Title: Pegfilgrastim enhances the antitumor effect of therapeutic monoclonal antibodies

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Financial support
This work was supported by the Lyric Grant INCa-DGOS-4664 (to C. Dumontet).

Conflict of Interest
C. Dumontet has previously received research funding from Roche.
Abstract

Therapeutic monoclonal antibodies (mAbs) exert antitumor activity through various mechanisms including apoptotic signalization, complement dependent cytotoxicity and antibody-dependent cellular cytotoxicity (ADCC) or phagocytosis (ADCP). G-CSF and GM-CSF have been reported to increase the activity of antibodies in preclinical models and in clinical trials. In order to determine the potential role of pegfilgrastim as an enhancer of anticancer antibodies we performed a comparative study of filgrastim and pegfilgrastim. We found that pegfilgrastim was significantly more potent than filgrastim in murine xenograft models treated with mAbs. This was observed with rituximab in CD20+ models and with trastuzumab in HER2+ models. Stimulation with pegfilgrastim was associated with significant enhancement of leukocyte content in spleen as well as mobilization of activated monocytes/granulocytes from the spleen to the tumor bed. These results suggest that pegfilgrastim could constitute a potent adjuvant for immunotherapy with mAbs possessing ADCC/ADCP properties.
Introduction

Monoclonal antibodies (mAbs) are increasingly used for the treatment of cancer patients. Their mechanisms of action are complex since antibodies can either target the tumor cells themselves or the microenvironment, including tumor vasculature or the surrounding immune cells. Among antibodies targeting tumor cells directly, various mechanisms of action are likely to be involved including direct apoptotic signalization after recognition of the cognate antigen and extracellular effector mechanisms such as complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) or its closely related phenomenon antibody-dependent cellular phagocytosis (ADCP) (1). Despite this diversity of mechanisms of antitumor activity, treatment by mAbs has not allowed to obtain cure in most of the approved indications, either as a single agent or in combination with other agents. The case of follicular non-Hodgkin’s lymphoma (NHL) is exemplary since certain patients can benefit from single agent therapy but resistance to treatment will eventually develop. In addition, a significant proportion of responding patients will no longer benefit of retreatment with this monoclonal antibody after relapse (2).

Most clinically used mAbs belong to the IgG subclass and their Fc domain interacts with IgG Fc receptors (Fcγ receptors). The human IgG receptor family includes several members, most of which are activating (CD64 or FcγRI, CD32a or FcγRIIa, CD16a or FcγRIIIa, CD16b or FcγRIIIb) and one which is inhibitory (CD32b or FcγRIIb) with various affinities for the IgG subclasses. Several of the mAbs currently used in the clinic rely at least partly on Fcγ receptor-mediated mechanisms and novel antibodies, such as obinutuzumab have been specifically engineered to possess enhanced ADCC properties (3). Fc receptor polymorphisms that affect affinity for IgG (FcγRIla-131H/R and FcγRIIIa-158V/F) have been found to influence the clinical response to rituximab (anti-CD20) (4), cetuximab (anti-EGFR)5 or trastuzumab (anti-HER2; human epidermal growth factor receptor 2) (5) therapy in lymphoma, colorectal and breast cancer patients, respectively, suggesting that Fcγ receptor-mediated effector functions play an important role in cancer treatment.
The nature of the Fcγ receptor-bearing cells involved in cytotoxic activity is not completely elucidated. Classically, Natural Killer (NK) cells have been considered as the main effector cells for ADCC (6). NK cells express the high affinity receptor FcγRIIIa, and the association between Fc receptor alleles and clinical success of mAb therapy has been attributed to NK cells (7). However, other cell populations can act as potential effector cells for mAb-mediated tumor regression, including myeloid effector cells such as monocytes/macrophages and neutrophils (8).

Neutrophils are the most abundant circulating leukocyte subpopulation in peripheral blood. In addition, this population can be amplified and/or mobilized by the administration of granulocyte (macrophage)-colony stimulating factors (G-CSF and GM-CSF) (9-11). Neutrophils have been described as potent cytotoxic effectors, able to produce many cytotoxic molecules (12) and exert direct tumoricidal activity (13). Their ability to directly recognize tumor cells is limited but is highly enhanced in the presence of mAbs (14).

Fcγ receptors expressed on neutrophils are FcγRIIa, FcγRIIIb, and FcαRI. FcγRIIIb is the most abundant Fcγ receptor on neutrophils, but evidence suggests that it is not involved in efficient tumor cell cytotoxicity (15). Antibody-dependent killing of tumor cells by neutrophils in the absence of G-CSF is described to be non-existent or weak (16, 17) and is likely mediated through FcγRIIa (18). Therefore, specific targeting of FcγRI has been proposed, as this may overcome potential inhibitory signals through FcγRIIb when IgG mAb are employed (19).

In order to improve antibody dependent cytotoxicity of leukocytes, co-administration of monoclonal antibodies and different cytokines has been performed, in particular with GM-CSF and G-CSF. Cartron et al (20) performed a phase II clinical study combining GM-CSF and rituximab in patients with relapsed follicular lymphoma. This study reported an improvement in the expected complete response rate with the combination compared to monotherapy (39% vs 6%, respectively) (21). The authors explain this result by the recruitment and activation of granulocytes and monocytes, leading to an increased antitumor potential, involving both ADCC and ADCP (22). While increasing the dose of GM-CSF appears to decrease the ADCC exerted by peripheral mononuclear cells (23), G-CSF or IFNγ-primev primed neutrophils have an increased potential for monoclonal antibody mediated cytotoxic activity through upregulated expression of FcyRI both in vitro and in vivo.
(24). Van der Kolk et al showed in a phase I/II clinical trial that, when G-CSF was combined with rituximab in CLL, the median time to progression observed for the combination was 24 months, and only 13 months for rituximab as a single agent although the overall response rate was comparable in both groups (25).

Recombinant G-CSF (filgrastim) used to treat patients has a short half-life of in the blood stream (3-4 hours). Pegfilgrastim, a filgrastim analog carrying a 20kDa polyethylene glycol molecule on its N-terminus has a half-life of 15 to 80 hours in blood. Pegylation increases the molecular weight of filgrastim above the threshold for renal clearance. Consequently, the drug is believed to be mostly cleared by neutrophils. Neutrophil-mediated clearance takes longer than renal clearance, thereby increasing the half-life of the drug. We show that filgrastim and pegfilgrastim display similar effects on normal human polymorphonuclear cells in vitro. However, in vivo, pegfilgrastim proved to be significantly more potent than filgrastim when combined with therapeutic monoclonal antibodies such as rituximab and trastuzumab. This in vivo effect was associated with a strong increase of spleen size as well as recruitment of neutrophils and activated monocytes in various compartments including the tumor site.
Materials and Methods

Reagents

Rituximab (MabThera, Roche) a chimeric monoclonal antibody directed against CD20, and trastuzumab (Herceptin, Genentech) a chimeric monoclonal directed against HER2/neu receptors were purchased as their commercial formulations. Filgrastim (Granocyte 34M, Chugai Pharmaceuticals), a recombinant granulocyte colony-stimulating factor synthesized in Chinese Hamster Ovary cells (CHO cells), and pegfilgrastim (Pegfilgrastim, Amgen) a recombinant human granulocyte colony-stimulating factor (filgrastim) coupled with a 20kDa polyethylene glycol (PEG) molecule to the N-terminus were also used as their commercial formulations.

Cell lines and culture

RL (ATCC: CRL-226), a human non-Hodgkin’s lymphoma B cell line expressing CD20 and two HER2 positive lines, the human lung adenocarcinoma epithelial cell line A549 (ATCC: CCL-185), and the breast cancer cell line MDA-MB361 (ATCC: HB-27), were purchased directly from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were authenticated by ATCC by generating human short tandem repeat profiles by simultaneously amplifying multiple STR loci and amelogenin (for gender determination) using the Promega PowerPlex Systems. These cells were cultured in the laboratory for less than 6 months in culture medium consisting of RPMI-1640 (Life Technologies), 10% fetal calf serum (Integro), 100 units/mL of penicillin and 100 μg/mL of streptomycin (Life Technologies). NFS-60 cells, kindly provided by Dr J. Ihle (St. Jude Children’s Research Hospital, Memphis, TN) were maintained in RPMI-1640 with 10% of fetal calf serum, 1% L-Glutamine (2mM), 1% β-mercaptoethanol (Sigma Aldrich), and 20 units/ml of Il-3 (Sigma-Aldrich). All cells were cultured at 37°C in a 5% CO2 atmosphere.

NK-92 is an interleukin-2 (IL-2) dependent natural killer cell line derived from peripheral blood mononuclear cells from a 50 year old male with rapidly progressive non-Hodgkin’s lymphoma generously provided by Conkwest which was transfected using pMX/CD16 plasmid as previously described (26). NK-92-CD16 cells were used as control effector cells in ADCC assays. Cell lines morphology was checked and the expression of cell surface markers CD20 and HER2 were assessed by flow cytometry on RL, A549 and MDA-MB361 cell lines.
respectively. Using MycoAlertKit (Lonza, base, Switzerland) all cell lines used in this study were tested mycoplasma-negative.

**Cell assays**

NFS-60, a murine myeloid leukemia cell line which proliferates in response to G-CSF, was used for cell cycle experiments (27). Cells were starved for 18 hours (no IL3 nor G-CSF) in order to block NFS60 cells in G0/G1 phase, then various concentrations of filgastrim or pegfilgastrim (from 5pmol/L to 1nmol/L) were added to the cells for 18 hours. Cell cycle distribution was estimated by flow cytometry using propidium iodide as previously described (28).

**Neutrophil preparation**

Blood samples from healthy donors were provided by the Lyon Blood Bank. Neutrophils were obtained by performing a density gradient centrifugation (Pancol, Pan-Biotech) followed by a dextran separation (3% Dextran, Sigma Aldrich). Remaining red blood cells were removed using a lysis solution (BD Pharm Lyse, BD Bioscience) and purity of neutrophils was evaluated by flow cytometry using CD45/SSC gating.

**Leukocyte subpopulations in mice**

The phenotype of leukocyte subpopulations in the blood, spleen, bone marrow, and tumor were studied after four injections with rituximab combined or not with filgrastim or pegfilgrastim. Briefly, spleen and tumor were cut in small pieces and incubated in PBS containing 0.5% FCS and 1mg/ml collagenase A (Roche Diagnostics GmbH, Mannheim, Germany), 0.1mg/ml hyaluronidase and 10μg/ml DNase (Sigma, Saint Quentin Fallavier, France) at 37°C for 45–60 min. Digested fragments were filtered through a stainless-steel sieve and cell suspensions washed in PBS 0.5% FCS. Blood and bone marrow samples were processed directly with no prior treatment. Cells were stained in 100μl PBS for 30 minutes at 4°C with anti-CD11b Alexa Fluor 488 (clone M1/70), anti-CD16/CD32 PerCP-Cy5.5 (clone 2.4G2), anti-CD49b PE-CF594 (clone DX5), anti-Ly-6C APC-Cy7 (clone AL-21), anti-Ly-6G (clone 1A8), purchased from BD Pharmingen, and anti-f4/80 Pe-Cy7 (clone BM8) purchased from eBioscience. For intratumoral leukocytes, anti-human CD20 V450 (clone L27, BD Pharmingen) was used to distinguish human cells and murine leukocytes. The cells were
washed once in PBS supplemented with 2% FCS and immediately analyzed in a LSR II flow cytometer (Becton Dickinson, San Jose, CA, USA). Data were analyzed with BD DIVA7.

**Human leukocytes**

Leukocyte populations of healthy donors used for *in vitro* experiments were assessed by flow cytometry using anti-CD13 VioBlue (clone BW264/56), anti-CD16 (VEP13), anti-CD14 APC-Vio770 (clone TUK4) anti-CD45 APC-vio700 (clone 5B1), anti-CD19 Pe-Vio770 (LT19), anti-CD15 APC (clone VIMC6) and anti-CD56 PE (AF12-7H3) obtained from Miltenyi Biotec.

**Human granulocyte studies**

Effect of G-CSF formulations on the phagocytic activity of normal human leukocytes was assessed with two different assays. Phagotest (Glycotope Biotechnology), which measures the ingestion of one or more labeled and opsonized bacteria, E.coli-FITC per cell, was used to determine the phagocytic properties of granulocytes, lymphocytes and monocytes. FagoFlowEx Kit (Exbio Diagnostics) was used to measure the respiratory (oxidative) burst after their stimulation with E. coli bacteria in human heparinized whole blood using flow cytometry.

**Antibody-dependent cellular phagocytosis (ADCP) assay**

Antibody dependent cellular phagocytosis mediated by neutrophils was evaluated by fluorescence transfer of target cells to effector cells. The assay was adapted from McEarchern et al (29). Tumor cells were labeled with PKH26, a fluorescent cell membrane dye, according to the manufacturer's instructions (Sigma, St Louis, MO). Neutrophils stimulated or not with filgrastim or pegfilgrastim (at a concentration of 10ng/ml during 2 hours at 37°C) were used as effector cells. Effectors and target cells were co-incubated at an E:T ratio of 2:1 overnight at 37°C 5% CO2, in the presence or absence of monoclonal antibodies. After incubation, cells were washed once in PBS 2% FCS stained with 3μl of anti-CD15-APC (clone HI98, BD Bioscience) for 30 minutes at 4°C and analyzed by flow cytometry.

**In vivo experiments**

Six-week-old female CB17 severe combined immunodeficient (SCID) mice purchased from Charles River Laboratories (L'Arbresle, France) were bred under pathogen-free conditions at the animal facility of our institute. Animals were treated in accordance with the European Union guidelines and French laws for the laboratory animal care and use. The animals were
kept in conventional housing. Access to food and water was not restricted. This study was approved by the University of Lyon Animal Ethics committee. Continuous health monitoring was carried out on a regular basis, with daily monitoring of clinical symptoms and adverse effects. Animals of an average weight of 20 g were inoculated subcutaneously with tumor cells (3 to 5 × 10^6 cells) into the right flank. When subcutaneous tumors were established (median volume, 100-150 mm^3) animals were randomized and treatments were initiated. Primary tumor volume (TV) was calculated according to the National Cancer Institute (NCI; Bethesda, MD) protocol \( \text{TV} = \frac{\text{length} \times \text{width}^2}{2} \), where “length” and “width” are the long and short diameters of the tumor mass. Measurements were performed twice a week. Treatments were administered once a week: monoclonal antibodies were injected intraperitoneally and G-CSF formulations intravenously on the same day.

To define optimal doses of filgrastim and pegfilgrastim for tumor growth inhibition experiments, a dose-response experiment was performed using various quantities of filgrastim and pegfilgrastim (3 μg to 60 μg per injection per mouse) combined with rituximab. Optimal doses were then used for \textit{in vivo} experiments to evaluate their effect when co-injected with different doses of monoclonal antibody (data not shown).

**Statistical analysis**

\textit{In vitro} data were analyzed using Student’s t test. For tumor growth inhibition assays, one way ANOVA was used to compare treatment groups. Analyses were performed with Statistica 8.0 (StatSoft, Inc.).
Results

Effect of filgrastim and pegfilgrastim on the antitumor activity of therapeutic mAbs in vivo

The efficacy of the combination of pegfilgrastim, filgrastim and therapeutic antibodies was compared in the RL, MDA-MB431 and A549 models. For the evaluation of tumor growth, calculations started the first day of treatment until sacrifice of the control group. Values were documented as medians and standard deviations (SD). The tumor growth inhibition (TGI) for volume (T/C) was calculated according to the NCI formula:

\[
\frac{TV_{treated}(day \, dd-xx) + 100}{TV_{control}(day \, dd-xx) + 100}
\]

In the first study, SCID mice bearing RL tumors were randomized into six groups, with five mice per group: controls, filgrastim, pegfilgrastim, rituximab, filgrastim + rituximab, pegfilgrastim + rituximab. As shown in Fig. 1A, at day 42, TGI values calculated as indicated previously were 95, 78 and 80% for pegfilgrastim/rituximab, filgrastim/rituximab and rituximab alone, respectively. In addition, a one way ANOVA showed that pegfilgrastim/rituximab treatment significantly decreased tumor size when compared to filgrastim/rituximab and rituximab alone, whereas no difference could be measured between filgrastim/rituximab and rituximab alone. In this model, pegfilgrastim effect on tumor size was equivalent to that observed with rituximab treatment. This may be the consequence of the significant changes in immune cells number and function observed after pegfilgrastim inoculation (30).

In the MDA-MB361 model, with three mice per group (Fig. 1B), TGI values were 17, 94 and 112% for pegfilgrastim alone, trastuzumab alone and pegfilgrastim/trastuzumab, respectively. In addition to significant differences between the treatment groups, a complete tumor remission was observed in the pegfilgrastim/trastuzumab group but not in the other groups. Similar observations were obtained with a third model (A549 cell line, Supplementary Fig. S1). Furthermore, in the RL model, an increased mice survival was observed in the pegfilgrastim/rituximab treated group (Supplementary Fig. S2).

Effect of filgrastim and pegfilgrastim on cell cycle distribution of NFS60 cells

Cell cycle experiments comparing variable concentrations of filgrastim and pegfilgrastim
showed that both molecules induce cell cycle entry of starved NFS60 cells, with a decrease in the percentage of cells in G0/G1 and a corresponding increase of the cells in G2/M phase. In this assay filgrastim was more potent than pegfilgrastim over the range of concentrations tested (0.1-2 ng/ml) with no dose-response effect for either molecule. Similar results were observed at concentrations of 5 ng/ml or greater (Fig. 2A and B).

**Effect of filgrastim and pegfilgrastim on phagocytic activity of normal human neutrophils**

Phagocytic activity of normal human neutrophils was explored in samples obtained from three healthy donors (Fig. 3A). Filgrastim induced a significant increase of the percentage of neutrophils performing phagocytosis in two out of three healthy donors (** p<0.005), whereas pegfilgrastim induced a significant increase of the percentage of neutrophils performing phagocytosis in three out of three healthy donors (* p<0.05). However no significant difference could be measured between samples stimulated with filgrastim or pegfilgrastim. Evaluation of ROS production by neutrophils using Fagoflow showed similar results. As shown in Fig. 3B, filgrastim and pegfilgrastim induced a significant increase of the percentage of neutrophils producing ROS (* p<0.05) while no significant difference of ROS production could be measured between leukocytes stimulated with filgrastim or pegfilgrastim. These two functional assays confirmed the ability of filgrastim and pegfilgrastim to enhance phagocytic and ROS-producing properties of neutrophils, with no significant difference observed between the two molecules.

**Effect of filgrastim and pegfilgrastim on ADCP properties of normal human neutrophils**

Phagocytosis of target cells was measured by incorporation of the fluorescent lipophilic molecule PKH67 used to label target cells into neutrophils. While trogocytosis may occur, this assay is commonly used to evaluate antibody dependent cellular phagocytosis *in vitro* (31). As presented in Fig. 3C, the presence of monoclonal antibody induced a significant increase of phagocytosis of tumor cells by neutrophils. In addition, pre-incubation of neutrophils with filgrastim and pegfilgrastim significantly enhanced their capacity to perform phagocytosis. Moreover in 2 out of 3 donors, pegfilgrastim was more potent than filgrastim in inducing an increased phagocytosis by neutrophils (* p<0.05).
Effect of filgrastim and pegfilgrastim on leukocyte populations in vivo

Mice received repeated injections of rituximab combined or not with filgrastim or pegfilgrastim (n=5 mice per group). The cellularity of spleens was found to strikingly increase in mice treated with pegfilgrastim without rituximab in comparison to controls; with cell numbers increased five- to six-fold (Fig. 4A). There was also a significant increase of spleen size of mice treated with pegfilgrastim (with or without rituximab) compared to the other groups (p<0.05) (Fig. 4B and C). The ratio of spleen leukocyte subpopulations was also analyzed. For each subpopulation assessed, association of pegfigrastim and rituximab induced an increase in the number of cells when compared to rituximab alone. The activated monocyte population was multiplied 19-fold and the granulocyte population 18-fold (Supplementary Fig. S3).

We analyzed leukocyte subpopulations to identify those which were preferentially increased. As shown in Fig. 5A, four leukocyte subpopulations were evaluated, including NK cells, resting monocytes, activated monocytes and granulocytes. None of the treatments induced variation of the percentage of NK cells, identified with CD49b+, among CD11b- cells. Among the monocytic population (Ly6G- Ly6C<sub>high</sub>or <sub>low</sub>), the percentage of activated monocytes (Ly6G- Ly6C<sub>high</sub>) was slightly but significantly increased after pegfilgrastim / rituximab injections (p<0.05), whereas resting monocytes (Ly6G- Ly6C<sub>low</sub>) were significantly decreased (p<0.05). The percentage of the Ly6G+ Ly6C<sub>low</sub> population, usually described as granulocytes, was increased in the spleen of mice treated with filgrastim and pegfilgrastim compared to both untreated and rituximab-treated mice, but no significant difference was observed between filgrastim and pegfilgrastim treatment groups.

We also assessed absolute number of leukocyte subpopulations within the spleen (Fig. 5B). In parallel with the weight increase observed with pegfilgrastim treatment (with or without rituximab), a systematic and significant increase of the number of cells of all leukocyte subpopulations was observed (p<0.05). In blood (Fig. 5C), significant differences were only observed for the pegfilgrastim groups (with or without rituximab) with a significant increase of the Ly6G+ Ly6C<sub>low</sub> population and a significant decrease of the monocytic populations (both Ly6G- Ly6C<sub>high</sub> and Ly6G- Ly6C<sub>low</sub>). Within the tumor no significant variation of NK cells or monocytes (both Ly6C<sub>high</sub> and Ly6C<sub>low</sub>) could be observed, but a significant increase of the percentage of neutrophils within CD11b+ cells up to 18% (vs. 2.85% in controls) was detected in mice receiving pegfilgrastim (*p<0.05) (Fig. 5D).
Discussion

Our results show that pegfilgrastim enhances the antitumor activity of ADCC/ADCP-performing antibodies such as rituximab and trastuzumab in preclinical models and that this effect was greater than that observed with filgrastim. This immuno-adjuvant effect was associated with the amplification and recruitment of neutrophils, including in the tumor site. Clinical studies, such as the one currently being conducted combining rituximab and pegfilgrastim in patients with NHL (NCT01682044) should determine whether this combination is beneficial in the clinic. Possible differences between filgrastim and pegfilgrastim may be the kinetic of stimulation of accessory cells, which is expected to be more prolonged in the case of pegfilgrastim or enhanced uptake of pegfilgrastim by accessory cells.

Some attempts have been made to potentiate the effect of mAbs in the clinic with growth factors targeting potential effector cells. An immunocytokine fusion protein combining interleukin 2 with an anti-fibronectin antibody in combination with rituximab was found to eradicate B cell lymphoma xenografts (32). Antibodies directed against the inhibitory Kir molecules expressed by Natural Killer cells have been evaluated in combination with rituximab (33). IL-15 has been shown to induce NK-mediated CLL depletion in vitro, with a potentiation of rituximab (34). IL-21 has been shown to enhance the activity of rituximab in Cynomolgus monkeys (35). Interferon gamma has been reported to enhance antibody-mediated toxicity of neutrophils against a colorectal model (36). Currently none of these approaches have yet been validated in the clinic.

Some investigators have used G-CSF or GM-CSF in an attempt to enhance Mab potency in preclinical models and patients. Hernandez-Ilizaliturri combined murine G- or GM-CSF with rituximab in a murine xenograft model in SCID mice and found that these molecules significantly increased the antitumor effect of the antibody in this setting (37). These authors also showed that neutrophil depletion in a murine model was associated with a loss of antitumor activity of rituximab (38). Van der Kolk administered G-CSF 5 μg/kg/day for 3 days with rituximab infusion on day 2 in 26 patients with relapsing low-grade lymphoma (25). Tolerance was satisfactory and time to progression was considered to be unexpectedly long in some patients. Cartron et al. treated 33 patients with relapsed follicular lymphoma with
GM-CSF 5 μg/kg/d on days 1 to 8 and rituximab 375 mg/m² on day 5 of each 21-day cycle for four cycles (20). They observed a 70% overall response rate and tolerance was satisfactory with 39% complete responses, which appears to be greater than what is expected with rituximab as single agent therapy.

Several issues remain to be addressed concerning the potential clinical benefit of combining a leukopoietic growth factor with ADCC/ADCP-performing mAbs. One question deals with the sequence of administration. In our study both molecules were administered simultaneously. This may prove to be an issue if patients receive simultaneous cytotoxic chemotherapy as it may sensitize hematopoietic cells to myeloid toxicity. A small yet provocative study by Gruber et al. on 32 CLL patients receiving the fludarabine/cyclophosphamide/rituximab combination with or without G-CSF, administered in case of profound neutropenia, reported much better outcomes in the G-CSF group (39). One possible explanation is that G-CSF potentiated rituximab in this setting, since the half-life of rituximab in patients is in the order of 15 days. Another potential issue is that of the effective dose. It is possible that the dose required to obtain an immunoadjuvant effect in combination with mAbs may be different from that classically used to prevent or treat neutropenia.

Another important aspect to consider when administering neutrophil growth factors is the existence of tumor-associated neutrophils with potential tumor-promoting properties. Fridlender et al. described the polarization of intratumoral neutrophils by TGF-beta, with the induction of a pro-tumor phenotype by this cytokine (40). More recently, colon epithelial cells exposed to mutagenic agents have been shown to produce GM-CSF, promoting an inflammatory pro-carcinogenic environment (41).

In conclusion pegfilgrastim appears to be an attractive candidate to potentiate the antitumor activity of monoclonal antibodies with ADCC or ADCP properties such as rituximab and trastuzumab. Further studies are required to determine whether these observations can be generalized to other target antigens and other monoclonal antibodies.

Supplementary information is available at Molecular Cancer Therapeutic's website.
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Figure Legends

Figure 1. Effect of filgrastim and pegfilgrastim on the antitumor activity of therapeutic mAbs in vivo.

A, efficacy of combination of pegfilgrastim and rituximab on RL xenograft growth in mice. The tumor growth inhibition (TGI) calculated on day 42 confirmed the potentiation of rituximab antitumor activity when co-injected with pegfilgrastim. Co-treatment with pegfilgrastim and rituximab significantly decreased RL growth when compared to untreated, rituximab alone and filgrastim with rituximab groups according to one way ANOVA (p<0.001). No significant differences were observed between rituximab and filgrastim with rituximab. B, efficacy of combination of pegfilgrastim and trastuzumab on MDA-MB 361 xenograft growth in mice. The tumor growth inhibition (TGI) calculated on day 62 showed the potentiation of trastuzumab efficacy when co-injected with pegfilgrastim. With a complete relapse in the MDA-MB 361 model, co-treatment with pegfilgrastim and trastuzumab significantly decreased tumor growth when compared to untreated and trastuzumab alone according to one way ANOVA (p<0.001).

Figure 2. Effect of filgrastim or pegfilgrastim on cell cycle of NFS60 cells. After 18 hours of G-CSF starvation of NFS60 cells in order to stop their proliferation, NFS60 cells were stimulated with different concentrations of filgrastim or pegfilgrastim. Both filgrastim and pegfilgrastim induced a re-entry into the cell cycle of NFS60 cells (p<0.05) compared to unstimulated NFS60 (p<0.005 for filgrastim and p<0.05 for pegfilgrastim). Figures 2A and B are representative of the percentage of NFS60 re-entry into the cycle phases S/G2 and G0/G1, respectively. In addition, filgrastim was significantly more potent to induce re-entry of NFS60 cells compared to pegfilgrastim (p<0.05) except at the highest concentrations of pegfilgrastim (no significant difference at 100 pmol/L and above (data not show).

Figure 3. Effect of filgrastim and pegfilgrastim on phagocytic and ADCP activity of normal human neutrophils.

A, effect of filgrastim or pegfilgrastim on phagocytic activity of neutrophils using FITC E coli from PhagoTest. Compared to unstimulated samples, filgrastim induced a significant increase of the percentage of neutrophils performing phagocytosis in two out of three healthy donors (** p<0.005), whereas pegfilgrastim induced a significant increase of the
percentage of neutrophils performing phagocytosis in three out of three healthy donors (* p<0,05 and ** p<0,005). B, effect of filgrastim or pegfilgrastim on production of reactive oxygen species (ROS) by granulocytes in presence or absence of E coli using FagoflowEx kit. Results shown are median values obtained with four different healthy donors. Filgrastim and pegfilgrastim induced a significant increase of percentage of neutrophils producing ROS (p<0,05). C, effect of filgrastim or pegfilgrastim on ADCP activity of fresh human neutrophils. Compared to unstimulated samples, filgrastim and pegfilgrastim induced a significant increase of the percentage of neutrophils performing phagocytosis (p<0,005). A significant difference was measured for some of the healthy donors (2 out of 3) between samples stimulated with filgrastim and pegfilgrastim (p<0,005). (p<0,005 comparison between samples with or without rituximab).

Figure 4. Effect of filgrastim and pegfilgrastim on spleen cellularity, spleen size and leukocyte subpopulations.
A, effect of filgrastim and pegfilgrastim on spleen cellularity. Spleens were obtained two days after the last treatment from five mice which were either not treated or treated weekly with rituximab (30mg/kg), filgrastim (375 mg/kg) with or without rituximab (30mg/kg) and pegfilgrastim (750mg/kg) with or without rituximab (30mg/kg), during 4 weeks. Spleen were dissociated into single-cell suspensions and leukocytes counted by trypan blue exclusion. Results are the mean ± SEM of five mice per group (*, p<0,005). B, effect of filgrastim and pegfilgrastim on spleen size and leukocyte subpopulations. Spleens were obtained two days after the last treatment from five mice which were either not treated or treated weekly with rituximab (30mg/kg), filgrastim (375 mg/kg) with or without rituximab (30mg/kg) and pegfilgrastim (750mg/kg) with or without rituximab (30mg/kg), during 4 weeks. Spleen size was not increased in mice exposed to filgrastim + rituximab in comparison to mice exposed to rituximab alone but was significantly increased in mice exposed to pegfilgrastim + rituximab (up to six-fold when compared to rituximab alone (* p<0,005), and up to four-fold (* p<0,005) when compared to filgrastim + rituximab. C, pictures of spleens of mice treated with rituximab, filgrastim + rituximab or pegfilgrastim + rituximab after four weeks of treatments.
Figure 5: Effect of treatments on leukocyte subpopulations

A, spleen leukocyte subpopulations. After four weeks of treatment, the percentage of activated monocytes (Ly6G⁻ Ly6C<sup>high</sup>) was significantly increased after pegfilgrastim/rituximab injections (p<0.05), whereas resting monocytes (Ly6G⁻ Ly6C<sup>low</sup>) were significantly decreased (p<0.005). The percentage of the granulocytes (Ly6G<sup>+</sup> Ly6C<sup>low</sup>) was increased in the spleen of mice treated with filgrastim and pegfilgrastim compared to both untreated and rituximab-treated mice, but no significant difference was observed between filgrastim and pegfilgrastim treatment groups. B, Absolute number of leukocyte subpopulations in spleens. Considering the important weight variation of spleens depending on treatment, we assessed absolute cell number in the different treatment groups. For each studied subpopulation, pegfilgrastim with rituximab treatment induced an increased number of cells (p<0.05 when compared to rituximab ± filgrastim). C, Leukocyte subpopulations within blood. After four weeks of treatments with pegfilgrastim with rituximab, the percentage of granulocytes within myeloid cells significantly increased compared to mice treated with rituximab alone or filgrastim with rituximab (p<0.05). In parallel, pegfilgrastim/rituximab-treated mice presented a significant decrease of monocytic populations (both activated Ly6C<sup>high</sup> and resting Ly6C<sup>low</sup>) (p<0.05). D, Leukocyte subpopulations within tumors. After four weeks of treatments with pegfilgrastim with rituximab, the percentage of granulocytes within CD11b<sup>+</sup> cells significantly increased in comparison to mice treated with rituximab alone or filgrastim with or without rituximab (p<0.05). In parallel, pegfilgrastim/rituximab-treated mice presented a significant decrease of activated monocytes (Ly6G⁻ Ly6C<sup>high</sup>) in comparison to rituximab treated mice (p<0.05).
Figure 2

A

% of N560 cells in S/G2 phase

- Filgrastim
- Pegfilgrastim

Stimulation

Unstimulated, 5pmol/L, 25pmol/L, 50pmol/L, 100pmol/L

B

% of N560 cells in G0/G1 phase

- Filgrastim
- Pegfilgrastim

Stimulation

Unstimulated, 5pmol/L, 25pmol/L, 50pmol/L, 100pmol/L
Molecular Cancer Therapeutics

Pegfilgrastim enhances the antitumor effect of therapeutic monoclonal antibodies

Sébastien Cornet, Doriane Mathé, Kamel Chettab, et al.

Mol Cancer Ther Published OnlineFirst March 17, 2016.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-15-0759

Supplementary Material
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