded in 1 ml of RPMI 1640 medium, with 10% FCS, in the presence of rmIL-5 (1 ng/ml), or rmGM-CSF (1 ng/ml) and incubated at 37 °C, 5% CO2/95% air for 7 (for eosinophils) or 6 (for neutrophils) days. Cells were resuspended, counted, cytocentrifuged and stained (for eosinophils, for eosinophil peroxidase, or EPO, with counterstaining by Harris’ Haematoxylin (Ten et al. 1989); for neutrophils, Wright–Giemsa (Gaspar-Elsas et al. 2009), before differential counts. We have previously reported that: a) EPO-staining closely matches immunostaining for CCR3 and Wright–Giemsa staining (Gaspar-Elsas et al. 2009); and b) these culture conditions support eosinophil proliferation and terminal differentiation, allowing detection of both enhancing (Gaspar-Elsas et al. 2000a) and suppressive (Gaspar-Elsas et al. 2000b) effects. BALF eosinophils and neutrophils were stained with Hemacolor Kit (Merck, Rio de Janeiro, Brazil).
Janeiro, Brazil). Colony formation assays were used to evaluate G-CSF effects on granulocyte and mononuclear phagocyte progenitors, because they: a) allow precise identification of the cytokine target (Gaspar-Elsas et al. 2000b); and b) eliminate mature macrophages, present in liquid culture, even when no cytokine is added (Gaspar-Elsas et al. 1997). Briefly, semisolid clonal cultures were seeded in triplicate (2×10^4 cells, 35 mm culture dishes, 1 ml total culture volume), in MethoCult® (StemCell Technologies) medium, supplemented with 15% FCS, 1% BSA, 10 μg/ml Insulin, 200 μg/ml Transferrin, 50 ng/ml rmSCF, 10 ng/ml rmIL-3, 10 ng/ml rhIL-6, and colonies at Day 14 were scored under an inverted microscope, following the manufacturer’s instructions (Mouse Colony-Forming Cell Assays Using MethoCult® TECHNICAL MANUAL, Stem Cell Technologies).

Statistical analysis

The significance of the differences observed among groups was determined by ANOVA with Bonferroni’s correction, using Prism 4.00 for Windows (GraphPad Software, San Diego, California, USA).

Results

G-CSF effects on inflammation and specific immune responses

Groups of OVA-sensitized mice were treated with G-CSF or vehicle, and challenged with OVA or SAL. 24 h after the last challenge, all mice were sacrificed and BALF was collected to monitor leukocyte accumulation. Large inflammatory leukocyte counts were obtained in BALF of OVA mice (Fig. 1A). As expected, very small numbers were recovered from the SAL mice (unchallenged control). Contrary to expectations, G-CSF-treated OVA-G mice presented a very significant decrease in BALF leukocyte counts relative to OVA mice (challenged control). By contrast, G-CSF had no significant effect in unchallenged mice (SAL-G versus SAL). Eosinophils were the major leukocyte class in BALF from OVA mice (1B), but only a minor component of BALF leukocytes in OVA-G mice (p<0.001 relative to OVA controls) as well as in unchallenged controls. In all groups but OVA mice, the predominant BAL leukocyte populations were lymphocytes and mononuclear phagocytes.
Significant differences in blood total leukocyte (1C), eosinophil (1D) and neutrophil (1E) counts were also observed following G-CSF administration. G-CSF pretreatment significantly increased blood eosinophil counts in OVA-G mice, relative to the three other groups. Furthermore, G-CSF pretreatment also increased blood neutrophil counts in the OVA-G and SAL-G groups, relative to the respective vehicle-treated controls, OVA and SAL. Importantly, this increase in blood neutrophils was not paralleled by BALF neutrophil counts (see above).

Fig. 2 shows representative views of H&E-stained lung sections from mice of the different groups. SAL (2A) and SAL-G (2B) mice had no detectable pulmonary inflammatory infiltrates. By contrast, extensive inflammatory infiltration, both perivascular and peribronchial, was detected in the lungs of OVA mice (2C, 2E, and 2G). These infiltrates often bridged small vessels and airways (2E). In OVA-G mice (2D), these infiltrates were considerably smaller, but not completely eliminated (2F). Eosinophils were numerous in the lung infiltrates from OVA mice (2G). In OVA-G mice, infiltrates still presented a minor eosinophil component (2H). Only in OVA-G mice eosinophils were observed inside blood vessels (2H).

A small number of goblet cells were evidenced through PAS staining among the epithelial cells of the airways, in mice of the SAL (2I) and SAL-G (2J) groups. By contrast, they were strikingly increased in number, and more intensely stained, in mice of the OVA (2K) and OVA-G (2L) groups.

As shown in Fig. 3A, pulmonary resistance was significantly increased in mice of the OVA group, relative to the negative (SAL and SAL-G) controls, after exposure. By contrast, it was brought back to the negative control level in OVA-G mice. Furthermore, as shown in 3B, enhanced pause (Penh) measurements provided similar findings.

Samples collected 24 h after last challenges were assayed for a wide panel of cytokines, and significant differences between groups are shown here for BALF and serum. BALF levels of inflammatory cytokines (IL-1α, IL-β and MIP-1α) were reduced by G-CSF (Fig. 4A).

Fig. 2. Effect of G-CSF pretreatment on challenge-induced inflammatory infiltration of the lungs. Representative views of the lungs of mice belonging to the indicated groups (n = 3 for all groups), following staining with H&E (A–H) and PAS (I–L): A, I, SAL; B, J, SAL-G; C, E, G, K, OVA; and D, F, H, L, OVA-G. Magnification: A, B, C, D, 100× (Bar = 500 μm); E, F, 400× (125 μm); G, H, 1000× (50 μm); and J, K, L, 200× (250 μm). G and H show details of the images in E and F, respectively.
Furthermore, both IL-12 p40, which induces Th1 cell differentiation, and eotaxin, which interacts with IL-5 and IL-13 to sustain eosinophilia in asthma models (Pope et al. 2001) were similarly reduced in BALF. Serum cytokine levels also differed between groups (4B). Importantly, G-CSF did not decrease serum eotaxin levels in OVA-G mice relative to OVA controls, as it did in BALF. By contrast, G-CSF had no impact (SAL versus SAL-G). By contrast, in vitro neutrophil counts (6C) did not follow this pattern, being comparable in all groups except SAL-G, which had significantly increased counts relative to OVA mice.

G-CSF effects on eosinopoiesis

Total and differential cell counts in freshly collected bone-marrow (Day 0) were used to evaluate G-CSF effects on hematopoiesis in vivo. Total nucleated cell counts were significantly decreased in bone-marrow of OVA-G mice relative to all other groups (Fig. 6A). In OVA-challenged mice, G-CSF-induced decreases bone-marrow cell counts were associated with increased blood leukocyte counts (1C). By contrast, no significant effect of G-CSF was found between unchallenged control groups. As expected, eosinophil-lineage (EPO+) cell counts were significantly increased by OVA-challenge (6B; OVA versus SAL). Importantly, the increase in eosinophils following challenge was prevented by G-CSF treatment (OVA versus OVA-G).

To confirm the lineage selectivity of these effects, we examined whether G-CSF would similarly suppress production of mononuclear phagocytes, which, like eosinophils, share an immediate common ancestor with neutrophils, the primary G-CSF target [30]. CFU-M (7A), CFU-GM (7C) and total colony (CFU-C, 7D) counts differed significantly between groups (Fig. 7). As expected, G-CSF treatment significantly increased the numbers of CFU-G in both challenged (OVA-G) and unchallenged (SAL-G) mice relative to their respective untreated controls. In SAL-G mice, the significant increase in all colony forming cells detected (CFU-C) could be accounted for by the increase in CFU-G alone. By contrast, in OVA-G mice, the increase in CFU-G was not accompanied by significant increases in CFU-C. In no case, however, significant suppression of CFU-M and CFU-GM due to G-CSF was observed.

Discussion

This study documents a novel modulatory effect of G-CSF in a model of allergic pulmonary inflammation, accompanied by a marked suppression of bone-marrow eosinophil production. OVA-G mice, as compared to the OVA controls, presented: a) decreased pulmonary infiltration; b) reversal of the challenge-induced changes in airway resistance; and c) down-modulation of cytokines associated with innate immune responses (IL-1α, IL-1β and MIP-1α), induction of Th1...
responses (IL-12 p40) and eosinophilia (IL-5, eotaxin). However, contrary to the assumptions commonly held regarding the mechanism of G-CSF beneficial actions in GVHD, no clear enhancement of Th2 cytokine production was observed. Indeed, a decrease in IL-5 was the only significant impact of G-CSF on Th2 cytokines.

In humans, G-CSF is believed to promote a tolerogenic environment through multiple regulatory effects on T lymphocyte gene expression and transcription factor activity (Toh et al. 2009). It was therefore important to define, in our study, whether suppression of specific antibody production or changes in infiltrating T lymphocyte populations underlie the attenuated allergic reaction in the lungs. No decrease in either OVA-specific serum IgG or IgE was found in OVA-G relative to OVA mice. The only significant change was increased serum IgG in SAL-G relative to SAL mice, but this observation is not relevant to the pulmonary inflammatory reaction, which requires challenge. The lung-infiltrating CD4+/CD8+ T cell populations were similarly unaffected by G-CSF. Overall, effects on specific immune responses do not account for the attenuation of challenge effects in the lungs.

These results ruled out the possibility that G-CSF, by promoting production of IL-5, IL-13 and other Th2 cytokines, would increase eosinophil numbers, independently of airway OVA-challenge (Xavier-Elsas et al. 2007). G-CSF had no impact of its own on the eosinophil lineage: regarding total cells and eosinophils in BALF, infiltrating eosinophils, release of eosinophil chemotactants, or bone-marrow eosinophil production, in vivo and ex vivo, SAL-G mice were indistinguishable from SAL controls. This cannot be ascribed to ineffectiveness of G-CSF pretreatment in unchallenged mice, because G-CSF strongly increased CFU-G counts in SAL-G mice, leading to overall increase in CFU-C.

By contrast, G-CSF strongly affected eosinophils when administered before OVA challenge. Challenge, therefore, unveils an important ability of G-CSF to regulate eosinophilia and eosinopoiesis, which is not apparent in unchallenged controls. G-CSF pretreatment reduced: a) eosinophil numbers in lungs and bone-marrow; b) pulmonary eosinophil accumulation; and c) eotaxin content of BALF. By contrast, eotaxin in blood was unaffected. Therefore, reduction of eosinophil infiltrates coincided with a tissue-to-blood eotaxin concentration gradient unfavorable to eosinophil recruitment. G-CSF might, therefore, work in part by suppressing chemoattractant generation at the challenge site.

However, G-CSF also decreased eosinophil numbers in OVA-G bone-marrow. Because a reduction in eotaxin-driven mobilization would likely increase, not decrease, bone-marrow eosinophils, a separate effect of G-CSF on eosinopoiesis should be investigated.

The impacts of G-CSF on bone-marrow eosinophil counts in vivo and on responses to IL-5 ex vivo were evaluated in parallel, since both parameters are upregulated in vivo (Ten et al. 1989) by allergen challenge. Both assays show a strong suppressive impact of G-CSF on eosinopoiesis. By contrast, no suppressive effect on the closely related mononuclear phagocyte lineage-selective modulation mechanism, rather than a generalized myelosuppressive activity, accounts for the scarcity of eosinophils in OVA-G bone-marrow and lungs. Importantly, this mechanism requires interaction between challenge and G-CSF pretreatment, because suppressive effects were always observed in challenged mice but not unchallenged controls.

In humans, G-CSF treatment induces overexpression of negative regulators of Th17 differentiation, ultimately decreasing the numbers of T cells with a Th17 phenotype to one-third of those found in normal controls (Toh et al. 2009). Because IL-17 is important in neutrophil recruitment and inflammation, a possible impact of G-CSF...
on IL-17 production might be relevant to the interpretation of our findings. In our study, unlike that of Toh et al., G-CSF had no impact on IL-17 levels. This apparent inconsistency may originate in several factors, including both species (humans versus mice) differences and the level of analysis (gene expression versus secreted cytokines). However, one should bear in mind that the relationship of G-CSF and IL-17 is not always antagonical, for G-CSF is believed to mediate some of the effects of IL-17 on neutrophil production in vivo (Stark et al. 2005).

G-CSF increased neutrophils in blood and neutropoiesis in culture, as expected from expression of its receptor in the neutrophil lineage (Toba et al. 1999). However, since eosinophils lack G-CSF receptors (Toba et al. 1999), and G-CSF does not stimulate eosinophil colony formation (Enokihara et al. 1988) by itself, an indirect mechanism is likely to account for its effects. Although G-CSF might indirectly affect the eosinophil lineage by favoring neutrophils in competition for critical resources in a restricted bone-marrow environment, shared by these closely related lineages (Iwasaki et al. 2005), the same considerations would apply to mononuclear phagocytes, which were unaffected by G-CSF in this study. Alternatively, G-CSF could equally well suppress the eosinopoietic response to challenge by preventing generation of a systemically acting signal induced by airway allergen challenge of sensitized mice (Gaspar Elsas et al. 1997). This hypothesis would be consistent with the observation that G-CSF only affected eosinophils in challenged animals, which generate this signal, unlike unchallenged controls (Gaspar Elsas et al. 1997). Following our original demonstration by transfer experiments (Gaspar Elsas et al. 1997), a number of defined stimuli have been shown to upregulate eosinophil production in vivo and in vitro, including glucocorticoids, either exogenously administered (Gaspar-Elsas et al. 2009) or endogenously released from the adrenal glands of surgically stressed mice (Elsas et al. 2004). However, while glucocorticoid-induced eosinophilopoiesis has been shown to be blocked by the glucocorticoid receptor antagonist, RU486 (mifepristone) (Gaspar-Elsas et al. 2009), the nature of the mediator induced by allergen challenge remains undefined. G-CSF, the first

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**Fig. 6.** G-CSF treatment effectively suppresses eosinophil production. Data (Mean±SEM) are number of total cells (A) EPO+ cells (eosinophil-lineage cells at all stages of maturation; (B and D) and neutrophils (C and E), present in bone-marrow, either examined without further culture, 24 h after the last challenge (Day 0; A, B and C), or cultured for 7 days in the presence of the eosinophil-selective growth factor, IL-5 (1 ng/ml) (Day 7; D), or cultured for 6 days in the presence of GM-CSF 1 ng/ml (E). (*) p<0.05, (**) p<0.01 and (***) p<0.001 for the differences indicated. n=3 for all groups.
immunomodulatory cytokine able to prevent the effects of allergen challenge on bone-marrow eosinophilopoesis, may either prevent the generation of this systemic mediator, or interfere with its activity.

**Conflict of interest statement**

The authors state that they have no conflict of interest that might influence the design, interpretation or conclusions of this study, which was publicly funded and unrelated to the pharmaceutical/biotechnology industry.

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