A Cell-Based Assay for Detection of Neutralizing Antibodies against Peg-Filgrastim in Human Serum

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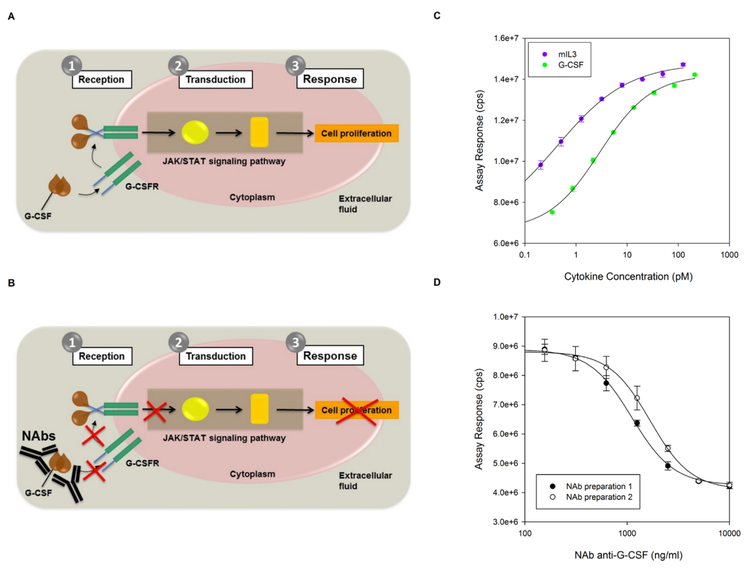
Applied Translational Medicine

Background

- Colony Stimulating Factors (CSFs) were first identified as factors required in vitro to grow colonies of granulocytes and monocytes from isolated mouse bone marrow or spleen cells
- 4 distinct CSFs with different colony-stimulating activities were isolated: multi-CSF (aka IL3, broad range of hematopoietic cells), G-CSF (granulocyte), GM-CSF (granulocyte and macrophage), and M-CSF (macrophage)
- G-CSF acts on stem cells in the bone marrow to induce proliferation/ differentiation into neutrophils; Neutrophils are most abundant white blood cells that specifically target infection (e.g. bacteria in wounds)
- Recombinant G-CSF is used for treatment of low neutrophil counts (aka neutropenia), either congenital or induced by chemotherapy treatment e.g. cancer prophylaxis. Infection is the primary life-threatening event of any chemotherapy course
- Bacterially expressed human G-CSF is commercially available under the INN Filgrastim, either unmodified or in pegylated form (PEG-Filgrastim, for extended half-life)
- Assay goal: detect neutralizing antibodies (NAbs) against PEG-Filgrastim in clinical samples (serum) as they may impact drug efficacy and drug half-life (e.g. via antibody mediated clearance)

Assay Principle

Figure 1. G-CSF Mode of Action and Assay Principle



- G-CSF binds to the monomeric G-CSF receptor (G-CSFR) on the cell surface and induces dimerization of the receptor (Figure 1A) which in turn allows transphosphorylation of the receptor associated Janus kinases, this phosphorylation event initiates the cascade of activation resulting in hematopoietic stem cell proliferation/differentiation into neutrophils (Figure 1A)
- Neutralizing antibodies to G-CSF could (i) prevent binding of G-CSF to G-CSFR or (ii) prevent G-CSFR dimerization by steric hindrance (Figure 1B)
- A cell-based assay for detection of NAbs to PEG-Filgrastim was selected as it will pick up both (i) and (ii); whereas a ligand binding assay will only pick up (i)

Method Development

Selection of a Suitable Cell Line and Assay Endpoint

- The NFS-60 cell line (murine leukemia) expresses G-CSFR and proliferates in response to G-CSF (Figure 1C); this proliferation can be inhibited in a dose-dependent manner by neutralizing antibodies to G-CSF (Figure 1D); this forms the basis of the **activity assay**
- Mouse interleukin 3 (mIL3) also induces cell proliferation (Figure 1C); this property of the NFS-60 cells was used to develop a **specificity assay** where mIL3 was used as an alternate stimulus to evaluate the specificity of the inhibition detected in the activity assay
- **Assay endpoint** is the number of cells in the assay following treatment; assay endpoint is measured indirectly using the non-toxic dye resazurin. Resazurin is reduced into a fluorescent product by the metabolic activity of the cells, fluorescence intensity (measured in counts per sec (cps)) is thus proportional to the cell number in the assay

Table 1. Assay Controls

	Cytokine (PEG-Filgrastim or mIL3)	Neutralizing Antibody Anti PEG-Filgrastim
Positive Control (PC)	✓	×
Negative Control (NC)	×	×
Quality Control (QC)	✓	✓

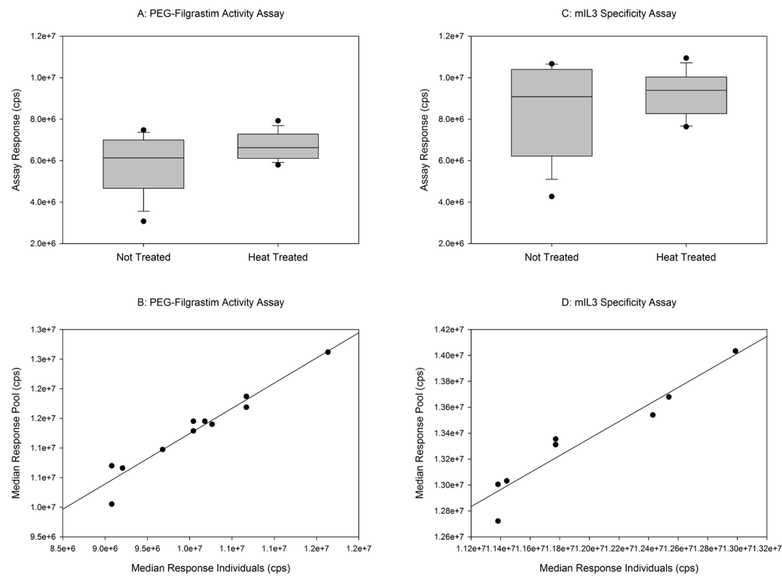
- All experiments were performed with 5% human serum in assay (matrix minimum required dilution of 20 fold); all concentrations are those in undiluted samples
- Samples and growth factors are pre-incubated for 1 hr before transfer onto the cells, all determinations are done in triplicate
- Samples pre-treatment: heat inactivation (56°C, 30 min)
- Sensitivity determination (Figure 4): human serum samples spiked with a high concentration of anti PEG-Filgrastim neutralizing antibodies were serially diluted in a normal human serum pool then measured in the activity and specificity assay
- Drug tolerance experiment (Figure 5): human serum samples containing 2000 ng/mL or 4000 ng/mL NAb to PEG-Filgrastim were spiked with increasing PEG-Filgrastim concentrations and incubated for 1 hr at ambient temperature before processing in the activity and specificity assay
- Stability experiment (Figure 6): human serum samples spiked with 2000 ng/mL NAb to PEG-Filgrastim or at blank level were subjected to different stability conditions (storage for 10 days at -20°C, 5 freeze and thaw cycles, benchtop incubation at ambient temperature for 24 hr) and compared to freshly prepared samples at the same levels in the activity and specificity assay
- Hemolysis (Figure 6): human serum samples were fortified with 1% or 2% human whole blood and tested in the activity and specificity assay either spiked with 2000 ng/mL NAb to PEG-Filgrastim or at blank level

Method Qualification

Cut Point Determination

Regulatory authorities' recommendation for cell-based NAbs assays: calculate an assay cut point based on the statistical analysis of the assay response from samples obtained from drug naïve subjects.

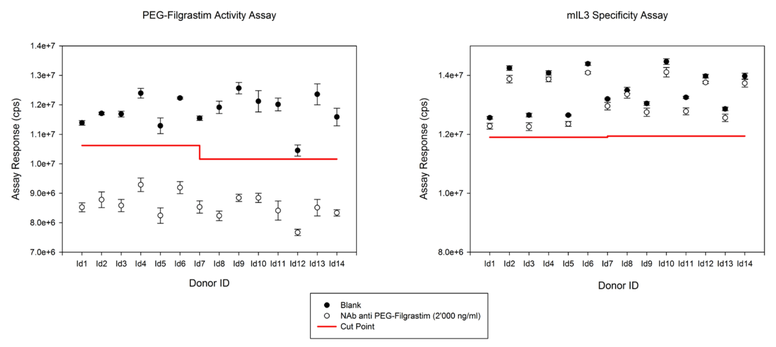
Figure 2. Feasibility of a Floating Cut Point



- Sample pre-treatment by heating reduced variability in response at PC level between individuals in both assays (Figure 2A and 2C), CV between individuals drops from 25% to less than 10% after sample pre-treatment
- Following pre-treatment, median response in normal human serum (NHS) pool versus median response in 14 NHS individuals at PC level shows good correlation in both assays (Figure 2B and 2D); distribution of individuals response at PC level was normally distributed (not shown)
- Based on these results, a parametric statistical approach was used to calculate a floating cut point for both assays based on the response in the NHS pool at PC level multiplied by a correction factor; this correction factor corresponds to a 12% and 11% inhibition of the PC level in the activity and specificity assay respectively

Selectivity and Specificity

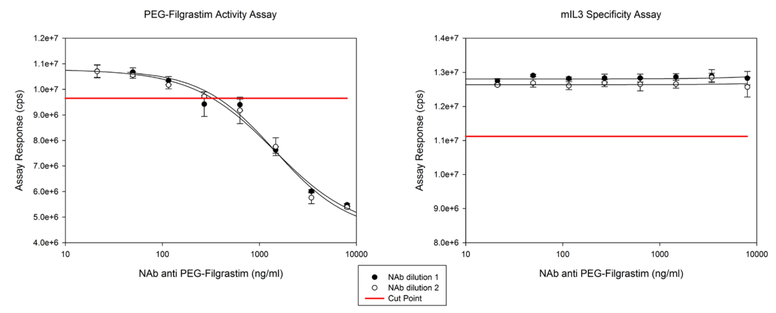
Figure 3. Assay Selectivity and Specificity in Individuals



- 14 individual donors from both genders were evaluated in the activity and specificity assay, at unspiked (blank level) or spiked with 2000 ng/mL NAb to PEG-Filgrastim; individuals were tested over 2 assay plates, therefore the cut point value varied slightly between assay plates (Figure 3)
- In the PEG-Filgrastim activity assay, all 14 individuals were detected as positive for NAb at spiked level and negative for NAb at blank level (Figure 3, left); individuals were all negative at both levels in the mIL3 specificity assay (Figure 3, right)
- NAbs to PEG-Filgrastim can thus be selectively and specifically detected in individual serum samples by testing in both assays

Sensitivity and Drug Tolerance

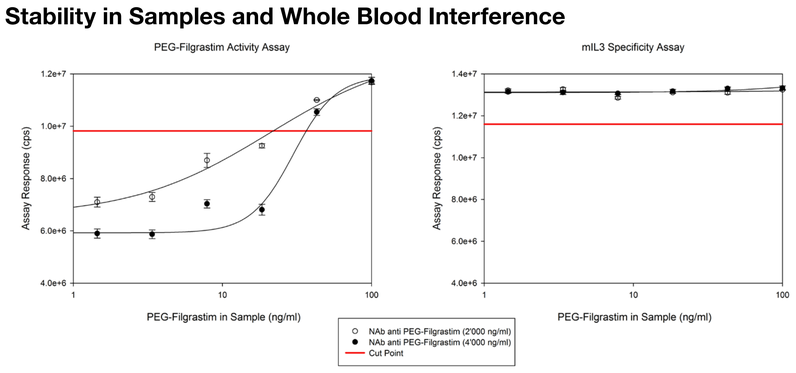
Figure 4. Assay Sensitivity



- Sensitivity of the activity assay at cut point was 288 ng/mL NAb
- No interference was observed in the specificity assay even at high NAb concentration

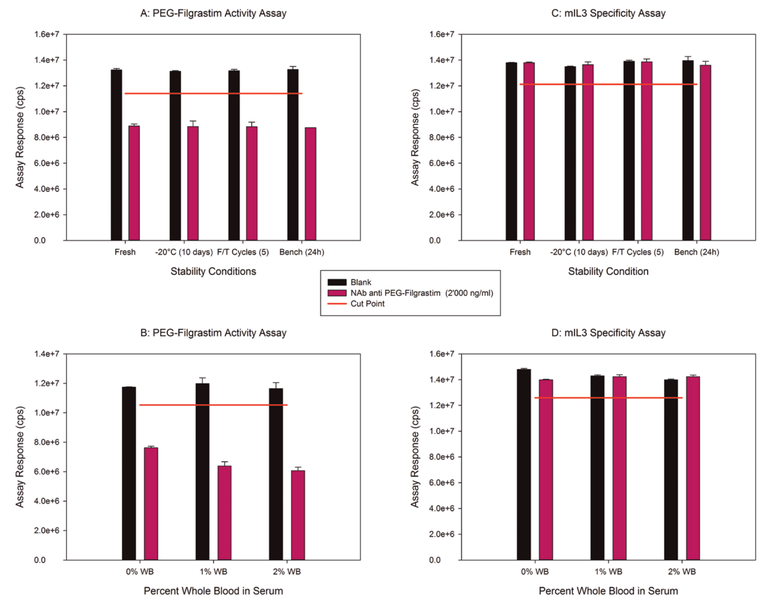
Figure 5. Assay Drug Tolerance

Stability in Samples and Whole Blood Interference



- Assay drug tolerance is higher at higher NAb concentration, as expected
- Measured assay drug tolerance (25-35 ng/mL free drug in sample) is 10-20 fold above the expected PEG-Filgrastim concentration at typical first sample collection time for antibodies (1-2 ng/mL drug expected, day 8)

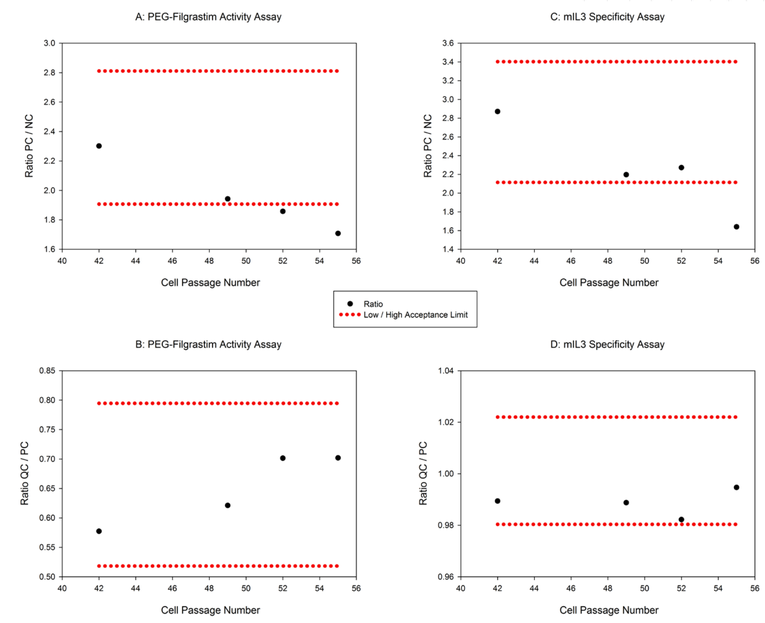
Figure 6. Stability and Hemolysis



- Sample stability was demonstrated in both assays at all conditions tested
- No differences were observed when heat treatment of the samples was performed at the beginning or at the end of the stability period under consideration (not shown)
- No interference in either assay was detected when analysing hemolyzed samples up to 2% whole blood

Assay Robustness

Figure 7. Cell Passage Number



- Cells at a different passage number were frozen then evaluated in one run in both assays (Figure 7); note that cells were received from a supplier at passage number 37 and additional passages were required for preparation of cell banks in-house
- Acceptance limit (red dotted line, Figure 7) is calculated based on the 95% confidence interval of the inter-assay mean ratios
- The response ratio PC/NC in the activity assay falls outside of acceptance limits (Figure 7A) for cells above passage 49; a decrease in neutralizing activity is also observed in cells above passage 49 as reported by an increase in the response ratio QC/PC ratio in the activity assay (Figure 7B)
- Control of cell passaging is critical for cell-based assay robustness and only cells in optimal physiological conditions should be used

Results

Table 2. Assay Parameters and Qualification Results

	Activity Assay PEG-Filgrastim	Specificity Assay mIL3
Cell Line	NFS-60 (murine leukemia)	
Effector Concentration	6 pM	1 pM
Assay Matrix	Human serum	
Minimum Required Dilution	20 fold (5% human serum)	
Intra-Assay Precision (% CV, PC level)	0.1 - 8.0	0.4 - 7.6
Inter-Assay Precision (% CV, PC level)	≤ 11.0	≤ 3.6
Sensitivity in Sample (Anti PEG-Filgrastim NAb)	288 ng/mL	No interference
Drug Tolerance (PEG-Filgrastim) at 2'000 ng/ml NAb	25 ng/mL	≥ 100 ng/mL
Correction Factor for Cut Point (% Inhibition PC)	12%	11%
Stability, Freeze/Thaw	5 cycles	
Stability, Benchtop (Ambient Temperature)	24 hr	
Stability, -20°C	10 days	
Hemolysis	No interference up to 2% whole blood	
Robustness: Cell Passage	Up to passage 49	

Conclusions

An assay for the precise and sensitive detection of neutralizing antibodies against PEG-Filgrastim in human serum was successfully developed and qualified using a cell-based system. This method could be applied in the comparative analysis of the NAb response of PEG-Filgrastim and a biosimilar compound.

Poster presentation at EBF 2015 8th Open Meeting Barcelona, Nov 18-20, 2015

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