Evidence for Endotoxin Contamination in Plastic Na⁺-Heparin Blood Collection Tube Lots

Kathryn J. Newhall,¹, * Geoffrey S. Diemer,¹ Natalia Leshinsky,¹ Keith Kerkof,¹ Hilary T. Chute,² Chris B. Russell,¹ William Rees,¹ Andrew A. Welcher,² Scott D. Patterson,² and Gary D. Means¹

BACKGROUND: Biomarker assays are often conducted on whole blood samples in the course of drug development studies. Because bacterial lipopolysaccharide (LPS) (endotoxin) contamination is known to cause spontaneous cytokine production by monocytes, contamination of blood collection tubes may interfere with biomarker assay results.

METHODS: Whole blood from healthy donors was collected into plastic or glass sodium (Na⁺)-heparin Vacutainer™ blood collection tubes and heparinized syringes. Samples were analyzed for phosphoprotein response, cytokine production, and RNA expression. Tubes were tested for endotoxin contamination by use of the limulus amoebocyte lysate assay.

RESULTS: Results of phospho-flow cytometry, branched DNA (bDNA), and ELISA assays indicated that a specific lot (#5339582) of plastic Na⁺-heparin Vacutainer tubes was highly contaminated with an endotoxinlike substance, and contamination was confirmed by the limulus amoebocyte lysate assay. Analysis of multiple-analyte panels revealed that analytes whose changed expression was predictive of LPS stimulation were increased when whole blood was incubated in contaminated tubes for 6 or 18 h. Two additional lots of plastic tubes tested had detectable amounts of endotoxin sufficient to strongly alter phospho-flow cytometry analyses, as determined by the fold change in phosphorylation of p38 mitogen-activated protein kinase in response to tumor necrosis factor α and LPS. In contrast, 3 lots of glass tubes had substantially lower levels of spontaneous blood activation.

CONCLUSIONS: Endotoxin contamination associated with tubes from 3 lots of a particular type of plastic Na⁺-heparin Vacutainer tube dramatically affected biomarker assay measurements. Prescreening these tubes is suggested before their use in clinical sample analysis.

Biomarker identification is an essential component of drug development. Biomarker analysis may have an impact on understanding therapeutic action of a drug, dose range (and schedule) determination, and patient response stratification, and allows for the targeting of patient populations most likely to be treated with a new drug. The importance of biomarkers in drug development is outlined by the US Food and Drug Administration in its Critical Path Initiative (1). One important goal of the initiative is to encourage the use of measurements of biomarkers from patient samples to provide data on biological activities expected to correlate with clinical efficacy and safety.

Selection of appropriate specimens for biomarker measurement depends on the ease of sample acquisition and the willingness of individuals to supply the necessary samples. Most study participants will readily donate blood, a situation that is advantageous because a wide array of biomarker assays can be performed on blood samples. Assays designed to detect changes in expression of RNA, proteins (both secreted and intracellular), and phosphoprotein targets are now commonly used to analyze blood samples.

Recent technical advances have led to the application of flow cytometry for identification of intracellular phosphoprotein biomarkers. These phospho-flow cytometry assays have the advantage of allowing rapid assessment of changes in the phosphorylation of target-proximal biomarkers, typically within minutes of pathway activation. In these assays, the effect of perturbation may be measured by using fluorescently labeled monoclonal antibodies to identify changes in specific phosphoprotein concentrations. These assays are read in a cell-intrinsic fashion and therefore are as robust for rare cell types as for abundant cell populations.

For any biomarker assay, it is critical that the blood collection procedure not affect the readout. Substantial effort is expended to test and qualify assays, yet equal attention must be applied to preanalytical variables. Analysis of errors in laboratory medicine has demon-

¹ Amgen Molecular Sciences, Amgen, Seattle, WA and ² Thousand Oaks, CA.
* Address correspondence to this author at: 1201 Amgen Court West, Seattle, WA 98119. Fax 206-217-0346; e-mail knewhall@amgen.com.

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stated that a high percentage of errors (30%–75%) occur in the preanalytical phase rather than in the analytical phase (2–5). Preanalytical variables to consider include sample collection, handling, processing, storage, and transport to the analytical laboratory. It is therefore critical that standardized protocols be developed and submitted to the clinical sites where biomarker samples are collected. These protocols must be very detailed to prevent preanalytical errors. In fact, one of the more commonly reported sources of erroneous measurements has been the use of the wrong type of collection tube (3). In this study we examined the magnitude of measurement errors that might also easily arise as a result of variable blood activation within a single type of collection tube.

We tested and qualified assays with respect to a range of preanalytical variables, such as the effects of anticoagulants on whole blood assays. Because the chelation of calcium (Ca$^{2+}$) ions, which occurs with anticoagulants such as citrate and EDTA, affects some Ca$^{2+}$-sensitive signal transduction pathways, we chose to develop the majority of our assays in whole blood collected in Na$^+$-heparin. Here we report our findings that endotoxinlike contamination associated with tubes from 3 lots of a particular type of plastic Na$^+$-heparin Vacutainer blood collection tubes dramatically affected the outcome of biomarker assay measurements by flow cytometry, ELISA, and RNA analysis.

Materials and Methods

WHOLE BLOOD COLLECTION

Whole blood from healthy donors was collected through the Amgen Blood Donor Program (Seattle, WA, and Thousand Oaks, CA). This program is conducted in accordance with the Declaration of Helsinki and the International Conference on Harmonization Good Clinical Practice Guidelines and meets compliance requirements established by the Western Institutional Review Board. All donors gave written informed consent.

WHOLE BLOOD STIMULATIONS AND FIXATION

Blood was collected in 10-mL Na$^+$-heparin plastic [BectonDickinson (BD), cat #367874, lots #5339582, #6123786] or glass (BD, cat #366480, lot #5007997) Vacutainer tubes or 60-mL syringes (BD, cat #309653) containing certified endotoxin-free Na$^+$-heparin (10 000 U/mL, 1 μL/mL whole blood; Sigma-Aldrich, cat #H3393). Tumor necrosis factor α (TNF-α) (R&D Systems, cat #210-TA/CF, lot #AA335111, and AA335054) and lipopolysaccharide (LPS) (Sigma-Aldrich, cat #L6529, lot #015K4103, and lot #016K4021) were diluted in PBS + 0.1% endotoxinfree BSA (Calbiochem, EMD Chemicals, Merck KGaA, cat #126579). From each of the collection tubes 250 μL of whole blood was stimulated with TNF-α (2.5 μg) for 5 min or LPS (25 μg) for 7.5 min at 37 °C. After the treatment times elapsed, 5 mL of prewarmed (37 °C) 1 × BD Phosflow Lyse/Fix Buffer (BD Biosciences, cat #558049) was added to each sample to simultaneously lyse the red blood cells (RBCs) and fix the peripheral blood mononuclear cells (PBMCs). The Lyse/Fix Buffer–treated sample tubes were incubated for 20 min in a 37 °C water bath to allow for complete RBC lysis. After incubation the tubes were centrifuged at 1000g for 15 min at 4 °C to pellet the fixed whole blood PBMCs.

FLOW CYTOMETRY

The pelleted PBMCs were washed 3 times with ice-cold PBS + 1% FBS, with pelleting by centrifugation performed each time. Extracellular antigen detection was conducted for 30 min by using a cocktail of CD14-Pacific Blue (BD Biosciences, cat #558121) and NK Simultest™: CD3–FITC, CD56/16–PE (BD Biosciences, cat #340042) diluted in PBS + 1% FBS. After being labeled, the cells were pelleted and washed 3 times in PBS + 1% FBS. Cells were then permeabilized with 200 μL ice-cold 80% methanol per sample and incubated on ice for 20 min in the dark. Cells were again washed 3 times before intracellular antigen detection. Cells were then labeled with a mouse antihuman mitogen-activated protein kinase (MAPK) phospho-p38-AlexaFluor 647 antibody (BD Biosciences, cat #612595) diluted in PBS + 1% FBS. Cells were washed 3 times and resuspended in PBS + 1% FBS for analysis, performed by using the LSRII BD Flow Cytometer system with 4-color analysis. CD14-positive cells were gated and specifically analyzed for phospho-p38-AF647 fluorescence.

CYTOKINE PRODUCTION ASSAY AND MEASUREMENT OF INTERLEUKIN-1β AND TNF-α

Whole blood from 2 healthy donors was collected in 10-mL Na$^+$-heparin plastic (BD, cat #367874, lot #5339582) Vacutainer tubes, 2 different Na$^+$-heparin glass Vacutainer tubes (BD, cat #366480, lot #5311775), or Monocyte tubes (Covidien, cat #320751, lot #312120). LPS (List Biological Laboratories, cat # 201) was diluted in assay medium. Assay medium consisted of RPMI 1640 (Invitrogen Life Technologies...
until analysis for interleukin (IL)-1β and TNF-α could be performed. IL-1β and TNF-α were assayed by using the custom-coated MSD 96-well Multi-Bias® Human Cytokine Assay Ultrasensitive kit (Meso Scale Discovery, cat #N411B-1) according to the manufacturer’s kit instructions for serum/plasma samples. Data were analyzed in GraphPad Prism 4.0 (GraphPad Software) by using sigmoidal dose response (variable slope) with 1/y² weighting. The CV% was determined by (SD/mean) × 100.

Whole blood from 3 separate donors was also collected in either glass (BD, cat #366480, lot #5311755) or plastic Na+−heparin Vacutainer tubes (BD, cat #367874, lot #5339582) and allowed to incubate for 6 or 18 h. Plasma was removed and analyzed by using a multiple analyte panel (MAP) (Rules Based Medicine).

**BRANCHED-DNA ASSAY**

Cultures of 80% whole blood in RPMI medium were stimulated for 24 h in 96-well microtiter plates at 37 °C and 5% CO₂ in the presence of 3 μg/L TNF-α (R&D Systems, cat #210-TA) and 0−1000 μg/L IL-17 (R&D Systems, cat #317-ILB). Heparinized whole blood from Amsgen Washington Blood Donor Program volunteers was collected into either plastic (lot #5339582) or glass (lot #5007997) BD Na+−heparin Vacutainer tubes, and the cytokine stimulation was initiated <3 h after blood harvest.

Blood cell lysis and quantification of chemokine ligand (CCL) 2/monocyte chemotactic protein (MCP) 1, CCL7, pentraxin 3 (PTX3), and IL-6 mRNA were performed as outlined by the manufacturer of the branched DNA (bdNA) assay kit (Panomics). Briefly, 20 μL of each blood culture was transferred to a deep-well, 96-well plate and lysed after the addition of 76 μL of a proprietary lysis solution from Panomics to each well and incubation at 60 °C for 1 h with shaking at 100 rpm. Lysate from each well was hybridized with DNA probes specific for the genes for CCL2, CCL7, PTX3, and IL-6 and capture beads for 16 h at 58 °C with shaking at 600 rpm. Capture beads hybridized to DNA probes and lysate RNA were washed on a vacuum-assisted 96-well filter plate apparatus and further hybridized for 1 h at 54 °C with shaking at 600 rpm first to an Amplifier Diluent and subsequently to a biotinylated DNA Label Probe provided in the kit, with washing steps before and after the Label Probe hybridization. The capture beads ultimately associated with biotinylated label probe were labeled with phycoerythrin-conjugated streptavidin at room temperature for 30 min with 600 rpm shaking. After 2 additional washing steps, the fluorescence intensity of the labeled beads in each well of the 96-well plate was evaluated on a LiquiChip 200 Workstation (Qiagen).

**LIMULUS AMOEBOCYTE LYSATE ASSAY**

For the limulus amoebocyte lyase (LAL) assay, endotoxin-free magnesium chloride (MgCl₂) buffer was added to 6 different lots of Vacutainer tubes: plastic Vacutainer tubes (BD cat #367874, lots #5339582, 6123786, and 6100968) and glass Vacutainer tubes (BD cat #367874, lots #5007997, 5311755, and 6123675). Buffer was allowed to incubate in the tube for 24 h before endotoxin analysis using the Kinetic-QCL kit (Lonza).

**Results**

One assay for monitoring the activation of TNF receptor 1 in whole blood is the measurement of TNF-α−induced increased phosphorylation of p38 MAPK (Fig. 1). During routine biomarker development assays, this previously qualified assay began to perform erratically or failed completely. An extensive evaluation of reagents and buffers was conducted but yielded no insight into the apparent loss of activity. At this point, we recognized that the sample collection tubes for our whole-blood−based assays had been switched from glass (lot #5007997) to plastic Na+−heparin Vacutainer tubes (lot #5339582).

To compare the 2 types of blood collection tubes, we used a phospho-flow cytometry protocol to assay whole blood monocytes (which are very sensitive to endotoxin) for an increase in activated p38 MAPK (Fig. 1). During routine biomarker development assays, this previously qualified assay began to perform erratically or failed completely. An extensive evaluation of reagents and buffers was conducted but yielded no insight into the apparent loss of activity. At this point, we recognized that the sample collection tubes for our whole-blood-based assays had been switched from glass (lot #5007997) to plastic Na+−heparin Vacutainer tubes (lot #5339582).

Although baseline phosphorylation of p38 MAPK was similar in monocytes from blood collected in either glass or plastic Vacutainer tubes (Fig. 1), TNF-α treatment failed to induce phosphorylation of p38 MAPK in monocytes from whole blood collected in plastic tubes. In contrast, we observed a 3.5-fold increase in phosphorylated p38 in TNF-α−stimulated blood from the glass tubes or heparinized syringes. LPS stimulation
did lead to a 2.5-fold increase in p38 phosphorylation in stimulated blood collected in plastic tubes. However, this response was blunted compared with the LPS-induced stimulation of phosphorylated p38 in blood from the glass tubes or heparinized syringes (5.4-fold increase). Different lots of TNF-α/H9251 and LPS had similar effects on p38 phosphorylation regardless of the blood collection method used, indicating that these reagents were not contaminated.

In a concurrent set of bDNA-based assays, unexpected increases in IL-6, CCL7, PTX3, and CCL2 mRNA expression were observed in whole blood not stimulated with exogenous cytokines. In many respects, the results obtained were similar to that observed with LPS stimulation. To test the effects of blood collection tubes on the bDNA assay, whole blood from a single donor was drawn into either glass (lot #5007997) or plastic (lot #5339582) Vacutainer tubes. An increase in the basal expression of CCL2, CCL7, IL-6, and PTX3 mRNA was observed when blood was collected into plastic tubes (Fig. 2). LPS increased mRNA expression of these cytokines in blood collected into glass tubes but no additional increase was observed in blood collected in plastic tubes.

We assessed the production of 2 cytokines (IL-1β and TNF-α) known to be induced in whole blood exposed to LPS to confirm that the observed effects on p38 phosphorylation in whole blood collected in plastic Vacutainer tubes was affecting events downstream of signal transduction. Blood was collected from 2 different donors into plastic Na⁺-heparin Vacutainer tubes (lot #5339582), glass Na⁺-heparin Vacutainer tubes (lot #5311755), or plastic Monoject® blood collection tubes (Kendall, lot #312120). Cytokine production was measured by ELISA (Table 1). IL-1β production from blood collected in the glass Vacutainer tubes or the Monoject tubes was below the lower limit of quantification of the assay (1.56 ng/L; Table 1). In contrast, blood from both donors collected in the plastic Vacutainer tubes displayed increased IL-1β concentrations, ranging from 2.4 to above the upper limit of quantification of the assay. Similarly, TNF-α production was below the lower limit of quantification in blood from both donors collected in the glass Vacutainer tubes or the Monoject tubes, but TNF-α concentrations were increased in blood collected by using the plastic Vacutainer tubes. TNF-α production in blood from the plastic tubes ranged from 2 to 3 μg/L (Table 1).

To further investigate the analytes affected by the collection tube choice, we analyzed blood using the MAP. Whole blood from 3 separate donors was collected into glass or plastic Na⁺-heparin Vacutainer tubes (lot #5339582) and allowed to incubate for 6 or 18 h. Plasma was removed and analyzed using the MAP. The expression of at least 20 different analytes was increased when blood was collected in the plastic tubes (Fig. 3). Most of the analytes that were increased in the blood collected in the plastic tubes were also increased when the blood was stimulated with LPS at either 6 or 18 h (data not shown). Analytes such as TNF-α, MCP-1/CCL2, and IL-6, which would be predictive of LPS stimulation, were increased. However, there were also some analytes that had an unexpected increase as a result of LPS stimulation or suspected en-
Endotoxin contamination. These included α-fetoprotein and brain-derived neurotrophic factor. These results further support the likelihood that the contaminant in the plastic tubes was endotoxin.

Taken together, the results of the phospho-flow cytometry data, the bDNA data, and the cytokine production data all indicate that the particular lot of 10-mL plastic Na$_2$-heparin tubes we used was contaminated with endotoxin or a substance that acted in a manner similar to endotoxin. At this point we tested additional lots of Vacutainer tubes for evidence of similar activity. In an attempt to quantify the concentrations of endotoxins in the different tube lots, we added endotoxin-free MgCl$_2$ buffer to 6 different lots of Vacutainer tubes. Three lots of plastic Vacutainer tubes (BD cat #367874, lots #5339582, 6123786, and 6100968) and 3 lots of glass Vacutainer tubes (BD cat #367874, lots #5007997, 5311755, and 6123675) were tested for endotoxin by using the LAL assay. As indicated in Table 2, endotoxin concentrations varied...
Proinflammatory Cytokines Are Induced in Vacutainer Tubes Containing Trace Endotoxin

Fig. 3. Analyte expression in unstimulated whole blood.
Whole blood was collected from 3 donors in either glass or plastic tubes and incubated for 6 or 18 h. Plasma was removed and analyzed by using the MAP. Fold changes vs the 6-h control are shown within each heat-map panel. Yellow, increase from baseline; blue, decrease from baseline; white, no change from baseline. MIP, macrophage inflammatory peptide; ENA, epithelial neutrophil-activating protein; G-CSF, granulocyte colony-stimulating factor.

In a final effort to compare the sensitivities of the methods used to test for possible endotoxin contamination, we identified a plastic Vacutainer tube lot that seemed to be uncompromised according to results of IL-1β and TNF-α expression measured after incubation (see Table in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol56/issue9). This lot of plastic Vacutainer tubes induced the lowest cytokine increase widespread in plastic tubes tested in this assay, ranging from 0.53 to 7.7 k endotoxin units/L. Although the concentrations varied, all 3 lots of plastic tubes that were tested had detectable concentrations of endotoxin. It is not known if the variation was due to the LAL assay or the tubes themselves. In contrast, endotoxin concentrations were below the limit of detection in all the glass tubes tested except one, and it is possible that this increase in endotoxin was due to contamination during the LAL assay.
we had observed in any of the lots of plastic tubes (<1% of that observed in the severely contaminated lot). The small amount of contamination present that induced slight changes in IL-1β and TNF-α was confirmed by the effects on both basal and induced p38 phosphorylation (Fig. 4). In this case the phospho-flow cytometry analysis was sufficiently sensitive to be strongly affected by even a very low amount of contaminating endotoxin as measured by the fold change in the phosphorylation of p38 in response to either TNF-α or LPS. Therefore, even low amounts of contamination can have a profound effect, depending on the technologies used and on the analytes measured.

Discussion

It has been recognized since the 1980s that bacterial LPS (endotoxin) contamination results in the spontaneous production of cytokines by monocytes (6, 7). Efforts were subsequently made to produce endotoxin-free reagents for use in cellular immunological assays. However, these reagents are useless if the sample col-

### Table 2. Summary of endotoxin concentrations in plastic and glass Vacutainer tubes.

| Tube sample | Lot number | Tube type | LAL, kEU/L | Tube sample | Lot number | Tube type | LAL, kEU/L |
|-------------|------------|-----------|------------|-------------|------------|-----------|------------|
| 1           | 5339582    | Plastic   | 2.13       | 1           | 5007997    | Glass     | <0.50      |
| 2           | 5339582    | Plastic   | 1.38       | 2           | 5007997    | Glass     | <0.50      |
| 3           | 5339582    | Plastic   | 1.95       | 3           | 5007997    | Glass     | <0.50      |
| 4           | 5339582    | Plastic   | 7.71       | 4           | 5007997    | Glass     | <0.50      |
| 5           | 5339582    | Plastic   | 6.85       | 5           | 5007997    | Glass     | <0.50      |
| 6           | 5339582    | Plastic   | 6.38       | 6           | 5007997    | Glass     | <0.50      |
| 7           | 5339582    | Plastic   | 6.41       | 7           | 5007997    | Glass     | <0.50      |
| 8           | 5339582    | Plastic   | 7.42       | 8           | 5007997    | Glass     | <0.50      |
| 9           | 5339582    | Plastic   | 0.98       | 9           | 5007997    | Glass     | <0.50      |
| 10          | 5339582    | Plastic   | 0.71       |              |            |           |            |
| 11          | 5339582    | Plastic   | <0.50      | 1            | 5311755    | Glass     | <0.50      |
| 12          | 5339582    | Plastic   | 0.66       | 2            | 5311755    | Glass     | <0.50      |
| 13          | 5339582    | Plastic   | 0.98       | 3            | 5311755    | Glass     | <0.50      |
| 14          | 5339582    | Plastic   | 0.71       | 4            | 5311755    | Glass     | <0.50      |
| 15          | 6100968    | Plastic   | 0.65       | 1            | 6123675    | Glass     | <0.50      |
| 16          | 6100968    | Plastic   | <0.50      | 2            | 6123675    | Glass     | <0.50      |
| 17          | 6100968    | Plastic   | 1.15       | 3            | 6123675    | Glass     | <0.50      |
| 18          | 6100968    | Plastic   | 0.55       | 4            | 6123675    | Glass     | <0.50      |
| 19          | 6100968    | Plastic   | 0.74       | 5            | 6123675    | Glass     | <0.50      |
| 20          | 6100968    | Plastic   | <0.50      | 6            | 6123675    | Glass     | <0.50      |
| 21          | 6100968    | Plastic   | <0.50      | 7            | 6123675    | Glass     | <0.50      |
| 22          | 6123786    | Plastic   | <0.50      | 8            | 6123675    | Glass     | <0.50      |
| 23          | 6123786    | Plastic   | 0.54       |              |            |           |            |
| 24          | 6123786    | Plastic   | <0.50      |              |            |           |            |
| 25          | 6123786    | Plastic   | 0.62       |              |            |           |            |
| 26          | 6123786    | Plastic   | 0.57       |              |            |           |            |
| 27          | 6123786    | Plastic   | <0.50      |              |            |           |            |
| 28          | 6123786    | Plastic   | <0.50      |              |            |           |            |

*Endotoxin-free MgCl₂ buffer was added to 6 different lots of Vacutainer tubes. Three lots of plastic Vacutainer tubes (BD cat #367874, lot #5339582, #6123786, and #6100968) and 3 lots of glass Vacutainer tubes (BD cat #367874, lot #5007997, #5311755, and #6123675) were tested for endotoxin by using the LAL assay. EU, endotoxin units.
lection tubes are contaminated. Sporadic reports of contamination in blood collection tubes can be found in the literature. It was previously reported that 10-mL Na$_2$-heparin Vacutainer tubes contained a mean (SD) LPS/tube of approximately 315 (95) pg as measured by the LAL assay (8). In addition, tubes contaminated with endotoxin can stimulate cytokine production in whole blood. Vacutainer tubes containing contaminated lithium heparin strongly stimulated IL-1, IL-6, and TNF-$\alpha$ production in whole blood after as little as 2 h of incubation (9). EDTA Vacutainer tubes or tubes containing endotoxin-free heparin had no effect on cytokine production, indicating that the choice of anticoagulant was not the factor contributing to spontaneous cytokine production. Another group found similar results when whole blood was collected in Na$_2$-heparin Vacutainer tubes that contained 10 ng/L of endotoxin (10).

The results of this study also mirror the well-documented phenomenon of endotoxin tolerance. In endotoxin tolerance, repeat exposure to endotoxin alters the signaling network within a cell, leading to a marked decrease in responsiveness to LPS stimulation (11). This hyporesponsiveness is usually manifested as diminished secretion of cytokines, particularly TNF-$\alpha$ and IL-6 (12). However, changes in signaling molecules downstream of toll-like receptor 4 have also been observed (11). In the current study, we observed changes in both signaling, as detected by phospho-flow cytometry, and cytokine release in whole blood as a result of prolonged exposure to endotoxin in the contaminated tubes. This in vitro tolerance alters the inflammatory response in the cells and therefore masks the overall patient response, making it impossible to draw conclusions from clinical trials.

Our data point to the conclusion that there may be a systemic problem with the use of plastic Na$_2$-heparin Vacutainer tubes, in that blood collected in any of 3 lots of extensively tested tubes consistently behaved as if stimulated with endotoxin. Endotoxins stimulate cellular responses through the toll-like receptor system in blood, which is sensitive to a wide variety of chemicals that often resemble molecules released from pathogens. The observed effects could therefore be due to contamination with endotoxin during the manufac-
turing process, or possibly even to cellular recognition of other synthetic chemicals incorporated into the plastic and then released into the blood. Highly sensitive phospho-flow cytometry assays are useful for evaluating drug development projects and determining on-target assessments, which inform the further clinical development of drug candidates. These kinds of assays are also critical for understanding disease etiology on a molecular level. They provide on-target assessment of therapeutic processes, and have even been used to provide information used to determine patient-dosing concentrations. The observation that the gold standard LAL assay and the relatively more sensitive cytokine detection assays were unable to detect low-level endotoxin contamination illustrates the need for rigorous evaluations of novel approaches for identifying and evaluating new whole blood biomarkers because strong responses are readily detectable with the use of the more sensitive phospho-flow cytometry assay. As we continue to develop these powerful and innovative assays it is necessary to keep a critical eye on sources of variation that may be introduced by something as ostensibly innocuous as the type of blood collection tube used. Caution is recommended in assessing the potential effects of this preanalytical variable before collecting and analyzing clinical samples.

Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.

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