To the editor:

Circulating microparticles do not all share biophysical light scatter properties with immune complexes when analyzed by flow cytometry

Recent discussions have been published on the flow cytometric analysis of cell-derived microparticles (MPs) and the confounding effects of insoluble immune complexes (ICs) on the interpretation of data, with György et al stating that MPs and ICs share biophysical light scatter properties when analyzed by flow cytometry (FCM). Although MPs and ICs overlap in size, we propose not all MPs would share biophysical light scatter properties with ICs because of the different constituents of each: MPs being a lipid-bilayer vesicle, with derived cellular components bound and internal to the vesicle, which is plastic in nature, whereas ICs are a result of multimolecular antibody-antigen bindings with a packed, rigid composition and are less prone to morphological change. The size of cell-derived MPs range from 0.1-1 micron in diameter, placing analysis at the very limits of flow cytometry (FCM) detection. Collecting at these detection limits, small differences in instrument design, maintenance, and operation can lead to contrasts in quality of data. Although FCM is used extensively for this application, standardization is very much in its infancy.

Following procedures described by György et al, artificial, insoluble ICs were created, cell-derived MPs were isolated from the serum of healthy donors after an established protocol. The samples were analyzed by FCM and Dynamic Light Scatter (DLS). In addition, György et al proposed antibody aggregation of certain antibodies leads to microvesicle-mimicking signals. We followed procedures as described to investigate the signal of antibody aggregate versus MPs.

As the DLS data shows (Figure 1A), the MPs and ICs overlap in size, but when analyzed by FCM the populations are distinct (Figure 1B). In addition, we did not see antibody aggregates produce a microvesicle-mimicking signal, but they did coincide with the IC population (Figure 1C), which is not unexpected based on the comparable constituents.

**Figure 1. DLS and FCM analysis of IC and MP samples.** (A) DLS analysis of ICs and MPs, showing both samples are of overlapping size. (B) FCM analysis of ICs, MPs, and ICs spiked with ICs. It can be seen the ICs and MPs are distinguished by light scatter alone. (C) FCM analysis of antibody (Ab) aggregate, where Ab aggregate overlaps with the IC population but does not create microvesicle-mimicking signals. For FCM analysis, a Coulter Epics XL instrument was used for acquisition, triggering of side scatter and using a Nano Fluorescent Particle Size Standard Kit; NFPPS-52-4K (Spherotech) for instrument calibration (data not shown). For DLS, a Coulter N4 plus instrument was used, acquiring the data at 90° angle, experiments carried out at 22°C.
Following the protocol from Amabile et al., who propose systematic triton lysis as a control to prove the vesicular nature of the MP population, we agree that the use of triton lysis can be a validating step for many MP-IC applications by FCM, as we also saw MP population dissolve from the MP gate. However, using the triton lysis we observed an impact on the IC population (data not shown). Under our conditions, as both populations are distinguishable by light scatter alone, the use of triton lysis in this instance added an unnecessary and complicating step. Here we have shown that MPs and ICs do not share biophysical light scatter properties when analyzed by FCM, but because of the small size of both, and the limits of some FCM instruments, maintenance, and operation, they may not appear discrete.

Acknowledgments: The authors thank Greg Noel and Xiaoyang Qi for technical assistance. Flow cytometry was performed at Shriners Hospital for Children supported by a grant from the Shriners of North America SSF 84070.

To the editor:

Ruxolitinib inhibits transforming JAK2 fusion proteins in vitro and induces complete cytogenetic remission in t(8;9)(p22;p24)/PCM1-JAK2–positive chronic eosinophilic leukemia

The JAK1/JAK2 inhibitor ruxolitinib was recently approved for intermediate and high-risk myelofibrosis. However, for malignancies with JAK2 rearrangements, known for their rapid evolution and dismal prognosis, the potential of JAK inhibitor therapy is not established. We investigated the efficacy of ruxolitinib against JAK2 fusions in vitro and in a patient with t(8;9)(p22;p24)/PCM1-JAK2 chronic eosinophilic leukemia (CEL).

A 72-year-old male presented with leukocytosis of 49 × 10^9/L, absolute eosinophil count of 23 × 10^9/L and circulating myeloid progenitors. Specific symptoms were absent and clinical examination was unremarkable. Bone marrow aspirate and biopsy were hypercellular with prominent myelopoiesis, pronounced eosinophilia, and hypergranulated granulocyte precursors (Figure 1A). Cytogenetics showed 46,XY,t(8;9)(p22;p24) in 33 of 35 metaphases, and FISH confirmed the breakpoints in 8p22 (PCM1) and 9p24 (JAK2; Figure 1B). RT-PCR and Sanger sequencing showed an in-frame fusion between PCM1 exon 36 and JAK2 exon 9, as in the first case reported.

This PCM1-JAK2 fusion was cloned and additional ETV6-JAK2 and SEC31A-JAK2 constructs were generated. In Ba/F3 cells, PCM1-JAK2, ETV6-JAK2, and SEC31A-JAK2 induced growth factor independent growth, which was suppressed by ruxolitinib with IC50 values of 57, 46, and 92nM, respectively (Figure 1C). Western blotting showed dose-dependent inhibition of phosphorylation of JAK2, indicating a direct and specific action of ruxolitinib, and of downstream STAT5 (Figure 1C). For comparison, the growth of JAK2-V617F–expressing Ba/F3 was inhibited with 106nM as IC50, in line with published data. Similar data were obtained for JAK inhibitor I, a JAK1/JAK2/JAK3/TYK2 inhibitor (not shown).

Given this, and lacking effective medical treatment options, ruxolitinib was initiated after approval from the ethical committee and patient informed consent. Since diagnosis, the leukocyte count had been controlled with hydroxyurea 250-2000 mg/d, but the peripheral eosinophilia remained elevated. Multiple transfusions and dose adjustments were required for anemia, with corresponding fluctuations in leucocytes and eosinophils. After 15 months, ruxolitinib was started at 10 mg twice daily. At this point, 25 of 30 marrow metaphases were t(8;9)-positive (Figure 1D-E). Ruxolitinib was cautiously increased to 15-20 mg bid and hydroxyurea could be discontinued 6 months later. By then, the eosinophil count had normalized, and the thrombocytopenia partially recovered. Moreover, the number of t(8;9) metaphases had decreased to 10 of 30. Continuing ruxolitinib as single agent, the eosinophil count remained normal. Marrow examinations after 11 and 15 months ruxolitinib therapy were hypocellular without evidence of CEL. After 15 months, a complete cytogenetic response (0 abnormal of 26 analyzed metaphases) was reached; and the number of interphase nuclei with a JAK2/9p24 breakpoint in FISH had equally decreased to less than 5%.

References

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